MONASH University

Adenosine A_{2B} Receptor Pharmacology and its Role in Pathophysiology

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B.Pharm (Hons)

A thesis submitted for the degree of Doctor of Philosophy

at Monash University in 2017

Drug Discovery Biology

Monash Institute of Pharmaceutical Sciences

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Abstract

The adenosine A_{2B} receptor ($A_{2B}AR$) is one of four adenosine receptor subtypes belonging to the Class A G protein-coupled receptor (GPCR) family. Until recently, the $A_{2B}AR$ remained poorly characterised, in part due to its relatively low affinity for the endogenous agonist adenosine and therefore presumed minor physiological significance. However, the substantial increase in extracellular adenosine concentration and the upregulation of $A_{2B}AR$ expression under conditions of hypoxia and inflammation, suggest the $A_{2B}AR$ as an exciting therapeutic target in a variety of pathophysiological states. In order to confirm the full therapeutic potential of the $A_{2B}AR$ and to facilitate drug discovery efforts, a deeper understanding of its complex pharmacology has been required. Therefore, the focus of this thesis was to investigate new paradigms of $A_{2B}AR$ behaviour and signalling within the context of pathophysiology, including constitutive activity, dimerization and biased agonism.

In Chapter 2, we first identified the $A_{2B}AR$ had an elevated basal level of activity, illustrating for the first time that the human wild type receptor displays appreciable constitutive active. The discovery that an $A_{2B}AR$ inverse agonist was able to reduce baseline cAMP and basal cell growth of prostate cancer cells indicated that the receptor can signal even in the absence of agonist activation and demonstrated a possible role for $A_{2B}AR$ constitutive activity in prostate cancer cell proliferation.

Recent work in the field has focused on the role of the $A_{2B}AR$ in ischaemia-reperfusion injury and cardiac fibrosis, as such, subsequent chapters investigated $A_{2B}AR$ signalling in the context of cardiovascular disease. In Chapter 3, this involved examining the molecular basis for $A_{2B}AR$ modulation of A_1AR-mediated cardioprotection at the cellular level and demonstrated potential

heteromerization between adenosine receptor subtypes. Chapter 4 and Chapter 5 reclassified two novel cardioprotective A_1AR agonists, capadenoson and VCP746 as additionally displaying unique signalling profiles at the A_{2B}AR. In Chapter 6, VCP746 was used to further interrogate the role of A_{2B}AR signalling in normal and diseased (+TGF-β1) cardiac fibroblasts in an effort to understand how both pro- and anti-fibrotic signalling could be downstream of A_{2B}AR activation. In addition to uncovering a novel signalling pathway involving protein kinase G cross-talk with G_q-mediated IP_3 accumulation in primary neonatal rat fibroblasts, it was identified that VCP746 displayed context-dependent bias, maintaining potency even when prototypical A_{2B}AR signalling was diminished under diseased (+TGF-β1) fibroblast conditions.

Collectively, these studies provide comprehensive insights into new paradigms of A_{2B}AR signalling in pathophysiology and have expanded the pharmacological toolbox of potential ligands which can be used to investigate this important adenosine receptor subtype.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes four original papers published in peer reviewed journals. The core theme of the thesis is "adenosine A_{2B} receptor pharmacology and its role in pathophysiology". The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Drug Discovery Biology theme of the Monash Institute of Pharmaceutical Sciences, under the supervision of A/Prof Paul White and Dr Lauren May.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 2, 4 and 5 and Appendix 1, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Nature of co-author's contribution
Chapter 2	Ligand-independent adenosine A_{2B} receptor constitutive activity as a promoter of prostate cancer cell proliferation	Published	Performed the majority of experiments, analysed data and wrote and proofed the manuscript (80%)	CYT performed RT-PCR experiments. KJG and AC contributed to writing of the manuscript. PJW and LTM conceived and supervised the project, performed data analysis and wrote the manuscript.

Chapter 4	Capadenoson, a clinically trialed partial adenosine A_1 receptor agonist, can stimulate adenosine A_{2B} receptor biased agonism	In Press	Performed the majority of experiments, analysed data and wrote and proofed the manuscript (50%)	JAB performed cAMP, pERK1/2 and Ca^{2+} assays in A_{2B}AR-FlpInCHO cells and experiments in cardiomyocytes, analysed data and wrote the manuscript. MAC and AC contributed to writing the manuscript. CXC and RHR supervised isolation of primary cardiac cells. PJW and LTM conceived and supervised the project, performed data analysis and wrote the manuscript.
Chapter 5	The hybrid molecule, VCP746, is a potent adenosine A_{2B} receptor agonist that stimulates anti-fibrotic signalling	Published	Performed the majority of experiments, analysed data and wrote and proofed the manuscript (50%)	CHC performed [^{3}H]proline incorporation and RT-PCR assays, analysed data and wrote the manuscript. LF and PJS conducted and supervised chemical synthesis. JAB and AC contributed to writing the manuscript and data analysis. BHW supervised [^{3}H]proline incorporation and RT-PCR experiments and wrote the manuscript. PJW and LTM conceived and supervised the project, performed data analysis and wrote the manuscript.
Appendix 1 & Section 1.4.2 of Chapter 1	Targeting adenosine receptor for the treatment of cardiac fibrosis	In Press	Wrote, edited and proofed manuscript (80%)	PJW and LTM contributed to the writing and revision of the manuscript and approved it for publication

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** 27/04/2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 27/04/2017

Publications

Publications included in thesis:

Vecchio, E. A., Tan, C. Y. R., Gregory, K. J., Christopoulos, A., White, P. J., and May, L. T. (2016). Ligand-independent adenosine A2B receptor constitutive activity as a promoter of prostate cancer cell proliferation. *J. Pharmacol. Exp. Ther.* 357(1): 36–44.

Vecchio, E. A.*, Chuo, C. H.*, Baltos, J.-A., Ford, L., Scammells, P. J., Wang, B. H., Christopoulos, A., White, P. J., and May, L. T. (2016). The hybrid molecule, VCP746, is a potent adenosine A2B receptor agonist that stimulates anti-fibrotic signalling. *Biochem. Pharmacol.* 117: 46-56 * Co-first author

Baltos, J.-A.*, **Vecchio, E. A.***, Harris, M. A., Qin, C. X., Ritchie, R. H., Christopoulos, A., White, P. J., and May, L. T. (2017). Capadenoson, a clinically trialed partial adenosine A1 receptor agonist, can stimulate adenosine A2B receptor biased agonism. *Biochem. Pharmacol.* In Press * Co-first author

Vecchio, E. A., White, P. J., and May, L. T. (2017). Targeting adenosine receptors for the treatment of cardiac fibrosis. *Front Pharmacol*. In Press

Publications arising during PhD but not included in thesis:

Nguyen, A. T. N., **Vecchio, E. A.**, Thomas, T., Nguyen, T. D., Aurelio, L., Scammells, P. J., White, P. J., Sexton, P. M., Gregory, K. J., May, L. T., and Christopoulos, A. (2016). Role of the second extracellular loop of the adenosine A1 receptor on allosteric modulator binding, signaling, and cooperativity. *Mol. Pharmacol.* 90(6): 715–725.

Jörg, M., Glukhova, A., Abdul-Ridha, A., **Vecchio, E. A.**, Nguyen, A. T. N., Sexton, P. M., White, P. J., May, L. T., Christopoulos, A., and Scammells, P. J. (2016). Novel irreversible agonists acting at the A1 adenosine receptor. *J. Med. Chem.* 59(24): 11182-11194.

Glukhova, A., Thal, D. M., Nguyen, A. T. N., **Vecchio, E. A.**, Jörg, M., Scammells, P. J., May, L. T., Sexton, P. M., and Christopoulos, A. (2017). Structure of the adenosine A1 receptor reveals the basis for subtype selectivity. *Cell* 168(5): 867–877.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors A/Prof Paul White and Dr Lauren May for their patience, guidance, immense knowledge and support throughout my PhD. Their continual encouragement and enthusiasm kept me motivated and inspired and played a great role in shaping the scientist I am today.

I would like to thank my thesis panel members: Dr Michelle Halls, A/Prof Bing Wang and Prof Arthur Christopoulos and temporary supervisor, Dr Karen Gregory for their insights and expertise throughout my candidature. I would like to acknowledge Prof Peter Scammells and his team for the provision of the VCP compounds used within my studies.

This research wouldn't have been possible without the financial support of the Australian Government Research Training Program Scholarship and the generous top up scholarship provided by Cancer Therapeutics Australia.

I would like to extend my thanks to my peers in the adenosine group: Jo-Anne Baltos, Frank Chuo, Dr Anh Nguyen, Dr Alisa Glukhova, Matt Harris, Dr Alaa Abdul-Ridha and Dr Christina Tan for their practical support and robust discussions which resulted in a number of fruitful publications. To the friends and colleagues at Monash University and the Drug Discovery Biology laboratory, especially Souhiela Fawaz, Angelina Lim, Jo-Anne Baltos and Amy Chen for being there through the highs and lows, this journey was made immeasurably easier having their support and listening ear to lighten the load. To all my other long-suffering friends including the Mingers and the Pharm Gang, thank you for providing much needed distraction, encouragement and good humour to help get me through the rollercoaster ride.

I am forever grateful to my Mum, Dad, Chris, Siobhan, Tim and Katherine for their love and support and never doubting for a moment that I could do this. I feel incredibly lucky to have been blessed with such an exceptional, caring and inspiring family.

And last, but not least, I would like to thank my incredible husband Adrian, for his unwavering belief in me, for picking me up every time I fell, for celebrating every little milestone and victory, for proofreading my thesis and for making every day seem just a little bit brighter. I won the lottery of life with you.

List of Abbreviations

[^{3}H]DPCPX	[^{3}H]cyclopentyl-1,3-dipropylxanthine 8 dipropyl-2,3
α-SMA	α-smooth muscle actin
A_1AR	adenosine A_1 receptor
A_{2A}AR	adenosine A_{2A} receptor
A_{2B}AR	adenosine A_{2B} receptor
A_3AR	adenosine A_3 receptor
AC	adenylyl cyclase
ADA	adenosine deaminase
ADK	adenosine kinase
ADP	adenosine diphosphate
AMP	5'-adenosine monophosphate
ANGII	angiotensin II
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAY60-6583	2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide
BrdU	5-Bromo-2'-deoxyuridine
BRET	bioluminescence resonance energy transfer
CADO	2-chloroadenosine
cAMP	cyclic adenosine monophosphate
CD 39	ecto-nucleoside triphosphate diphosphohydrolase 1
CD 73	ecto-5'-nucleotidase
CFTR	cystic fibrosis transmembrane conductance regulator
CGS-21680	4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride
CHO	Chinese hamster ovary
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CTGF	connective tissue growth factor
DAG	diacylglycerol

DMEM	Dulbecco's modified Eagle's medium
ECL	extracellular loop
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ENT1/2	equilibrative nucleoside transporter 1 & 2
eNTPD1/CD39	ecto-nucleoside triphosphate diphosphohydrolase 1 (also known as CD39)
Epac	exchange protein directly activated by cAMP
ERK1/2	extracellular signal-regulated kinase 1 & 2
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
Fra-1	fos-related antigen-1
FRET	fluorescence resonance energy transfer
FSK	forskolin
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HEK	human embryonic kidney
HIF-1α	hypoxia-inducible factor 1α
HMC	human mast cells
HTRF	homogenous time-resolved fluorescence
IBD	inflammatory bowel disease
IFN	interferon
IL	interleukin
IP_1	inositol monophosphate
IP_3	inositol triphosphate
IRI	ischaemia-reperfusion injury
ISO	isoprenaline
JNK	c-Jun N-terminal kinase
K_{ATP}	ATP-sensitive K^+ channel
LAD	left anterior descending artery
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MeCCPA	2-chloro-*N*-cyclopentyl-2'-methyladenosine
MI	myocardial infarction

min	minute
mPTP	mitochondrial permeability transition pore
NECA	5'-N`-ethylcarboxamidoadenosine
NF-κB	nuclear factor-κB
Nox4	NADPH oxidase 4
NT	non-transfected
NVCF	neonatal ventricular cardiac fibroblast
NVCM	neonatal ventricular cardiomyocyte
PBS	phosphate buffered saline
PGE2	prostaglandin E2
PI	propidium iodide
PI3K	phosphoinositol-3 kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PSB-603	8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl))-1-propylxanthine
PTX	pertussis toxin
RMC	renal mesangial cells
ROS	reactive oxygen species
RT-PCR	reverse-transcription polymerase chain reaction
SAH	*s*-adenosylhomocysteine
SCH 442416	2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H- pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine
SFM	serum free media
shRNA	short hairpin ribonucleic acid
SLV320	*trans*-4-((2-phenyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino) cyclohexanol
TGF-β1	transforming growth factor-β1
TM	transmembrane
TNF	tumour-necrosis factor
VCP171	(2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl) methanone

VCP746	4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-*N*-(6-(9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxylmethyl) tetrahydro-furan-2-yl)-9*H*-purin-6-ylamino)hexyl)benzamide
VEGF	vascular endothelial growth factor
ZM241385	4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol

Chapter 1:

General Introduction

1.1 Adenosine and Adenosine Receptors

Adenosine is an endogenous purine nucleoside present both intracellularly and extracellularly in living cells. It is comprised of an adenine group attached to a ribose sugar by a glycosidic bond (Figure 1.1) (Shyrock and Belardinelli, 1997). Adenosine as both a precursor and metabolite of adenine nucleotides, provides the structural building block of adenosine triphosphate (ATP) and thus plays a central role in the basic energy transfer of all living organisms (Fredholm, 2007; Layland et al., 2014). Adenosine also acts as a ubiquitous extracellular signalling molecule to exert a plethora of physiological actions throughout the body. It is often described as a 'retaliatory metabolite' owing to the fact that adenosine reduces cellular work and restores energy balance in the very same cells within which it is produced (Newby, 1984; Shyrock and Belardinelli, 1997). Adenosine was first identified as a mediator of coronary vascular tone, heart rate and blood pressure in 1929 (Drury and Szent-Györgyi, 1929) and is still used as mainstay clinical therapy for patients with supraventricular tachycardia today (Eltzschig, 2009). Much of the early understanding of the physiological role of adenosine comes from the cardiovascular system, however it has since been recognised to have critical roles in nearly every organ system and tissue. This includes as a regulator of the; i) central nervous system (Dunwiddie and Masino, 2001; Fredholm et al., 2005), ii) inflammatory and immune response (Fredholm, 2007; Haskó et al., 2008), iii) endocrine system (Dong et al., 2001; Figler et al., 2011), and as an endogenous modulator of; i) pain (Sawynok, 2016; Zylka, 2011), ii) lung function (Wilson et al., 2009; Zhou et al., 2009) and iii) kidney function (Roberts et al., 2014a).

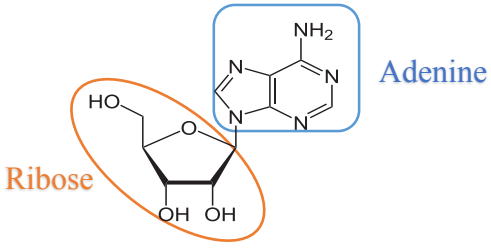


Figure 1.1. Chemical structure of adenosine.

1.1.1 Sources of Endogenous Adenosine

Under normal physiological conditions, adenosine is continuously formed and metabolised intracellularly as well as extracellularly. Intracellular adenosine is produced primarily through the catabolism of nucleotides, with the sequential dephosphorylation of ATP to 5'-adenosine diphosphate (ADP) to 5'-adenosine monophosphate (AMP) and lastly to adenosine. The hydrolysis of intracellular AMP is mediated via cytosolic 5'-nucleotidase with the rate of adenosine formation dictated by levels of AMP and hence is directly determined by cellular workload and oxygenation (Fredholm, 2014; Newby, 1984; Schubert et al., 1979). To a lesser extent intracellular adenosine can also be formed by the enzyme *S*-adenosylhomocysteine (SAH) hydrolase which catalyses the hydrolysis of SAH to adenosine and L-homocysteine (Deussen et al., 1988). Subsequently, adenosine can move into the extracellular compartment (and vice versa) via specific 'equilibrative nucleoside transporters', ENT1 and ENT2, which allow for the diffusion of adenosine across the cell membrane (Young et al., 2008). Extracellular adenosine is also formed from the extracellular hydrolysis of adenine nucleotides that have been released from intracellular stores. This is regulated by a two-step process by surface bound enzymes: first ATP/ADP is rapidly converted to AMP by ecto-nucleoside triphosphate diphosphohydrolase 1 (eNTPD1; also known as CD39). Secondly AMP is hydrolysed to adenosine by ecto-5'-nucleotidase (also known as CD73) (Eltzschig, 2009; Fredholm et al., 2001a). (See Figure 1.2). The other pathway that contributes to the fine-tuned regulation of extracellular adenosine levels in many cells, is the cAMP-adenosine cycle. Upon activation of adenylyl cyclase and the subsequent formation of cyclic adenosine monophosphate (cAMP) there is the robust and immediate efflux of cAMP from the cell (Gödecke, 2009; Jackson and Raghvendra, 2004). This is then broken down to AMP by ecto-phosphodiesterases and finally converted to adenosine by ecto-5'-nucleotidase in the extracellular space (Fredholm et al., 2001a; Jackson and Raghvendra, 2004; Jackson et al.,

2007). Once in the extracellular compartment, adenosine stimulates purinergic signalling through the occupancy of specific cell-surface adenosine receptors (described in detail in section 1.1.3).

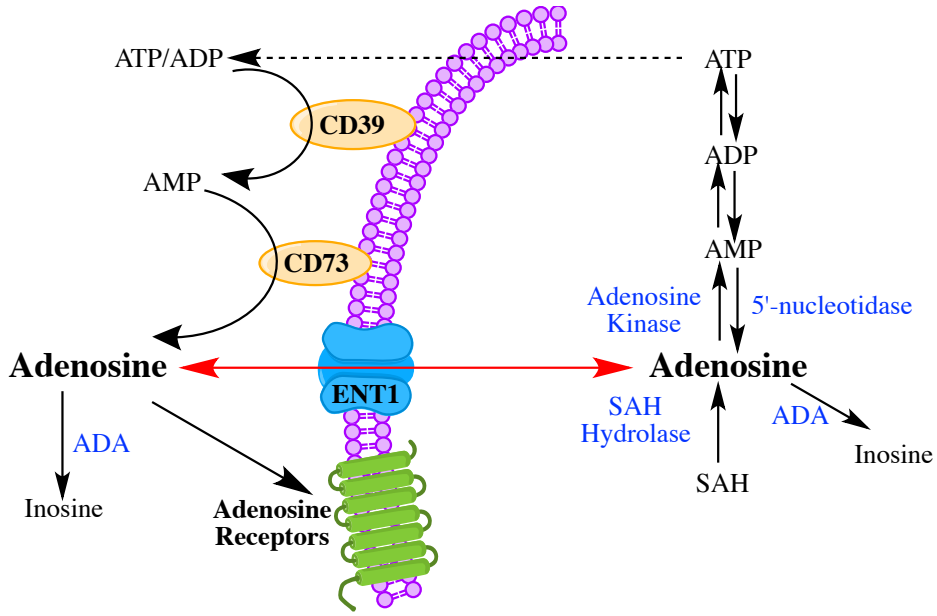


Figure 1.2. Schematic illustration of adenosine generation and metabolism. Adenosine is formed predominantly by degradation of adenine nucleotides intracellularly and extracellularly. ATP; adenosine triphosphate, ADP; adenosine diphosphate, AMP; adenosine monophosphate, CD39; ecto-nucleoside triphosphate diphosphohydrolase 1, CD73; ecto-5'-nucleotidase, ENT; equilibrative nucleoside transporter, SAH; *S*-adenosylhomocysteine, ADA; adenosine deaminase

1.1.2 Adenosine Uptake and Metabolism

The concentration of free adenosine inside, as well as outside the cell is finely regulated and is largely determined by energy consumption. Extracellular adenosine concentrations can rise from baseline (20-300nM) under normal physiological conditions (Ballarín et al., 1991; Chen et al., 2013), up to the low micromolar range in response to hypoxia, ischaemia or other stressed conditions (Dux et al., 1990; Hagberg et al., 1987). Excess adenosine is transported intracellularly by the bi-directional concentration-dependent transporters ENT1 and ENT2. Following uptake into the intracellular compartment, adenosine is rapidly metabolised by phosphorylation to AMP or degradation to the metabolite inosine, by adenosine kinase (ADK)

and adenosine deaminase (ADA) respectively (Lloyd and Fredholm, 1995; Shyrock and Belardinelli, 1997) (Figure 1.2). Adenosine deaminase also exists in the extracellular space to remove excess adenosine. Thus deficiency of adenosine deaminase activity through genetic deletion or the pharmacological inhibition of nucleoside transporters, such as with the use of dipyridamole will prolong the actions of extracellular adenosine (Eltzschig, 2009; Noji et al., 2004).

1.1.3 Classification and Pharmacology of Adenosine Receptors

Despite being recognised as an endogenous regulator of the cardiovascular system in 1929 (Drury and Szent-Györgyi, 1929), it took some 40 years of scientific discovery before it was postulated that these actions were due to adenosine's occupancy of specific cell surface receptors (Sattin and Rall, 1970). Today it is recognised that adenosine mediates its myriad of physiological (and pathological) actions via activation of adenosine receptors, which belong to the superfamily of G protein-coupled receptors (GPCRs).

1.1.3.1 G Protein-Coupled Receptors

GPCRs, also known as seven-transmembrane receptors, constitute the largest family of mammalian cell-surface receptors (Fredriksson et al., 2003). They mediate cellular responses to a vast array of extracellular ligands including hormones, neurotransmitters, calcium ions, peptides, small molecules and even photons of light. Extracellular stimuli are transduced into a cellular response via a conformational change of the ligand-bound receptor which activates the intracellular heterotrimeric G protein. Once activated, GDP is released from the G protein and replaced by GTP which leads to the dissociation of the Gα subunit and Gβγ dimer. This unmasks interactive domains that are capable of modulating a variety of signal transduction pathways through activation of effector proteins including adenylyl cyclase, ion channels and

phospholipase C (Neer, 1995; Pierce et al., 2002; Rosenbaum et al., 2009). Characterised by a seven-transmembrane α-helical core structure (Figure 1.3), GPCRs are grouped into five distinct classes on the basis of sequence conservation and structural similarity. These are the rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and frizzled/taste2 receptor families (Lagerström and Schiöth, 2008; Rosenbaum et al., 2009). The rhodopsin-like family A, which includes the adenosine receptors, represents the largest and most rigorously studied of these receptor classes. The therapeutic importance of GPCRs is highlighted by the fact that up to 40% of current marketed pharmaceuticals target these ubiquitous proteins (Tyndall and Sandilya, 2005).

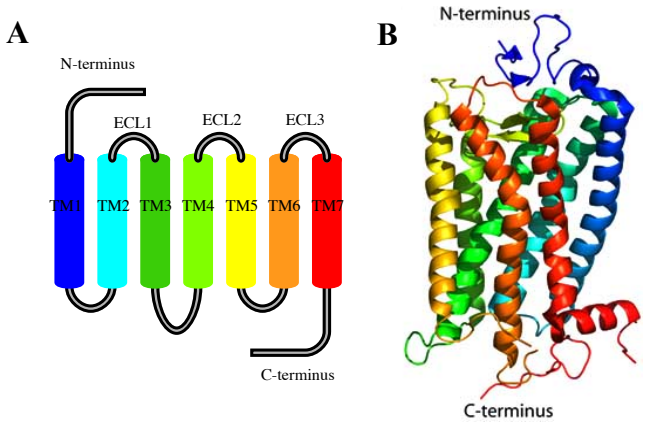


Figure 1.3. A) Schematic representation of the general structure of GPCRs showing 7 transmembrane (TM) domains linked by extracellular loops (ECL). B) Crystal structure of bovine rhodopsin, which serves as a model for Class A GPCRs (PDB ID: 1f88. (Palczewski et al., 2000)). Figure adapted from (Tyndall and Sandilya, 2005).

1.1.3.2 Classification of Adenosine Receptors

Adenosine binds to 4 structurally similar G protein-coupled adenosine receptors; the adenosine A_1 receptor (A_1AR), the adenosine A_{2A} receptor (A_{2A}AR), the adenosine A_{2B} receptor (A_{2B}AR) and the adenosine A_3 receptor (A_3AR) (Fredholm et al., 2000). Adenosine receptors were originally classified based on the effects of receptor stimulation to either inhibit (A_1 or R_i) or stimulate (A_2 or R_a) the activity of adenylyl cyclase (Londos et al., 1980; van Calker et al., 1979). Adenosine A_2 receptors were subsequently subdivided based on their capacity to stimulate cAMP accumulation at low (0.1-1 μM) or high (>10 μM) extracellular adenosine

concentrations in the rat brain (Daly et al., 1983) and were later defined as the A_{2A}AR and A_{2B}AR respectively (Bruns et al., 1986). The most recently discovered subtype, the A_3AR was identified from its sequence homology to other adenosine receptors during molecular cloning from rat testis (Zhou et al., 1992). All four adenosine receptors have since been cloned in humans (and many other species) with the greatest similarity between the A_1 and A_3ARs (49% sequence homology) and the A_{2A} and A_{2B}ARs (59% similarity) (Fredholm et al., 2000; 2001a; Jacobson and Gao, 2006).

1.1.3.3 Signalling of Adenosine Receptors

Classically, adenosine-mediated signalling is subdivided based on the effects of adenosine receptor activation on cAMP levels. The A_1AR and A_3ARs preferentially couple to pertussis toxin sensitive $G_{i/o}$ proteins to inhibit adenylyl cyclase whereas the A_{2A}AR and A_{2B}ARs stimulate adenylyl cyclase through activation of G_s proteins (Daly et al., 1983; Fredholm et al., 2000; Freissmuth et al., 1991; Palmer et al., 1995b). In addition to cAMP accumulation, it has since been recognised that a variety of other second messengers are modulated by adenosine receptors. The A_1AR activates potassium channels (including K_{ATP} channels in the myocardium and neurons), increases intracellular calcium and inositol triphosphate (IP_3) levels by activating phospholipase C (PLC) (via Gβγ), stimulates protein kinase C (PKC) activity and also inhibits N-type voltage-sensitive Ca^{2+} channels in neurons (Akbar et al., 1994; Freund et al., 1994; Gerwins and Fredholm, 1995; Trussell and Jackson, 1985). The A_{2A}AR almost exclusively couples to cAMP/PKA signalling via G_s, except for in the striatum where G_{olf} stimulation predominates (Corvol et al., 2001; Kull et al., 2000). The A_{2B}AR on the other hand, appears to be very promiscuously coupled, partnering with G_s to stimulate cAMP/PKA in most tissues but also interacting with $G_{q/11}$ to activate PLC and mobilisation of calcium in mast cells and cardiac fibroblasts (Cohen et al., 2010; Gao et al., 1999; Linden et al., 1999). (A_{2B}AR signalling is

discussed in more detail in sections 1.3.4 and 1.4). The A_3AR via $G_{i/o}$ coupling activates PLC and Ca^{2+} signalling through the Gβγ and activates K_{ATP} channel opening in the myocardium (Abbracchio et al., 1995; Englert et al., 2002; Tracey and Magee, 1998). The A_3AR has also been reported to increase IP_3 levels and activate PKC downstream of $G_{q/11}$ coupling in heterologous expression systems and human cancer cell lines (Kanno et al., 2012; Palmer et al., 1995a). In addition, all adenosine receptor subtypes have been demonstrated to activate mitogen-activated protein kinase (MAPK) pathways, including phosphorylation of extracellular signal-regulated protein kinases (ERK) via a variety of mechanisms (Schulte and Fredholm, 2000; 2003a). Though not traditionally associated binding partners of adenosine receptors, there is also evidence emerging of interactions with β-arrestin proteins, which adds another layer of complexity to adenosine receptor signalling (Jajoo et al., 2009; Klaasse et al., 2007; Mundell et al., 2000).

1.2 Signalling Paradigms in Adenosine Receptor Pharmacology

The ability of GPCRs to transduce external stimuli into intracellular activity has traditionally been explained by a two-state model of receptor activation, most commonly the ternary complex model. The classical model was first described by De Lean and colleagues (1980) and included the formation of a ternary complex consisting of the receptor, agonist and G protein (De Lean et al., 1980). Within this canonical model, receptor activation could only occur upon the formation of the ternary complex. Agonist binding enables the receptor to transition from an uncoupled inactive receptor state to an active receptor state coupled to the G protein (Park et al., 2008; Stallaert et al., 2011). While conceptually still helpful, further understanding of GPCR pharmacology over the past four decades has necessitated the evolution of traditional receptor signalling models in an attempt to explain complex receptor and ligand behaviour including constitutive activity, biased agonism and dimerization. These novel signalling paradigms and their influence on adenosine receptor signalling are discussed in detail below.

1.2.1 Constitutive Activity

The requirement for receptors to 'switch' from an inactive to active state allows for the inherent property of spontaneous isomerization in the absence of agonist activation (Ehlert et al., 2011; Lefkowitz et al., 1993). The ability of a GPCR to adopt an active conformation in the absence of agonist and induce ligand-independent signal transduction is termed constitutive activity. Evidence of GPCR constitutive activity was first identified for the δ-opioid receptor (Koski et al., 1982) and the β_2-adrenoceptor (Cerione et al., 1984) in the early 1980s and has since become a firmly established pharmacological phenomenon of GPCR signalling over the last three decades (Costa and Cotecchia, 2005; Parra and Bond, 2007). While recognised to be an oversimplification of a complex process, the two state model of receptor activation as described by Paul Leff (Leff, 1995) and the extended ternary complex model (Samama et al., 1993), still provide invaluable tools for understanding constitutive activity and in turn, inverse agonism

(Figure 1.4). Agonists are ligands that stabilise or selectively enrich the active state of the receptor. Neutral antagonists do not disrupt the equilibrium, but are able to block agonist-induced effects by binding with equal affinity for both receptor states (Milligan, 2003; Strange, 2002). In contrast, inverse agonists prefer the inactive state of the receptor over the activated state and will reduce the basal signal transduction of constitutively active systems. It has only been through the further understanding and detection of elevated basal activity that has enabled the reclassification of the great majority of clinically used antagonists as true inverse agonists (Bond and Ijzerman, 2006; Kenakin, 2004).

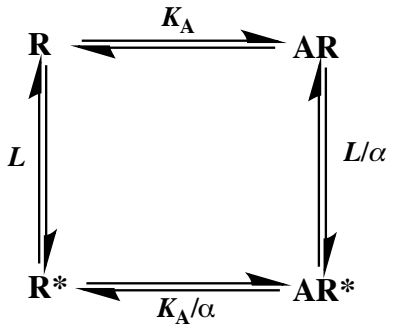


Figure 1.4. The classical two-state model of receptor activation. The receptor constitutively isomerises between the inactive state (R) and active state (R*). The equilibrium between these species is governed by its isomerization constant (L). Once the ligand (A) is bound, the receptor can exist in two states, occupied (AR) or occupied and activated (AR*), the latter being the species that couples to the G protein. An inverse agonist will preferentially bind the inactive receptor state (AR) whereas an agonist will have higher affinity for R*, pushing the equilibrium toward the active state (AR*). K_A and $K_{A/\alpha}$ are the equilibrium constants for agonist binding to the receptor conformations R and R* respectively; α denotes the efficacy of the ligand (A) (Leff, 1995; Samama et al., 1993).

To observe the constitutive or spontaneous state of signalling there is a requirement to overcome a detection threshold, with a critical number of receptors in the active form and there needs to exist a surmountable energy barrier for the transition between the inactive and active receptor conformations (Parra and Bond, 2007; Vilardaga et al., 2005). While practically all GPCRs can be engineered or modified to display some level of constitutive activity (Chalmers

and Behan, 2002), it was the discovery of disease-causing receptors with elevated basal activity that revealed a true therapeutic potential for inverse agonists (Seifert and Wenzel-Seifert, 2002). Constitutively active mutants and virally encoded GPCRs with grossly elevated basal activity, such as the pirated chemokine type 2 receptor that causes Kaposi's sarcoma in HIV-infected patients, provide an ideal platform for treating disease with the application of an inverse agonist (Milligan and IJzerman, 2000; Seifert and Wenzel-Seifert, 2002). Constitutive activity can also become a consideration in settings where there is receptor overexpression, for example in most heterologous systems (Milligan, 2003), or more importantly in pathological conditions such as tumour cells in which there is naturally occurring hypoxia-regulated overexpression and receptor upregulation (Dorsam and Gutkind, 2007).

1.2.1.1 Adenosine Receptor Constitutive Activity

Constitutive activity of adenosine receptors was first identified for the A_1AR. High density expression of the human A_1AR in Chinese Hamster Ovary (CHO) cells caused constitutive activation of G_i proteins and inhibition of adenylyl cyclase, which enabled the classification of 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) as an A_1AR inverse agonist (Shryock et al., 1998). It was also proposed that agonist-independent effects of the allosteric enhancer PD 81,723 were due to the potentiation of A_1AR constitutive activity (Kollias-Baker et al., 1997). These experiments were performed in the presence of sufficient concentrations of ADA to ensure the effects observed were not due to residual endogenous agonist. Mutagenesis studies identified substitution of a single glycine residue to a threonine at position 14 ($Gly^{14}Thr$) in helix I near the extracellular N-terminal domain resulted in a 'locked-on' constitutively active mutant of the A_1AR (de Ligt et al., 2005). The $Gly^{14}Thr$ mutant receptor was constitutively active in a [^{35}S]GTPγS binding assay with neither agonist nor inverse agonist capable of modulating basal activity, suggesting the receptor was physically trapped in its R*

conformation (de Ligt et al., 2005). While these studies demonstrate A_1AR constitutive activity is measurable in settings of heterologous overexpression or receptor mutation, evidence for physiologically relevant A_1AR constitutive activity has also been identified (He et al., 2013; Searl and Silinsky, 2012). Using osteoclasts derived from ecto-nucleotidase CD39 and CD73 knockout mice, it was demonstrated that rolofylline, a novel A_1AR inverse agonist, stimulated cAMP production and inhibited osteoclast formation by blocking basal A_1AR constitutive activity (He et al., 2013). In the central nervous system (CNS), A_1AR constitutive activity has also been suggested to control acetylcholine release at mouse motor nerve endings (Searl and Silinsky, 2012). In contrast, a study in the rat brain suggested that A_1ARs are not constitutively active, because the response evoked by the inverse agonist DPCPX could not be reversed in the presence of an A_1AR neutral antagonist N-0840 (Savinainen et al., 2003). The authors suggested tonic A_1AR-mediated G protein activity in brain membranes was due to an ADA-resistant adenosine pool (Savinainen et al., 2003), however failure to block DPCPX inverse agonism may also be due to the use of insufficient concentrations of the lower affinity antagonist N-0840 or that N-0840 may not behave as a true neutral antagonist.

The A_2ARs have also been demonstrated to display ligand-independent activity. The wild type A_{2A}AR can stimulate constitutive activation of cAMP accumulation in stably transfected HEK293 cells, an effect that is driven by the long carboxy terminus of the A_{2A}AR (Klinger et al., 2002). A_{2A}AR constitutive activity appears to be important in the regulation of neurotransmitter release and signalling in the CNS (Fernández-Dueñas et al., 2014; Ibrisimovic et al., 2012). In neuroblastoma SH-SY5Y cells, even in the absence of agonist, A_{2A}AR constitutive cAMP production enhanced noradrenaline release (Ibrisimovic et al., 2012). In addition, caffeine, acting as an inverse agonist at the A_{2A}AR, is thought to mediate its

antiparkinsonian effect by blocking the well-known A_{2A}AR-mediated tonic inhibition of dopamine D_2 receptor signalling in the striatum (Fernández-Dueñas et al., 2014).

To date, much of the work investigating A_{2B}AR constitutive activity has involved a mutagenesis approach. Mutational studies have identified constitutively active mutant A_{2B}ARs which, in a yeast growth assay, enabled the reclassification of commonly used adenosine receptor antagonists ZM241385, DPCPX and MRS1706 as inverse agonists at the A_{2B}AR (Beukers et al., 2004b; Li et al., 2006). Further yeast screening of constitutively active mutant receptors was able to identify important regions in extracellular loop 2 and transmembranes 4 and 5 responsible for A_{2B}AR activation and constitutive activity (Peeters et al., 2014). A role for A_{2B}AR constitutive activity has also been postulated in the regulation of inflammation, as immune cells from A_{2B}AR knock out mice have enhanced basal cytokine release and mast cell degranulation, even in the absence of extracellular adenosine (Haskó et al., 2009). This is of particular importance for the A_{2B}AR given that it was previously considered to remain silent in conditions of low adenosine concentration (Feoktistov and Biaggioni, 1997). Hence A_{2B}AR constitutive activity may expand the role of this receptor subtype in physiology and warrant the further investigation of its therapeutic potential throughout the body.

Confirming the presence of constitutive activity *in vivo* requires the removal of all endogenous agonist from the system, which in the case of adenosine receptors is exceedingly difficult due to the ubiquitous and continual production of extracellular adenosine. Nevertheless, current studies would suggest there is appreciable and physiologically relevant constitutive activity of the A_1, A_{2A} and A_{2B}ARs at least, and warrants the development of inverse agonists for conditions where blockade of adenosine receptor activity is beneficial.

1.2.2 Biased Signalling

Canonical signalling at GPCRs predicted that all agonists activate the receptor by stabilising a single active receptor conformation, resulting in uniform activation of the same subset of signalling pathways for that given receptor. Thus, according to classic receptor theory, agonist efficacy was simply based on the strength of the imparted signal, and as such, relative agonist potency ratios should be independent of the influence of stimulus-response coupling and receptor density (Kenakin, 2011; Kenakin and Christopoulos, 2013; Shonberg et al., 2014). Recent evidence from pharmacological, biophysical and biochemical experiments have demonstrated that structurally-distinct ligands occupying the same GPCR in the same cellular background can generate different functional outcomes in a manner that cannot be explained by simple differences in stimulus-response coupling (Luttrell et al., 2015; Shonberg et al., 2014). The ability of ligands to differentially influence receptor behaviour in a pathway-dependent manner is termed 'functional selectivity', 'signalling bias' or 'biased agonism' (Kenakin and Christopoulos, 2013). At a molecular level, biased agonism is a consequence of the fact that agonists stabilise different conformational active states of the receptor. This can lead to the engagement of an alternative subset of intracellular effectors, and in turn, the activation of differential signalling pathways (Hodavance et al., 2016; Shonberg et al., 2014). Much of the early work on GPCR bias examined G protein-dependent versus G protein-independent β-arrestin signalling (Reiter et al., 2012), however it is also recognised that ligand bias can be detected within G protein-dependent pathways (Baltos et al., 2016b).

The discovery that clinically efficacious drugs targeting the μ-opioid receptor (Sternini et al., 1996; Whistler and Zastrow, 1998) and β-adrenoceptors in particular (Reiter et al., 2012; Wisler et al., 2007), impart distinct physiological outcomes via unique biased signalling profiles, has revealed the novel opportunities for biased ligands in drug discovery (Bradley and Tobin,

2016). The ability of distinct GPCR-agonist complexes to differentially activate intracellular signals provides a new avenue for the development of drugs that are not only 'receptor subtype-selective', but also 'pathway-selective'. Biased agonism thus allows the opportunity to specifically design pathway-selective drugs that will separate on-target side effects from therapeutic effects mediated by the same receptor and is actively being pursued in drug discovery programs (Violin et al., 2014). While biased agonism offers great clinical potential, it also presents significant medicinal chemistry and pharmacological challenges. It necessitates the screening of ligands across multiple signalling endpoints. The selection of appropriate endpoints is complicated by the fact that the desirable signalling profile for most drug targets has not yet been established (Shonberg et al., 2014; Violin et al., 2014). In addition, biased agonism is highly dependent on the cellular background in which it is detected; which means a particular bias profile in a heterologous system does not guarantee the same signal bias profile will be observed in endogenous systems or indeed *in vivo*. The recognition that ligand bias is influenced by cellular context also gives rise to the idea of context-dependent bias; whereby conceivably the receptor bias can change with alterations in membrane composition, proteins and signalling partners, for example as consequence of disease progression. However bias fingerprinting does provide the opportunity to screen and identify compounds that display a distinct profile from the endogenous ligand and are therefore more likely to engender different pharmacological outcomes, providing a promising starting point with which to move lead compounds into more physiologically relevant *in vitro* and *in vivo* models (Bradley and Tobin, 2016; Khajehali et al., 2015; Luttrell et al., 2015).

1.2.2.1 Quantification of biased agonism

Methods that allow for the detection and accurate quantification of biased agonism are essential to inform structure-activity studies and subsequent drug candidate selection. A critical aspect

of the quantitative analysis of bias is the need to remove confounding factors of 'system bias', which reflects the differing coupling efficiencies of the receptor at a given pathway, and 'observational bias', which results from differing assay conditions and sensitivities. Once system and observational bias are excluded then the bias imposed by the ligand on the receptor can be revealed and reflects a molecular property of the ligand that can be exploited therapeutically (Herenbrink et al., 2016; Kenakin and Christopoulos, 2013; Kenakin et al., 2012; Rajagopal et al., 2011). Recent analytical advances that extend the classic operational model of agonism (Black and Leff, 1983) have been described to quantify ligand bias. The most robust and widely applicable method of these uses relative transduction ratios [$\Delta\Delta\log(\tau/K_A)$] (see Figure 1.5) (Kenakin et al., 2012; van der Westhuizen et al., 2014). Agonist concentration-response curves are fitted to the Black-Leff operational model using the equation as follows:

$$\text{response} = \frac{E_m[\text{A}]^n\tau^n}{[\text{A}]^n\tau^n + ([\text{A}] + K_\text{A})^n}$$

where E_m describes the maximal response of the system, [A] is the agonist concentration, n is the 'transducer slope' that links agonist concentration to measured response, τ is an index of the coupling efficacy of the agonist and K_A is the functional equilibrium dissociation constant. The ligand-specific parameters, K_A and τ can be derived as a composite parameter, termed the transduction coefficient $\log(\tau/K_\text{A})$ also known as the log$R$. The transduction coefficient is an overall measure of the relative 'power' of an agonist to induce a response and is used to quantify biased agonism (Kenakin et al., 2012). In order to eliminate the effects of system and observational bias, the $\log(\tau/K_\text{A})$ is first normalised to a reference agonist, yielding $\Delta\log(\tau/K_\text{A})$. These values for a given agonist can then be compared across two signalling pathways to obtain the relative transduction ratio, $\Delta\Delta\log(\tau/K_\text{A})$ which quantifies agonist bias. Taking the anti-

logarithm of $\Delta\Delta\log(\tau/K_A)$ generates the bias factor ($10^{\Delta\Delta\log(\tau/KA)}$) which can then be plotted on a 'web of bias' or equivalent to enable visualisation of ligand bias across multiple pathways (Kenakin et al., 2012; van der Westhuizen et al., 2014).

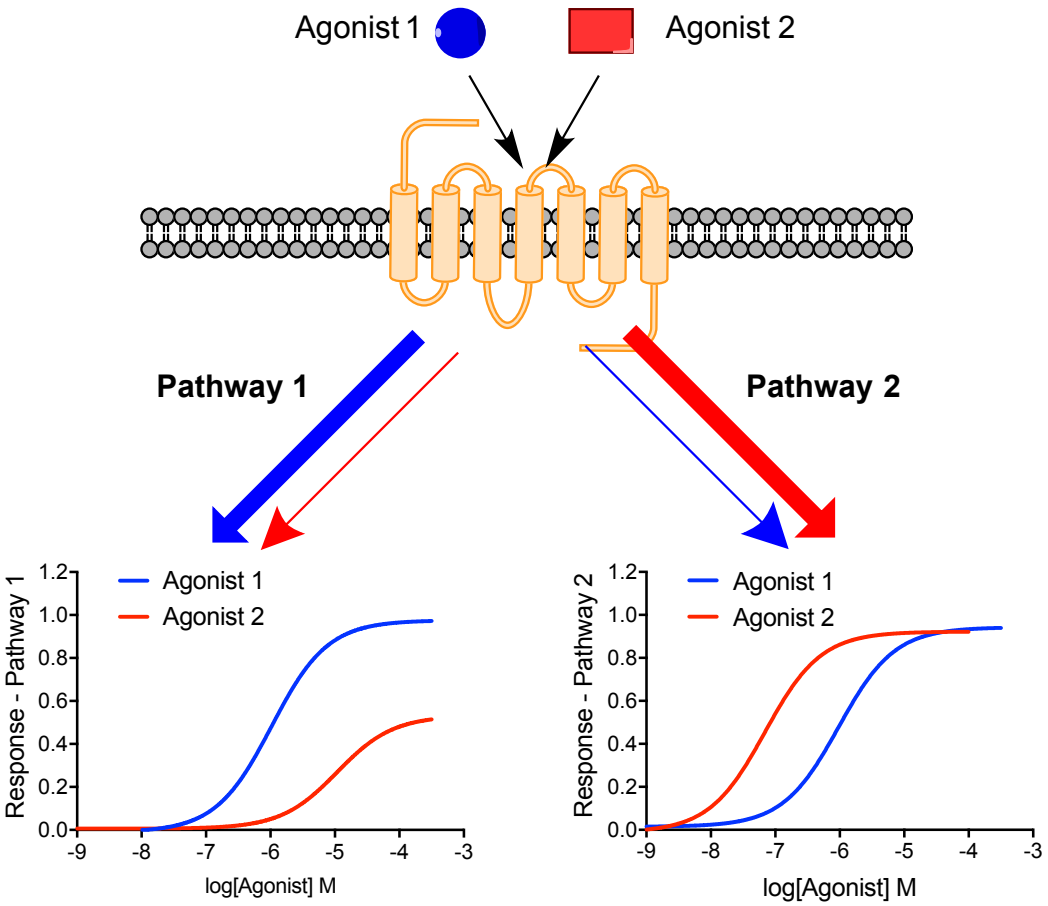


Figure 1.5. Schematic representation of biased agonism. Relative to agonist 2, agonist 1 produces a biased stimulus that favours stimulation of pathway 1 over pathway 2.

1.2.2.2 Biased agonism at adenosine receptors

Despite biased agonists being increasingly identified for a range of GPCRs (Kenakin and Christopoulos, 2013), relatively few studies have investigated the pharmacological phenomenon of signalling bias at the adenosine receptor family. An initial screen of over 800 compounds at the A_1AR identified only one ligand, LUF5589, that appeared to be functionally selective for the G protein-dependent pathway over β-arrestin-mediated signalling (Langemeijer et al., 2013). The authors concluded that biased ligands for the A_1AR are most

likely rare, if existing at all (Langemeijer et al., 2013). However, as recent work from our lab demonstrates, A_1AR bias may arise from differences *within* G protein-dependent pathways, with differential coupling to the various $G_{i/o}$ proteins in particular (Baltos et al., 2016b; Valant et al., 2014). This was highlighted by the rationally designed adenosine receptor agonist VCP746, which was shown to be significantly biased away from Ca^{2+} mobilisation compared to other G protein-dependent pathways; a profile postulated to underlie its novel cytoprotective actions in the heart in the absence of traditional A_1AR haemodynamic side effects (Baltos et al., 2016b; Valant et al., 2014). Similarly, capadenoson, an adenosine receptor agonist that has previously entered clinical trials for angina and atrial fibrillation (Bayer; Tendera et al., 2012), was also shown to be an A_1AR biased agonist within G protein-dependent pathways (Baltos et al., 2016b). This highlights that the ability to quantify any observed signal bias is highly dependent on the choice of pathways utilised to detect biased agonism. Which in the case of the A_1AR (and if not all GPCRs) may be lost if only examining G protein versus arrestin pathways (Baltos et al., 2016b). Another way in which stimulus bias may be engendered is through the use of small molecules, known as allosteric modulators, that bind to the receptor in a topographically distinct binding site from the orthosteric ligand (Shonberg et al., 2014). Allosteric modulators promote a conformational change in GPCR structure and as such have the capacity to stimulate biased agonism, either by themselves or by modulating the actions of the orthosteric ligand in a pathway-biased manner (May et al., 2007b; Shonberg et al., 2014). It was through the investigation for potential adenosine receptor allosteric modulators that within a series of 2-amino-3-benzoylthiophene derivatives, novel compounds that promoted pathway-biased allosteric modulation at the A_1AR were identified (Aurelio et al., 2009; Valant et al., 2012).

Biased agonism has also been reported at the other adenosine receptor subtypes. Inosine, the stable metabolite of adenosine was identified as a functional agonist of the A_{2A}AR with a bias for ERK1/2 phosphorylation over cAMP production when compared to the parent compound (Welihinda et al., 2016). A recent study characterising the structure-efficacy relationship of a diverse range of A_{2B}AR agonists identified BAY60-6583 as a biased A_{2B}AR agonist with a unique signalling profile, even appearing to act as an A_{2B}AR antagonist in low expressing MIN-6 mouse pancreatic β cells (Gao et al., 2014). Studies at the A_3AR have detected functional selectivity both within G protein-dependent pathways (Baltos et al., 2016a) and also with respect to β-arrestin translocation (Gao and Jacobson, 2008). Collectively these findings demonstrate that ligand bias can indeed be detected at each of the adenosine receptor subtypes. It is hoped that the further understanding of functional selectivity and the identification of novel ligands that selectively stimulate therapeutically beneficial pathways will offer exciting opportunities for targeting adenosine receptors in pathophysiology.

1.2.3 Influence of Dimerization on Adenosine Receptor Signalling

The traditional model for GPCR structure depicted them as monomeric units, singly interacting with their corresponding heterotrimeric G protein. However, over the last two decades this canonical thinking has changed, with much of the evidence suggesting GPCRs form dimers or higher order oligomers as part of their normal trafficking and receptor function (Angers et al., 2002; Dalrymple et al., 2008; Ferré et al., 2014; Prinster, 2005). Homodimerization, the self-association of the same receptor subunits and heterodimerization or oligomerization, the association of two or more different receptor subunits are phenomenon now well accepted in the GPCR field. While family C GPCRs are known to function as obligate dimers (Kniazeff et al., 2011), there is also a growing body of evidence to support the presence of family A GPCR oligomerization which can engender altered receptor pharmacology including changes to

receptor signalling, trafficking, desensitization and subcellular localization (Jordan and Devi, 1999; Milligan, 2007). Indeed, it has been proposed that oligomerization diversifies the number of receptor entities possible from the limited number of GPCR genes, adds to their pharmacological complexity and represents novel opportunities for drug discovery (Angers et al., 2002; Milligan, 2006; Park and Palczewski, 2005). Physiologically relevant adenosine receptor oligomeric interactions have been identified by evidence gathered largely within the central nervous system. Assembly of adenosine receptors into heteromers, with either closely related GPCRs or structurally distinct receptors, are proposed as a probable mechanism underlying functional cooperativity observed in the brain and also more recently in the heart (Chandrasekera et al., 2013; Franco et al., 2000; Prinster, 2005).

1.2.3.1 Adenosine Receptor Homomers

The ability of adenosine receptors to self-associate into homomers was first described for the A_1AR. The possibility of A_1AR dimers in the brain cortex was suggested some 20 years ago after antibody immunoprecipitation and immunoblotting revealed higher order bands that appeared to correspond to A_1AR homomers (Ciruela et al., 1995). More recent studies have confirmed the existence of A_1AR homomers at the plasma membrane using high powered biophysical techniques including bimolecular fluorescence complementation (BiFC) and fluorescence correlation spectroscopy (FCS) (Briddon et al., 2008). The presence of A_1AR homomers in the native environment may be used to explain the biphasic concentration-dependent effects of caffeine, an adenosine receptor antagonist, on locomotor activity and demonstrates the physiological relevance of A_1AR self-association (Gracia et al., 2013). The assembly of A_{2A}ARs into homomeric complexes has been predominantly studied through the use of recombinant receptors. Studies using resonance energy transfer (RET) between a donor and acceptor molecules in close proximity, including bioluminescence resonance energy

transfer, BRET; (where the bioluminescent catalytic activity of an enzyme acts as the donor) and fluorescence resonance energy transfer, FRET; (where both donor and acceptor moieties are fluorescently labelled) have demonstrated that A_{2A}ARs not only form dimers at the cell surface (Canals et al., 2004), they additionally associate into oligomers with three or more A_{2A}AR protomers (Gandía et al., 2008; Vidi et al., 2008b). Homomers of the A_3AR have only recently been identified with high powered microscopy techniques in combination with fluorescent ligand binding dissociation kinetics, demonstrating allosteric interactions across an A_3AR dimer in CHO cells (Corriden et al., 2014; May et al., 2011). In contrast to the A_1AR, despite evidence of A_{2A}AR and A_3AR homodimerization in heterologous expression systems, a physiological consequence of these homomeric pairs is yet to be fully elucidated. To date, direct evidence of A_{2B}AR homomers has not been investigated.

1.2.3.2 Adenosine Receptor Heteromers

Like many other rhodopsin-like family A GPCRs, there is growing recognition of adenosine receptor heterodimeric interactions with other receptors, in particular with members of the dopamine family (Franco et al., 2008; Fredholm et al., 2011; Milligan, 2006). Heterodimerization of adenosine receptors was first suggested as the basis of the negative functional cross talk displayed between A_{2A}AR and dopamine D_2 receptors in the striatum on locomotor activity, with implications in the treatment of Parkinson's Disease (Azdad et al., 2008; Ferré et al., 2004). This represents the most widely studied and accepted adenosine receptor heteromer to date. Debate remains over whether the A_{2A}AR-D_2 heteromer is constitutively formed or ligand-induced but appears to be reliant on the C-terminal of the A_{2A}AR for its structure (Canals et al., 2003; Vidi et al., 2008a). In addition, the A_{2A}AR-D_2 dimer has been reported to participate in higher order oligomeric complexes, interacting with both the cannabinoid CB_1 receptor and the metabotropic glutamate $mGlu_5$ receptor as

determined by sequential BRET-FRET techniques (Cabello et al., 2009; Carriba et al., 2008). The specificity of the adenosine-dopamine receptor interactions is highlighted by the ability of the A_1AR to form a functional dimer with the dopamine D_1 receptor, but not the dopamine D_2 receptor in co-transfected mouse fibroblasts (Ginés et al., 2000). Heteromeric interactions within the adenosine receptor family have also been identified. Heteromers of the A_1AR and A_{2A}AR have been detected in recombinant cells and human brain tissue with resonance energy and radioligand binding techniques and have also been implicated in the presynaptic control of glutamatergic neurotransmission (Casadó et al., 2010; Ciruela et al., 2006a). The A_{2A}AR has additionally been proposed to complex with the A_{2B}AR, providing the dominant forward transport signal for efficient cell surface expression of the A_{2B}AR, the importance of which has been demonstrated in splenocytes from A_{2A}AR knock out mice (Moriyama and Sitkovsky, 2010).

While the involvement of adenosine receptor heteromers in the exquisite control of neurotransmitter signalling in the brain is well studied, the role of such complexes in other systems including the heart is only just being realised. Functional interactions of the A_1AR with the δ and κ opioid receptors have been assessed by coimmunoprecipitation and are hypothesised to be involved in cardioprotection by remote ischaemic preconditioning (Surendra et al., 2013). Similarly A_1AR dimers with β_1 and β_2 adrenergic receptors demonstrate novel heteromers with altered ligand binding affinity and ERK1/2 phosphorylation (Chandrasekera et al., 2013). In addition, though while not directly detected, dimers of the A_1AR and A_2AR subtypes are proposed as explanation for the functional synergy observed in cardioprotection against ischaemia-reperfusion injury (Urmaliya et al., 2009; 2010b; Zhan et al., 2011).

1.2.3.3 Dimerization or Receptor Cross-Talk?

It must be acknowledged that evidence of receptor interactions occurring at downstream signalling pathways does not confirm the presence of direct interactions at a receptor level, nor does evidence of direct receptor association in recombinant cells constitute proof of physiological relevance. According to an International Union of Basic and Clinical Pharmacology committee, for an oligomeric interaction to be considered physiologically significant it must have evidence of physical association in native tissue or primary cells, demonstrate specific pharmacological properties unique to the dimer that is altered in the absence of one of the subunits, preferably validated with the use of knock out animals or RNA interference technology (Pin et al., 2007). Despite not all of the aforementioned examples of adenosine receptor heteromers fulfilling the criteria for true oligomeric classification, it is anticipated the increasing recognition of the importance of these unique receptor complexes to physiology and pathophysiology will provide novel opportunities for adenosine receptor drug discovery (Ferré et al., 2004; Franco et al., 2008; Milligan, 2006).

1.3 Adenosine A_{2B} Receptor

The A_{2B}AR is a GPCR of 332 amino acid length with a molecular weight of approximately 36 kDa (Stehle et al., 1992). At the time of its discovery, the A_{2B}AR was distinguished from the A_{2A}AR based on its ability to stimulate cAMP production at higher (>10 μM) adenosine concentrations (Daly et al., 1983). The A_{2B}AR was subsequently classified as a low-affinity receptor due to its modest-to-negligible affinity for adenosine and prototypical agonists and as such was initially presumed to be a low-affinity version of the A_{2A}AR (due to its sequence homology; see Figure 1.6), with lesser physiological relevance (Beukers et al., 2000; Feoktistov and Biaggioni, 1997; Fredholm et al., 2001b). It wasn't until the development of subtype-selective ligands (in particular antagonists) that the A_{2B}AR was demonstrated to couple to different intracellular signalling pathways and to exert distinct physiological effects from the A_{2A}AR (Beukers et al., 2006; Fredholm et al., 2001a; Ji et al., 2001; Linden et al., 1999; Yang et al., 2006). Today, the A_{2B}AR is increasingly being recognised as an important target in numerous pathologies, including ischaemia-reperfusion injury (Eltzschig et al., 2013; Zimmerman et al., 2013), fibrosis (Dubey et al., 1998), inflammation (Ham and Rees, 2008) and cancer (Cekic et al., 2011; Ma et al., 2010; Wei et al., 2013).

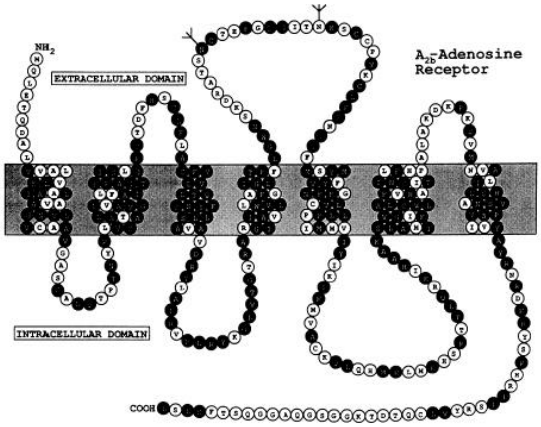


Figure 1.6. Snake diagram of the first cloned rat A_{2B}AR identifies strong sequence homology with the rat A_{2A}AR. Amino acids that are identical between the two receptors are shaded dark. Figure taken from Rivkees and Reppert 1992 (Rivkees and Reppert, 1992).

1.3.1 Tissue Distribution of Adenosine A_{2B} Receptors

Efforts to elucidate exact A_{2B}AR tissue expression were for a long time hampered by the lack of selective and useful radioligands and as such, much of the early work on A_{2B}AR distribution relied on data from the expression of the corresponding mRNA (Feoktistov and Biaggioni, 1997). The A_{2B}AR was first cloned from the rat hypothalamus and human brain in 1992 (Pierce et al., 1992; Rivkees and Reppert, 1992) and was demonstrated to be present in the caecum, large intestine and bladder. Lower levels were also demonstrated in the brain, spinal cord, lung, vas deferens and pituitary (Stehle et al., 1992). Later it was shown to have a fairly ubiquitous distribution with A_{2B}AR mRNA detected at various levels, though generally low abundance, in all rat tissues studied (Dixon et al., 1996). The recent development of the A_{2B}AR-knockout/reporter gene-knock-in mouse provided a valuable tool that enabled the determination of A_{2B}AR tissue distribution *in vivo* (Yang et al., 2006). The A_{2B}AR was demonstrated to be extensively distributed throughout the vasculature, smooth muscle, heart, lung, aorta, brain, kidney and large intestine (Sun and Huang, 2016; Yang et al., 2006). Furthermore, many studies have shown the A_{2B}AR is widely expressed in many diverse cell types including mast cells, neutrophils, macrophages, lymphocytes, neurons, glial cells, epithelial cells, myocardial cells and fibroblasts (Reviewed in: Feoktistov and Biaggioni, 1997; Sun and Huang, 2016). The functional expression of the A_{2B}AR in such a wide array of tissues and cell types, which can be further regulated by a diverse range of environmental cues (discussed in section 1.3.2), highlights the physiological and pathological importance of this receptor subtype.

1.3.2 Regulation of Adenosine A_{2B} Receptor Expression

A_{2B}AR expression is influenced by changes in the cellular microenvironment and is induced in response to inflammation, cell stress, ischaemia-reperfusion injury and hypoxia (Fredholm, 2007; Ham and Rees, 2008; Haskó et al., 2009). Hypoxia directly increases the expression of

the A_{2B}AR via a canonical hypoxia-inducible factor 1α (HIF-1α) binding site in the promoter (Kong et al., 2006). There is also a positive feedback mechanism whereby the A_{2B}AR increases HIF-1α through stabilisation of the circadian rhythm protein period 2 (Per2) (Eckle et al., 2012). Factors present in an inflammatory environment such as the pro-inflammatory cytokines tumour necrosis factor (TNF)-α (Kolachala et al., 2005), interferon (IFN)-γ (Xaus et al., 1999), interleukin (IL)-1β (Khoa et al., 2003), and free radicals such as Nox4 (St Hilaire et al., 2008) also upregulate A_{2B}AR expression at the mRNA and protein level. In addition, the endogenous agonist adenosine has been demonstrated to recruit A_{2B}AR to the plasma membrane on intestinal epithelial cells (Sitaraman, 2002; Wang, 2004) and may represent a more general mechanism of regulating A_{2B}AR expression at the cell-surface.

1.3.3 Adenosine A_{2B} Receptor Binding Partners

Besides heterotrimeric G proteins, the A_{2B}AR interacts with a number of proteins and binding partners that appear to have important roles in modulating receptor function (Sun and Huang, 2016). Adenosine deaminase (ADA), in addition to degrading extracellular adenosine, anchors to the cell's surface and even in the absence of enzymatic activity increases the binding affinity of the A_{2B}AR for a non-selective adenosine receptor agonist NECA (Herrera et al., 2001). ADA was later shown to co-localise with the A_{2B}AR on dendritic cells and this co-localisation markedly increased the production of pro-inflammatory cytokines (Pacheco et al., 2005). Similarly, intracellular protein kinase C increases the sensitivity of the A_{2B}AR and potentiates agonist activation of the pro-survival kinases, PI3K and ERK which contributes to ischaemic preconditioning in the heart (Kuno et al., 2007). Co-expression with the A_{2A}AR, possibly through heterodimeric interactions improves the cell-surface expression of the A_{2B}AR by providing the dominant forward-transport signal for export from the endoplasmic reticulum (Moriyama and Sitkovsky, 2010). Other proteins that have been implicated in the translocation

of the A_{2B}AR to the plasma membrane include the scaffold-based regulatory proteins E3KARP and ezrin and the trafficking soluble NEM-sensitive protein (SNAP)-23 (Sitaraman, 2002; Wang, 2004). These may help to transport and stabilise the receptor in a signalling complex at the plasma membrane, possibly through interaction with a PDZ-binding motif on the C-terminal end of the A_{2B}AR (Sitaraman, 2002). The PDZ-binding domain of the A_{2B}AR also interacts with the cystic fibrosis transmembrane conductance regulator (CFTR), leading to enhanced A_{2B}AR expression and agonist-mediated cAMP production which results in enhanced signalling of the innate lung defence system (Watson et al., 2011; 2016). In addition, the C-terminus of the A_{2B}AR is involved in complexing with the actin-filament-crosslinking protein, α-actinin-1, which stabilises the receptor's global and cell-surface expression (Sun et al., 2016). Like many GPCRs, the A_{2B}AR can also interact with arrestin proteins which promote agonist-induced desensitisation and internalisation to uncouple G protein-mediated signalling (Matharu et al., 2001; Mundell et al., 2000). From changes in receptor trafficking and stabilisation, signalling pathways and desensitisation, it is clear these binding partners and protein complexes may alter the pharmacology of the A_{2B}AR and may also contribute to explaining the sometimes paradoxical effects observed downstream of the A_{2B}AR (Sun and Huang, 2016).

1.3.4 Signal Transduction Pathways of Adenosine A_{2B} Receptors

Originally classified by its stimulation of cAMP/PKA pathways in the brain, the A_{2B}AR has been shown to activate adenylyl cyclase via interaction with G_s proteins in virtually every cell in which it is expressed (Feoktistov and Biaggioni, 1997). It has now been appreciated that a number of other intracellular signalling pathways are functionally coupled to the A_{2B}AR (Figure 1.7). The A_{2B}AR has been proposed to interact with G_q proteins to activate PLC leading to increased PKC activation and elevations of IP_3 and intracellular calcium (Linden et al., 1999). Nonetheless, such A_{2B}AR-mediated G_q protein recruitment remains to be evaluated using

techniques such as fluorescence or bioluminescence resonance energy transfer, which could directly demonstrate $A_{2B}AR$ coupling to G_q proteins (in addition to G_s proteins). Intracellular Ca^{2+} can also be mobilised via direct activation of calcium channels through a cholera toxin-sensitive mechanism likely downstream of Gβγ (Feoktistov et al., 1994) or indirectly via activation of PKA (Mogul et al., 1993). The $A_{2B}AR$ also stimulates ERK, c-Jun N-terminal Kinase (JNK) and p38 MAP kinase signalling (Feoktistov et al., 1999; Gao et al., 1999; Schulte and Fredholm, 2003b), however there is ongoing debate whether this is mediated through G_s- or G_q-coupling to the receptor (Aherne et al., 2011). Preferential G protein-coupling and intracellular signalling appears to be largely cell type dependent with G_q coupling particularly important for $A_{2B}AR$-mediated actions on immune cells including human mast cells (HMC-1) (Feoktistov and Biaggioni, 1995; Ryzhov et al., 2006) and also cardiac fibroblasts (Feng et al., 2009). The plasticity of $A_{2B}AR$ signalling and the relative role of intracellular signalling pathways in particular tissues and specific disease pathologies is explored in more detail in section 1.4.

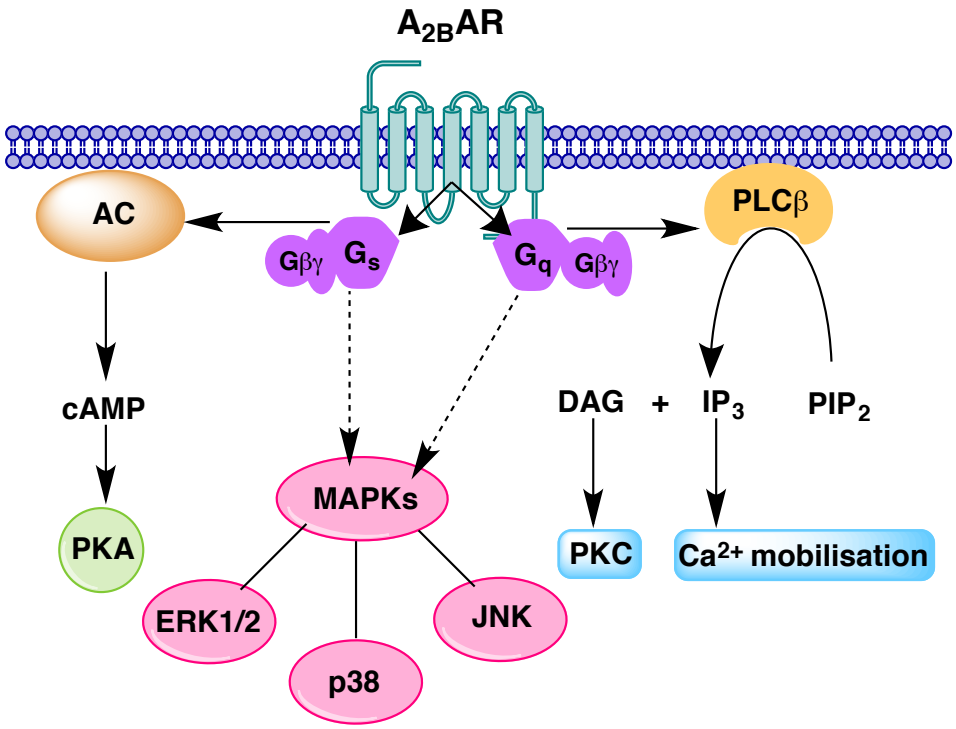


Figure 1.7. General signalling pathways mediated by the $A_{2B}AR$.

1.3.5 Adenosine A_{2B} Receptor Ligands

The pharmacological characterisation of the A_{2B}AR had languished behind the other adenosine receptor subtypes due to the paucity of potent and selective A_{2B}AR ligands (Fredholm et al., 2001a). However, an improved understanding of the A_{2B}AR in recent years has followed with the development of new pharmacological tools, in particular high affinity antagonists.

1.3.4.1 Adenosine A_{2B} Receptor Antagonists

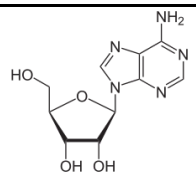
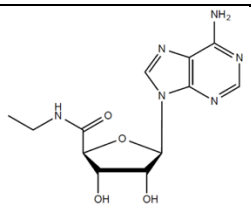
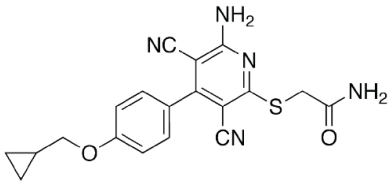
A_{2B}AR antagonists can be broadly divided into two classes, the xanthines and non-xanthine derivatives. Xanthines are based on the structure of the classic non-selective adenosine receptor antagonists caffeine (Table 1), theophylline and many of the current high affinity A_{2B}AR antagonists possess this same core structure (Baraldi et al., 2006; Kalla and Zablocki, 2009). One of the first discovered derivatives was DPCPX; (Table 1) which showed good affinity at the A_{2B}AR but had higher affinity at the A_1AR (Ortore and Martinelli, 2010). Lengthening of the alkyl substituents in the 1,3-positions or 1,8-disubstitution generated the first high-affinity A_{2B}AR-selective antagonists MRS1754 (Kim et al., 2000) and PSB-1115 (Hayallah et al., 2002) (Table 1). Further modification generated PSB-603 (Table 1) which shows high affinity and selectivity not only in humans but also in rodents. It is commonly used as a pharmacological tool to study the A_{2B}AR due to its sub-nanomolar affinity (Borrmann et al., 2009). Modification of xanthines at the 8-position with certain aryl groups has given rise to high-affinity A_{2B}AR antagonists that have progressed as preclinical candidates. For example CVT-6883 (also known as GS-6201; Table 1) has been investigated for the treatment of asthma (Elzein et al., 2008). Non-xanthine derivatives include ZM241385 (which was initially characterised as an A_{2A}AR-selective antagonist) and OSIP 339391(Table 1), both of which have been used in a tritiated form as radiolabeled antagonists to investigate the A_{2B}AR *in vitro* (Ji and Jacobson, 1999; Stewart et al., 2004).

1.3.4.2 Adenosine A_{2B} Receptor Agonists

The goal of attaining selectivity for A_{2B}AR agonists has been even more challenging than for antagonists, however progress has been made in recent years (Feoktistov and Biaggioni, 2011). Agonists can be classified as adenosine-like or non-adenosine ligands based on the presence or absence of a nucleoside-like core respectively (Baraldi et al., 2009). Adenosine modifications focusing on the N^6 and C^2 positions of the purine heterocycle have generated agonists with increased potency for the A_{2B}AR and include the non-selective N^6-modified adenosine derivative, 5'-N-ethylcarboxamidoadenosine (NECA; Table 1) (de Zwart et al., 1998). Despite being non-selective across all adenosine receptor subtypes, NECA is still widely used as one of the most potent A_{2B}AR agonists (EC_{50} ≈150 nM) (Baraldi et al., 2009). Further modifications at the N^6 position yielded a novel series of NECA derivatives with higher potency but again low A_{2B}AR-selectivity (Baraldi et al., 2007). In pursuit of enhanced subtype-selectivity, drug discovery efforts shifted to the development of non-nucleoside agonists. A patent describing 2-aminopyridine-3,5-dicarbonitrile derivatives as adenosine receptor ligands identified 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio) acetamide (BAY60-6583; Table 1) as an A_{2B}AR agonist with potency in the low nanomolar range and high selectivity versus the other adenosine receptors (Rosentreter et al., 2001). BAY60-6583 has since been widely used in animal studies for the pharmacological characterisation of the role of the A_{2B}AR, however it has recently been identified to behave as a partial agonist in some assay systems (Hinz et al., 2014). A further series of 2-amino-4-phenyl-6-phenylsulfanylpyridine-3,5-dicarbonitriles identified agonists with enhanced A_{2B}AR, one of which behaved as a partial agonist (LUF5845) and another as a potent full agonist with an EC_{50} of 10 nM (LUF5835) (Beukers et al., 2004a). None of the compounds were as selective for the A_{2B}AR as BAY 60-6583, however one derivative LUF5834 was proposed to be of particular interest thanks to its high potency at the A_{2B}AR (EC_{50}=12 nM) and significant selectivity versus the A_3AR making

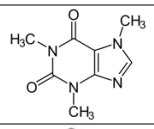
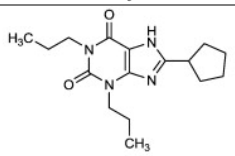
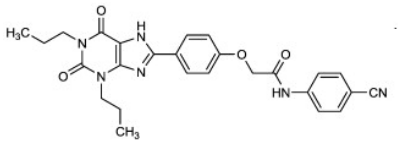
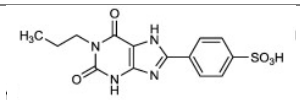
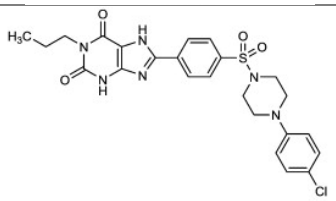
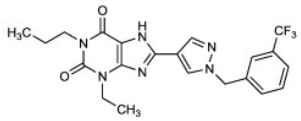
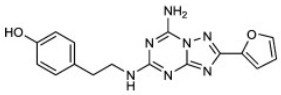
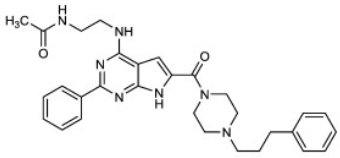
it a useful tool to distinguish the contributions of these two receptor subtypes in mast cells (Baraldi et al., 2009; Beukers et al., 2004a).

Table 1.1. Structure and binding affinity of commonly used agonists for the $hA_{2B}AR$.

Name	**Chemical Structure**	**$hA_{2B}AR$ Affinity pK_i (K_i nM)**	**References**
Adenosine		4.62 (24,000)	(Yan et al., 2003)
NECA		6.48 (330)	(Linden et al., 1999)
BAY 60-6583		6.67 (212)	(Hinz et al., 2014)

pK_i denotes the negative logarithm of the ligand equilibrium dissociation constant

Table 1.2. Structure and binding affinity of commonly used antagonists for the $hA_{2B}AR$.

Name	Chemical Structure	$hA_{2B}AR$ Affinity pK_i (K_i nM)	References
Caffeine		4.69 - 4.98 (10,400-20,500)	(Bertarelli et al., 2006; Kim et al., 2002)
DPCPX		7.19 - 7.29 (51-64)	(Kim et al., 2002; Weyler et al., 2006)
MRS1754		8.84 (1.45)	(Ji et al., 2001)
PSB-1115		7.28 (53)	(Hayallah et al., 2002)
PSB-603		9.26 (0.55)	(Borrmann et al., 2009)
CVT-6883 (GS-6201)		7.66 (22)	(Elzein et al., 2008)
ZM241385		6.84 (145)	(Ji et al., 2001)
OSIP 339391		9.39 (0.41)	(Stewart et al., 2004)

pK_i denotes the negative logarithm of the ligand equilibrium dissociation constant

1.4 Role of Adenosine A_{2B} Receptors in Pathophysiology

The A_{2B}AR is a low affinity receptor that until recently, was regarded as having minor physiological importance (Feoktistov and Biaggioni, 1997). However as illustrated below, the A_{2B}AR is upregulated and activated in numerous pathological conditions. It provides protection against ischaemia-reperfusion injury, promotes cancer growth and metastasis, modulates the fibrotic response in a variety of organs and tissues and is an important regulator of the immune and inflammatory response. This section will review the literature for the role of the A_{2B}AR and outline some of the controversies that still remain regarding A_{2B}AR signalling in pathophysiology.

1.4.1 Ischaemia-Reperfusion Injury

During an ischaemic event, an imbalance between energy supply and demand, usually from the disruption or reduction in blood flow, results in tissues being deprived of oxygen and nutrients. This results in a series of abrupt biochemical and metabolic changes. These changes are characterised by reduced aerobic glycolysis and energy production leading to mitochondrial membrane depolarization, ATP depletion, accumulation of metabolites and ions, Ca^{2+} overload and decreased intracellular pH, all of which contribute to cellular death (Frank et al., 2012; Hausenloy and Yellon, 2013). Therapy is aimed at restoring blood flow to the ischaemic area, however this unfortunately can elicit further tissue damage, collectively termed ischaemia-reperfusion injury (IRI) (Frank et al., 2012; Pantazi et al., 2016). During reperfusion there is further increases in intracellular Ca^{2+} and the opening of the mitochondrial permeability transition pore (mPTP), the production of reactive oxygen species (ROS), activation of the inflammatory cascade and intensified cell damage through necrosis and apoptosis. These events result in added organ injury, which in the case of the heart can account for up to 50% of the final infarct size after a myocardial infarction (MI) (Hausenloy, 2013; Mozaffarian et al., 2016;

Pantazi et al., 2016; Quintana et al., 2004). Extracellular adenosine concentrations rapidly rise with ischaemic insult and this functions as an endogenous distress signal (Headrick and Willis, 1988; Headrick et al., 2003). Unsurprisingly, adenosine represents one of the most powerful and well-studied cytoprotective agents, particularly in the area of cardioprotection. Adenosine's action, through A_{2B}AR activation, is increasingly being implicated as an important modulator against IRI (Eltzschig et al., 2009; Sommerschild and Kirkebøen, 2000).

1.4.1.1 A_{2B}AR in Myocardial Ischaemia-Reperfusion Injury

Stimulation of the A_1AR prior to ischaemia, termed ischaemic preconditioning, has long been acknowledged to protect the myocardium from IRI in a variety of animal models (Ashton et al., 2003; Regan et al., 2003; Reichelt et al., 2009; Yang et al., 2002). It has provided an attractive therapeutic target for myocardial IRI that has been pursued in human clinical trials without fruition, in part due to the dose-limiting haemodynamic effects on heart rate and blood pressure (Kloner et al., 2006; Kopecky et al., 2003; Ross et al., 2005). In addition, the potential requirement for A_1AR activation prior to the onset of ischaemia for optimal reduction in infarct size (Thornton et al., 1992), has led to the investigation of other adenosine receptor subtypes for their efficacy in limiting the reperfusion phase of injury. While selective A_{2A}AR activation prior to the ischaemic event does not appear to be beneficial (Lasley et al., 2006; Thornton et al., 1992), its cardioprotective role when administered at the onset of reperfusion has been well established (Jordan et al., 1997; McIntosh and Lasley, 2012; Methner et al., 2010). This has been primarily attributed to its anti-inflammatory and immune cell effects (McIntosh and Lasley, 2012; Yang, 2005). More recently, the A_{2B}AR has also been identified as a novel modulator of myocardial cell survival by enhancing ischaemic tolerance (Chen et al., 2013; Eltzschig et al., 2013). The cardioprotective effects of the A_{2B}AR were first demonstrated after the A_{2B}AR-selective antagonist MRS1754 was shown to block the infarct-sparing action of the

non-selective adenosine receptor agonist NECA in rabbit hearts (Philipp et al., 2006). The A_{2B}AR-selective agonist, BAY60-6583 was subsequently shown to reduce myocardial infarct size in isolated rabbit and rat hearts and intact mice when administered prior to, or at the onset of reperfusion (Grube et al., 2011; Kuno et al., 2007; Methner et al., 2010; Xi et al., 2009). These effects were not observed in the hearts of A_{2B}AR knock out mice (Eckle et al., 2007). Most often, this A_{2B}AR-mediated reduction in infarct size was associated with concomitant stimulation of the A_{2A}AR (Lasley et al., 2006; Methner et al., 2010; Xi et al., 2009). A requirement for cooperative activation of adenosine receptors has also been observed for A_1AR-mediated cardioprotection (Urmaliya et al., 2009; 2010b; Zhan et al., 2011), suggesting both A_1 and A_2AR subtypes are needed for the full effects of adenosine in protecting the heart against IRI. This may be a result of the requirement of receptor co-stimulation for pathway convergence and subsequent signal amplification (Xi et al., 2009), or alternatively heteromeric interactions between AR subtypes that allosterically modulate ligand binding and/or signal transduction (discussed in detail in Section 1.2.3) (Headrick et al., 2013; Moriyama and Sitkovsky, 2010).

A_{2B}AR-mediated cardioprotection has been suggested to involve a novel mechanism for improving oxygen-efficient metabolism through the stabilisation of the circadian rhythm protein period 2 (Per2) which mediates a metabolic switch that enhances myocardial glycolytic capacity, thereby providing enhanced ischaemic tolerance (Eckle et al., 2012). In addition to direct effects on the myocardium, possibly via sensitization by PKC (Kuno et al., 2007), A_{2B}AR reduction of IRI in the heart has been demonstrated to also involve modulation of the inflammatory response. *In vivo* studies using selective A_{2B}AR deletion on immune cells (Seo et al., 2015) or transplantation of wild-type bone marrow into A_{2B}AR knock out mice (Koeppen et al., 2012), demonstrated the A_{2B}AR dampens myocardial IRI via signalling on inflammatory cells. This appears to involve promotion of anti-inflammatory macrophage differentiation

downstream of PI3K/Akt activation (Tian et al., 2015) and inhibition of superoxide production, interestingly by a pertussis toxin sensitive $G_{i/o}$/ERK/PI3K pathway (Yang et al., 2011). The A_{2B}AR therefore represents an important modulator of adenosine-mediated cardioprotection against IRI. This is achieved via complex signalling on the myocardium and immune cells that possibly involves interplay with proteins such as Per2 and other adenosine receptor subtypes.

1.4.1.2 A_{2B}AR in Ischaemia-Reperfusion Injury of Other Organs

The A_{2B}AR has also been implicated as an important modulator of ischaemia-reperfusion injury in other organs and tissues, though these effects have not been as widely studied as in the heart. Acute gastrointestinal IRI caused by surgery, organ transplantation, sepsis or haemorrhagic shock frequently results in bowel necrosis and is associated with a high mortality (Eltzschig et al., 2009). A protective role of the A_{2B}AR was initially suggested after mucosal scrapings following murine gastrointestinal IRI showed selective induction of A_{2B}AR expression (Hart et al., 2009). Pharmacological inhibition or targeted deletion of the A_{2B}AR enhanced intestinal inflammation and injury during ischaemia-reperfusion, whereas activation of the A_{2B}AR with the selective agonist BAY60-6583 was protective (Hart et al., 2009). The A_{2B}AR was further demonstrated to dampen hypoxia-induced inflammation via a HIF-1α-dependent mechanism (Hart et al., 2011) and to modulate intestinal barrier function (Yang et al., 2014) which may be contributing to a protective role of the A_{2B}AR in gastrointestinal IRI. Similarly in the liver, signalling through hepatocellular-specific A_{2B}ARs attenuated nuclear factor NF-κB activation thereby dampening liver IRI (Zimmerman et al., 2013). In acute kidney injury, the A_{2B}AR affords protection via inhibition of neutrophil-dependent TNF-α release (Grenz et al., 2012) and preservation of post-ischaemic renal perfusion via endothelial cell A_{2B}ARs (Weinberg and Venkatachalam, 2012). In contrast, evidence in the lung suggests A_{2B}AR blockade significantly improves lung function and attenuates pro-inflammatory cytokine production following

ischaemia-reperfusion, pointing at a detrimental effect of A_{2B}AR activation in lung IRI (Anvari et al., 2010; Huerter et al., 2016). Collectively, these studies suggest a more widespread mechanism for A_{2B}AR-mediated protection from IRI throughout the body, except perhaps in the lung, though the exact signalling mechanisms still need to be elucidated.

1.4.2 Fibrosis

Fibrosis, characterised by the accumulation of extracellular matrix molecules that make up scar tissue, is a common feature of chronic tissue injury. Fibrosis underlies the disease pathology of many diseases including heart failure, chronic obstructive pulmonary disease (COPD) and chronic kidney disease, which collectively represent a large disease burden with a huge unmet clinical need (Friedman et al., 2013). Extracellular nucleotides and nucleosides have recently been implicated as important mediators of fibroblast homeostasis and as such purinergic signalling has been investigated for its role in fibrosis. Interestingly, both pro- and anti-fibrotic actions have been attributed to adenosine receptor activation, which highlights both the complexity and ensuing challenges faced when targeting adenosine receptors for the treatment of fibrosis (Chan and Cronstein, 2009; Cronstein, 2011; Karmouty-Quintana et al., 2013). This section will focus on cardiac fibrosis, for which the preponderance of evidence has implicated the A_{2B}AR (Epperson et al., 2009; Headrick et al., 2013; Novitskaya et al., 2016) and briefly discuss the role of A_{2B}AR signalling in augmenting or attenuating fibrosis in the lungs and kidney.

Note: Sections 1.4.2.1 – 1.4.2.3 are taken and amended from Vecchio et al. 2017, included in full in Appendix 1.

1.4.2.1 Cardiac Fibrosis

Cardiac fibroblasts form the largest population of interstitial cells in the adult mammalian heart (Chen and Frangogiannis, 2013). They have an essential role in the regulation of the extracellular matrix (ECM), which is crucial for maintaining the structural integrity of the myocardium and for electro-mechanical signal transduction (Camelliti et al., 2004; Souders et al., 2009). Cardiac fibroblasts are regulated by various mechanical and hormonal stimuli, in particular growth factors such as angiotensin II (ANGII) and the cytokine transforming growth factor β1 (TGF-β1). ANGII and TGF-β1 can activate fibroblast cell-surface receptors to promote differentiation to myofibroblasts, the pro-fibrogenic phenotype that express the contractile protein, α-smooth muscle actin (α-SMA) and exhibit enhanced secretory, migratory and proliferative properties (Leask, 2007; Lu and Insel, 2014; Petrov et al., 2002; Porter and Turner, 2009; Schnee and Hsueh, 2000). Following a MI, fibroblasts promote essential matrix deposition for proper tissue repair and scar formation to ensure structural integrity of the infarct zone. However, aberrant ECM deposition and excessive myofibroblast accumulation extending beyond the area of the original insult are responsible for maladaptive fibrosis leading to cardiac dysfunction, a hallmark feature of heart failure pathophysiology (Ferrari et al., 2016; See et al., 2005; Segura et al., 2012). Heart failure remains a major cause of mortality and morbidity in the western world with an estimated 50% 5 year survival rate after diagnosis (Mozaffarian et al., 2016). This highlights both the limitations of current therapeutic management and the crucial need for new and innovative therapies for the treatment and prevention of heart failure. Adenosine signalling via the A_{2B}AR represents one such novel pharmacological approach, however as outlined below, the precise role and timing of receptor activation remains controversial.

1.4.2.2 A_{2B}AR-mediated anti-fibrotic signal transduction in the heart

Studies in isolated rat cardiac fibroblasts first proposed the A_{2B}AR as the subtype responsible for mediating adenosine's inhibitory actions on foetal calf serum-stimulated fibroblast proliferation (Dubey et al., 1997) and collagen and protein synthesis (Dubey et al., 1998). The role of the A_{2B}AR in adenosine-mediated anti-fibrotic signal transduction was later confirmed via genetic abrogation of A_{2B}AR expression which resulted in increased cell proliferation and basal collagen synthesis in cardiac fibroblasts (Dubey et al., 2001b). Similarly, A_{2B}AR overexpression had the opposite effect, significantly decreasing collagen and protein synthesis (Chen et al., 2004). The second messenger cAMP, has been shown to have a central role in inhibiting fibroblast and myofibroblast activity (Lu et al., 2013; Swaney et al., 2005). Accordingly, A_{2B}AR-mediated cAMP accumulation stimulated in fibroblasts by the non-selective adenosine receptor agonist NECA (Epperson et al., 2009) can reduce ANGII-stimulated collagen synthesis. This occurs via an exchange protein directly activated by cAMP (Epac) and phosphoinositol-3 kinase (PI3K) dependent pathway (Figure 1.8) (Villarreal et al., 2009). In addition to effects on collagen synthesis, A_{2B}AR stimulation has been shown to decrease mRNA expression of pro-fibrotic gene markers including collagen I and connective tissue growth factor (CTGF) (Vecchio et al., 2016a). Of specific importance to adenosine receptors, a positive feedback loop has been identified whereby β-adrenoceptor-stimulated cAMP can be secreted by fibroblasts or cardiac myocytes and metabolised in the extracellular space to adenosine to activate A_2ARs. Thus exerting further inhibitory effects on fibroblast growth and function (Dubey et al., 2001a; Sassi et al., 2014).

Commensurate with the *in vitro* findings, an *in vivo* study in rats demonstrated chronic administration of the stable adenosine analogue, 2-chloroadenosine (CADO) or the adenosine uptake inhibitor, dipyridamole, initiated one week after permanent ligation of the left anterior

descending (LAD) coronary artery, protected against cardiac remodelling and reduced markers of fibrosis such as collagen volume fraction and matrix metalloproteinase gene expression (Wakeno et al., 2006). The effects of CADO on fibrotic and haemodynamic parameters were abolished in the presence of the selective A_{2B}AR antagonist MRS1754, but not selective antagonists for the other adenosine receptor subtypes (Wakeno et al., 2006). Together, these studies suggest a salutary effect of A_{2B}AR activation on cardiac fibrosis, an effect which may be lost upon A_{2B}AR downregulation as observed in hearts taken from human patients with chronic heart failure (Asakura et al., 2007).

1.4.2.3 A_{2B}AR-mediated pro-fibrotic signal transduction in the heart

While the majority of *in vitro* studies have identified an anti-fibrotic role for the A_{2B}AR, recent studies have demonstrated A_{2B}AR blockade appears to be beneficial within *in vivo* models of cardiac remodelling and fibrosis. In an *in vivo* mouse model of myocardial infarction involving permanent coronary artery ligation, chronic administration of a novel, highly selective A_{2B}AR antagonist, GS-6201, significantly reduced cardiac enlargement and dysfunction compared to vehicle-treated mice (Toldo et al., 2012). Similarly in an *in vivo* rat myocardial ischaemia-reperfusion model, GS-6201 improved ejection fraction and decreased fibrosis in the non-infarct and border zones. The greatest effect was observed when GS-6201 was given 1 week rather 1 day after MI (Zhang et al., 2014). A pro-fibrotic role for the A_{2B}AR has been supported by a study in A_{2B}AR knock-out ($A_{2B}AR^{-/-}$) mice that demonstrated the A_{2B}AR contributes to post-infarction heart failure (Maas et al., 2008). $A_{2B}AR^{-/-}$ mice had improved end diastolic pressure and reduced interstitial fibrosis when compared to wild type mice 8 weeks after permanent left coronary ligation. Systolic blood pressure and infarct size remained the same between knock-out and wild type animals, suggesting the A_{2B}AR contributes to heart failure pathology via post-infarction remodelling and reactive fibrosis rather than acute

cardioprotection (Maas et al., 2008). The mechanism underlying the pro-fibrotic activity of the A_{2B}AR may involve the pro-inflammatory effects mediated by this adenosine receptor subtype. Blockade of the A_{2B}AR inhibits caspase-1 activity and leukocyte infiltrate (Toldo et al., 2012), and attenuates secretion of pro-fibrotic and pro-inflammatory mediators such as TGF-β1, TNF-α and IL-6, likely via a G_q/PKC-δ pathway post MI (Feng et al., 2009; Toldo et al., 2012; Zhang et al., 2014). A pro-inflammatory role of the A_{2B}AR is reported by studies in other organ systems. In particular the lung where elevated adenosine concentrations and A_{2B}AR activity promotes chronic fibrosis and inflammation in asthma and chronic obstructive pulmonary disease (Chan and Cronstein, 2009; Karmouty-Quintana et al., 2013; Sun, 2006; Zhou et al., 2009). Given the inflammatory response is intricately linked to the regulation of tissue fibrosis, it is perhaps unsurprising therefore, that the A_{2B}AR has been implicated as a promoter of cardiac fibrosis *in vivo* (Kong et al., 2013; Stuart et al., 2016).

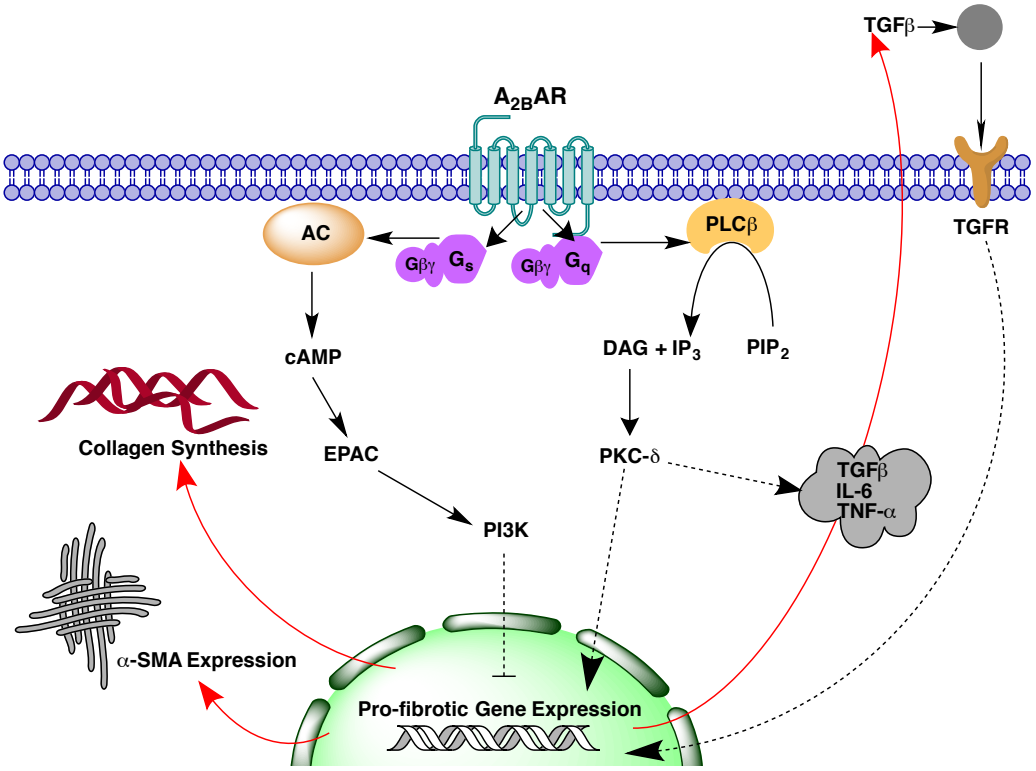


Figure 1.8. Proposed A_{2B}AR-mediated signalling pathways implicated in the regulation of cardiac fibrosis.

1.4.2.4 A_{2B}AR modulation of fibrosis in other organs and tissues

Opposing roles of A_{2B}AR activation has also been implicated in the fibrosis of other organ systems (Karmouty-Quintana et al., 2013). In the lung, A_{2B}AR stimulation is protective in acute-bleomycin-induced lung injury but actually promotes fibrosis in chronic models of lung disease (Zhou et al., 2009; 2011). Genetic ablation of the A_{2B}AR in myeloid cells protects mouse lung from chronic bleomycin exposure (Karmouty-Quintana et al., 2014), which suggests a pro-inflammatory role of the A_{2B}AR in the progression of pulmonary fibrosis. This is further supported by studies demonstrating the A_{2B}AR activates macrophages and stimulates IL-6 release from bronchial smooth muscle cells and lung fibroblasts (Zhong et al., 2004; 2005). Similarly in the kidney, A_{2B}AR activation is beneficial in attenuating acute kidney injury (Grenz et al., 2012) but prolonged A_{2B}AR signalling increases interstitial fibrosis and collagen deposition in renal tissue (Roberts et al., 2014b; 2014a).

Therefore, the central question that remains is how the same receptor subtype can have both pro- and anti-fibrotic activity in the same tissue. The opposing effects may reflect differences in underlying disease pathology due to the type and duration of insult; whereby A_{2B}AR activation appears to be largely anti-fibrotic in acute ischaemic events but potentially pro-fibrotic under conditions of chronic stress. The exact mechanism behind these paradoxical effects requires further elucidation, but may reflect changes in differential receptor coupling with changes in cellular background as the disease progresses. Certainly, this idea is readily foreseeable for the A_{2B}AR with its high degree of plasticity and ability to couple to multiple G proteins and intracellular signalling cascades (Cohen et al., 2010). In addition, it should be noted a great deal of our understanding of the A_{2B}AR's role in fibrosis, particularly in the heart, has come from *in vitro* studies. This may not reflect the true course of disease progression *in vivo* due to the exclusion of the inflammatory response and loss of organ complexity, including

cross-talk with other cell types. Therefore, while A_{2B}AR signalling appears to be a promising target in fibrosis, further studies are needed to fully appreciate the potential of A_{2B}AR therapeutics in chronic fibrotic diseases of the heart, lung and kidneys.

1.4.3 Cancer

A growing body of evidence suggests the A_{2B}AR may provide a novel therapeutic target for the treatment of cancer, for which the A_{2B}AR appears to play an important pathological role. The A_{2B}AR is upregulated by HIF-1α (Kong et al., 2006), so is often highly expressed in the cells and tissues from the hypoxic microenvironment of many solid tumours including colon carcinomas (Ma et al., 2010), prostate cancer (Mousavi et al., 2015; Wei et al., 2013), oral squamous cell carcinomas (Kasama et al., 2015), lung adenocarcinoma (Li et al., 2005; Ryzhov et al., 2008) and breast cancer (Mittal et al., 2016). The overexpression of the A_{2B}AR in different cancers relative to normal tissue, combined with the increase in adenosine concentrations in the tumour microenvironment, suggests a role in disease progression and highlights the therapeutic potential of A_{2B}AR antagonists as adjuvants in cancer therapy (Sepúlveda et al., 2016).

1.4.3.1 A_{2B}AR and Cancer Cell Proliferation and Tumour Growth

One of the key roles of the A_{2B}AR in cancer pathophysiology is the promotion of cancer cell proliferation and tumour growth. Activation of the A_{2B}AR with the subtype-selective agonist BAY 60-6583 increased tumour growth in a mouse model of melanoma (Iannone et al., 2013). Conversely application of an A_{2B}AR-selective antagonist decreased proliferation of prostate cancer cell lines (Vecchio et al., 2016b; Wei et al., 2013) and colon cancer cells (Ma et al., 2010) and reduced tumour volume of both bladder cancer (Cekic et al., 2011) and melanoma in mice (Iannone et al., 2013). These results could be recapitulated with genetic knockdown or

knockout of the A_{2B}AR (Cekic et al., 2011; Kasama et al., 2015; Ryzhov et al., 2008), confirming the role of this receptor subtype in cancer cell proliferation and growth. This likely involves G_s-mediated cAMP activation of PKA and ERK by Epac as these pathways are known to be involved in A_{2B}AR-mediated proliferation of non-cancerous endothelial cells (Figure 1.9) (Fang and Olah, 2007; Grant et al., 2001). In addition, increases in cAMP have been demonstrated to promote proliferation of various cells, including progression of prostate cancer (Flacke et al., 2013; Merkle and Hoffmann, 2011), which would support the role of A_{2B}AR-G_s signalling in mediating cancer cell growth in the general sense.

1.4.3.2 A_{2B}AR and Metastasis

The A_{2B}AR also modulates the ability of cancer cells to metastasise and migrate which contributes to disease progression (Sepúlveda et al., 2016; Sun and Huang, 2016). The A_{2B}AR is higher expressed in metastatic versus non-metastatic derived colorectal cancer cell lines (Futschik et al., 2002). It has also been shown to promote tumour-cell chemotaxis *in vitro* and lung metastasis *in vivo* in models of breast cancer and melanoma resulting in poorer prognosis (Cekic et al., 2011; Mittal et al., 2016; Stagg et al., 2010). Recent data also shows that experimental and spontaneous lung metastasis could be suppressed by the use of an A_{2B}AR-selective antagonist or genetic knockdown with shRNA (Desmet et al., 2013; Mittal et al., 2016). The enhanced metastasis may involve A_{2B}AR-increased gene expression of a key metastatic transcription factor, Fos-related antigen-1 (Fra-1) (Desmet et al., 2013), and the suppression of Rap1 protein activity which has been associated with reduced cell adhesion and increased tumour cell migration through a PKA-dependent mechanism (Ntantie et al., 2013). Hence the A_{2B}AR appears to enhance tumour cell metastasis by promoting migration and induction of an invasive, metastatic phenotype.

1.4.3.3 A_{2B}AR and Angiogenesis

Tumour growth is also enhanced by the A_{2B}AR via promotion of angiogenesis. The A_{2B}AR induces vascular endothelial growth factor (VEGF) (Feoktistov, 2002; Ryzhov et al., 2008; 2013) and interleukin 8 (IL-8) production in human endothelial and cancer cells (Merighi et al., 2007; 2009), which are essential for tumour angiogenesis. Stimulation of adenosine receptors has been shown to increase VEGF production five-fold in tumour-associated $CD45^+$ immune cells, an effect that is not observed in $CD45^+$ cells from A_{2B}AR knockout mice (Ryzhov et al., 2008). Unlike the effects on cancer cell proliferation, the induction of pro-angiogenic factors via the A_{2B}AR appear to be largely mediated through PLC downstream of G_q-coupling (Feoktistov, 2002). VEGF in particular appears to be stimulated by a mechanism involving the transcription factor JunB downstream of A_{2B}AR-mediated PLC-Rap1-MEK activation (Figure 1.9) (Ryzhov et al., 2013).

In addition to direct effects on metastasis, proliferation and angiogenesis, the A_{2B}AR can have an indirect role on cancer progression via modulation of the immune system, which will be discussed in Section 1.4.4. Together, this highlights the importance of the A_{2B}AR in cancer pathophysiology and reveals the therapeutic potential of A_{2B}AR antagonists in the treatment of cancer, in particular solid tumours.

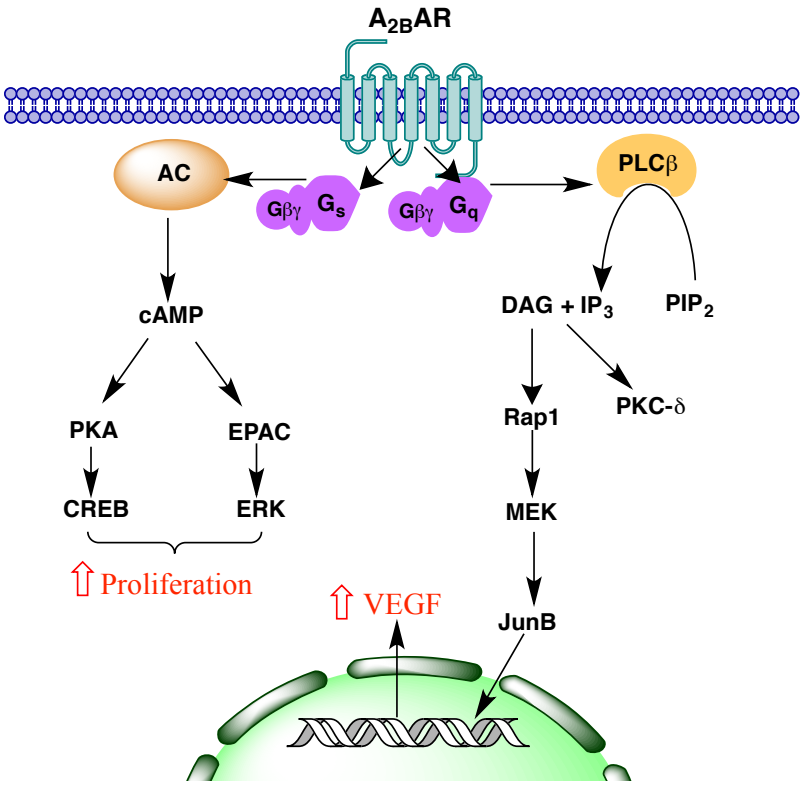


Figure 1.9. Involvement of $A_{2B}AR$ signalling in regulating cancer cell growth and angiogenesis. $A_{2B}AR$-mediated proliferation and vascular endothelial growth factor (VEGF) production are proposed to be downstream of G_s- adenylyl cyclase (AC) activity and G_q- phospholipase C (PLC) respectively.

1.4.4 Inflammation & the Immune Response

The $A_{2B}AR$ is increasingly recognised as an important mediator of the immune response and the inflammatory cascade, which contributes to the pathophysiology of the above discussed ischaemia-reperfusion injury, fibrosis, cancer and other disease states such as asthma, inflammatory bowel disease and diabetes (Haskó et al., 2009; Sun and Huang, 2016). The $A_{2B}AR$ is expressed on various cells of haematopoietic origin including macrophages, mast cells, lymphocytes, dendritic cells and neutrophils (Feoktistov and Biaggioni, 2011; Gessi, 2005; Mirabet et al., 1999; Novitskiy et al., 2008). As outlined previously, adenosine levels and $A_{2B}AR$ expression are markedly upregulated during hypoxia and cell injury (see section 1.3.2). Multiple mediators of the inflammatory response have also been demonstrated to increase $A_{2B}AR$ expression in numerous immune cell types, including bacterial product

lipopolysaccharide (LPS), pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ and the prostaglandin E2 (PGE2) (Aherne et al., 2011; Haskó et al., 2009). The expression and upregulation on immune cells therefore suggests the A_{2B}AR as an important therapeutic target in treating immune system dysfunction and inflammation.

1.4.4.1 A_{2B}AR Anti-Inflammatory Signalling

The A_{2B}AR was suggested to dampen the immune response after an A_{2B}AR knockout mouse model showed increases in vasculature leukocyte adhesion and the augmentation of pro-inflammatory cytokines, TNF-α and IL-6, accompanied by decreases in the plasma level of the anti-inflammatory IL-10, compared to wild-type mice (Eisenstein et al., 2015; Yang et al., 2006). An anti-inflammatory role for the A_{2B}AR was further demonstrated after stimulation of the A_{2B}AR was shown to increase the production of IL-10 from LPS-activated macrophages (Németh et al., 2005) and inhibit superoxide generation by neutrophils (van der Hoeven et al., 2011). In addition, studies have also shown that the A_{2B}AR limits leukocyte vascular permeability and neutrophil infiltration (Eckle et al., 2008; Haskó et al., 2009) and stimulates alternative macrophage activation (Csóka et al., 2012). This promotes an anti-inflammatory phenotype that protects against tissue injury and promotes tissue restitution (Haskó et al., 2009). The loss of A_{2B}AR on non-haematopoietic cells has also been shown to increase pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and NF-κB activation, suggesting the A_{2B}AR had a role in preventing sepsis-induced mortality (Csóka et al., 2010). Likewise, the immunosuppressive actions of adenosine, acting via the A_{2B}AR, provide a protective role in a model of type I diabetes mellitus (Németh et al., 2007). However, these same A_{2B}AR-mediated anti-inflammatory effects may help tumour cells evade natural defences and promote tumour growth (Sepúlveda et al., 2016). For example, in addition to the variety of immunomodulatory actions discussed above, A_{2B}AR activation on dendritic cells induces expression of pro-

angiogenic and immunosuppressant factors such as VEGF which promotes tumour growth and vascularisation (Novitskiy et al., 2008).

1.4.4.2 A_{2B}AR Pro-Inflammatory Signalling

In addition to anti-inflammatory effects, a pro-inflammatory role of the A_{2B}AR has also been demonstrated. A_{2B}AR activation leads to increased IL-6 cytokine release by bronchial smooth muscle cells (Zhong et al., 2004), primary murine alveolar macrophages (Pedroza et al., 2011) and lung fibroblasts (Zhong et al., 2005). In mast cells, primarily through coupling to G_q, stimulation of the A_{2B}AR induces degranulation and release of pro-inflammatory cytokines IL-4, IL-8 and IL-13, which in turn promotes immunoglobulin E (IgE) synthesis by B lymphocytes (Auchampach et al., 1997; Feoktistov et al., 1999; Ryzhov et al., 2006). These actions contribute to chronic inflammatory lung pathology and rationalise the use of A_{2B}AR antagonists in the treatment of asthma and chronic obstructive pulmonary disease (Wilson et al., 2009). Similarly, in intestinal epithelial cells, the A_{2B}AR increases IL-6 and keratinocyte-derived chemokine concentrations and contributes to murine colitis, suggesting A_{2B}AR blockade may be an effective strategy to treat inflammatory bowel disease (IBD) (Kolachala et al., 2008; Sitaraman et al., 2001).

These studies again highlight the complexity of A_{2B}AR signalling. Like the contrasting effects on fibrosis, A_{2B}AR modulation of the inflammatory response may be highly dependent on the cell type and tissue in which it is expressed and the duration and nature of receptor activation. Acute A_{2B}AR activation appears to dampen the immune response and provides protection against hypoxia and ischaemia-reperfusion injury, but this is contrasted by a pro-inflammatory role in more chronic models of disease, such as asthma or IBD (Eisenstein et al., 2015; Feoktistov and Biaggioni, 2011). Differences in A_{2B}AR immunomodulation may also arise

from the models used to detect physiological outcomes, with genetic knockout of the receptor not always predicting the response to pharmacological inactivation, possibly due to compensatory developmental processes (Eisenstein et al., 2015). So while the A_{2B}AR appears to represent an attractive target in treating immune system dysfunction and inflammation, a better understanding of the role of the A_{2B}AR in inflammatory responses in various tissues and at different time points of disease progression is required for effective translation into novel therapies.

1.5 Scope of Thesis

Despite widening appreciation of the contribution of the A_{2B}AR to numerous pathological conditions throughout the body, understanding of A_{2B}AR pharmacology still lags behind the other adenosine receptor subtypes. This has in part been due to the paucity of subtype selective ligands, in particular high affinity agonists and the previously presumed minor physiological importance due to low receptor occupancy at physiological concentrations of the endogenous agonist adenosine. However, with important roles in ischaemia-reperfusion injury, fibrosis, inflammation and cancer now firmly established, the focus of this thesis was to investigate new paradigms of A_{2B}AR biology including constitutive activity, dimerization and biased agonism. In each case the role of the unique behaviour was assessed in the context of pathophysiology, with an overarching aim to understand the complex pharmacology of this receptor in order to provide novel therapeutic insights for targeting the A_{2B}AR.

In **Chapter 2**, elevated basal cAMP production prompted the investigation and characterisation of constitutive activity of the human wild type A_{2B}AR in both heterologous and endogenously expressing cell lines. The discovery that A_{2B}AR inverse agonists were able to reduce baseline cAMP and basal cell growth of prostate cancer cells indicated that the receptor can signal even in the absence of agonist activation and demonstrated a possible role for A_{2B}AR constitutive activity in prostate cancer cell proliferation.

Moving into the context of cardiovascular disease, **Chapter 3** investigated the possible pharmacological explanation behind the requirement for activation of multiple adenosine receptor subtypes for full adenosine-mediated cardioprotection against ischaemia-reperfusion injury. Experiments in primary rat neonatal cardiomyocytes revealed functional interactions between the A_1AR and A_{2B}AR at the level of ERK1/2 phosphorylation. In addition, we

demonstrated alterations of A_1AR dissociation kinetics with blockade of the A_{2B}AR in cardiomyocyte membranes which suggested possible heteromeric A_1AR-A_{2B}AR interactions of in primary cells. Further investigation in model systems using dual receptor transfected CHO cells were unable to provide evidence of direct functional interactions, however this lack of effect may reflect the complex and possibly dynamic nature of adenosine receptor heteromeric interactions.

Chapter 4 and **Chapter 5** identified and characterised two novel agonists at the A_{2B}AR, capadenoson and VCP746, which had both previously been classified as A_1AR agonists. **Chapter 4** illustrated capadenoson displayed a unique bias signalling profile and strongly stimulated A_{2B}AR cAMP accumulation in primary cardiac myocytes and fibroblasts. In **Chapter 5** we showed that VCP746, through a novel bivalent mode of action, was a relatively high affinity, high efficacy A_{2B}AR agonist and displayed potent anti-fibrotic activity in cardiac fibroblasts. In **Chapter 6,** VCP746 was used to further interrogate the role of A_{2B}AR signalling in normal and diseased (+TGF-β1) cardiac fibroblasts in an effort to understand how both pro- and anti-fibrotic signalling could be attributed to this adenosine receptor subtype. In addition to uncovering a novel signalling pathway involving protein kinase G (PKG) cross-talk with G_q-mediated IP_3 accumulation in primary fibroblasts, it was identified that VCP746 displayed context-dependent bias, maintaining activity even when A_{2B}AR signalling was diminished under diseased (+TGF-β1) fibroblast conditions.

Chapter 2:

Ligand-Independent Adenosine A_{2B} Receptor Constitutive Activity as a Promoter of Prostate Cancer Cell Proliferation

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Journal of Pharmacology and Experimental Therapeutics **2016**, 357(1): 36-44

Supplemental material to this article can be found at:
http://jpet.aspetjournals.org/content/suppl/2016/01/20/jpet.115.230003.DC1

1521-0103/357/1/36–44$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
http://dx.doi.org/10.1124/jpet.115.230003
J Pharmacol Exp Ther 357:36–44, April 2016

The Journal of PHARMACOLOGY And Experimental Therapeutics

Ligand-Independent Adenosine A_{2B} Receptor Constitutive Activity as a Promoter of Prostate Cancer Cell Proliferation[S]

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Received October 13, 2015; accepted January 19, 2016

ABSTRACT

Aberrant ligand-independent G protein–coupled receptor constitutive activity has been implicated in the pathophysiology of a number of cancers. The adenosine A_{2B} receptor ($A_{2B}AR$) is dynamically upregulated under pathologic conditions associated with a hypoxic microenvironment, including solid tumors. This, in turn, may amplify ligand-independent $A_{2B}AR$ signal transduction. The contribution of $A_{2B}AR$ constitutive activity to disease progression is currently unknown yet of fundamental importance, as the preferred therapeutic modality for drugs designed to reduce $A_{2B}AR$ constitutive activity would be inverse agonism as opposed to neutral antagonism. The current study investigated $A_{2B}AR$ constitutive activity in a heterologous expression system and a native 22Rv1 human prostate cancer cell line exposed to hypoxic conditions (2% O_2). The $A_{2B}AR$ inverse agonists, ZM241385 [4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo [2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol] or PSB-603 (8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl)-1-propylxanthine), mediated a concentration-dependent decrease in baseline cAMP levels in both cellular systems. Proliferation of multiple prostate cancer cell lines was also attenuated in the presence of PSB-603. Importantly, both the decrease in baseline cAMP accumulation and the reduction of proliferation were not influenced by the addition of adenosine deaminase, demonstrating that these effects are not dependent on stimulation of $A_{2B}ARs$ by the endogenous agonist adenosine. Our study is the first to reveal that wild-type human $A_{2B}ARs$ have high constitutive activity in both model and native cells. Furthermore, our findings demonstrate that this ligand-independent $A_{2B}AR$ constitutive activity is sufficient to promote prostate cancer cell proliferation in vitro. More broadly, $A_{2B}AR$ constitutive activity may have wider, currently unappreciated implications in pathologic conditions associated with a hypoxic microenvironment.

Introduction

Ligand-independent activation of G protein–coupled receptors (GPCRs), known as constitutive activity, is an established biologic phenomenon that results from the spontaneous isomerization of receptors from inactive to active states (Lefkowitz et al., 1993; Parra and Bond, 2007). The two-state model of receptor activation (Leff, 1995), which condenses the vast array of possible receptor conformations into either an active (R*) or inactive (R) form, provides the simplest conceptual framework for understanding constitutive activity and, in turn, inverse agonism. Inverse agonists selectively stabilize the R state of the receptor and thus inhibit both ligand-dependent and ligand-independent signal transduction. By contrast, agonists preferentially stabilize the R* state of the receptor, whereas neutral antagonists have equal affinity for both receptor states and only inhibit ligand-dependent effects (Strange, 2002; Milligan, 2003). Over the past three decades, the experimental capability to detect constitutive activity has enabled the reclassification of many clinically used antagonists as inverse agonists (Bond and Ijzerman, 2006). Although many GPCRs can be engineered or overexpressed to display some level of constitutive activity (Chalmers and Behan, 2002), the realization that mutant GPCRs with aberrant intrinsic activity can have a critical role in disease progression

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia [1084487] and the Australian Research Council [DE130100117]. L.T.M. is an Australian Research Council (ARC) Discovery Early Career Researcher Award (DECRA) Postdoctoral Research Fellow [DE130100117], K.J.G. is an NHMRC CJ Martin Fellow [1013709], and A.C. is an NHMRC Principal Research Fellow [1041875]. E.A.V. holds an Australian Postgraduate Award and an Australian Cancer Therapeutics scholarship.
dx.doi.org/10.1124/jpet.115.230003.
[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: $A_{2B}AR$, adenosine A_{2B} receptor; ADA, adenosine deaminase; ANOVA, analysis of variance; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; Emax, maximal effect; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FlpInCHO, Chinese hamster ovary cells with FlpIn vector; GPCR, G protein–coupled receptor; [^{3}H]DPCPX, cyclopentyl-1, 3-dipropylxanthine, 8-[dipropyl-2, 3-^{3}H(N)]; HIF-1α, hypoxia inducible factor 1-α; HTRF, homogeneous time-resolved fluorescence; IP_1, inositol monophosphate; MTT, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NECA, 5′-*N*-ethylcarboxamidoadenosine; pEC_{50}, potency; pERK1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; PI, propidium iodide; PSB-603, 8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl)-1-propylxanthine; R, inactive receptor conformation; R*, active receptor conformation; RT-PCR, reverse-transcription polymerase chain reaction; SCH 442416, 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

revealed the therapeutic potential for inverse agonists (Seifert and Wenzel-Seifert, 2002). Indeed, the degree of potential inverse agonism should be considered in the rational design and screening of drug candidates for disease states in which constitutive activity is known to be important (Milligan and IJzerman, 2000; Bond and IJzerman, 2006). As constitutive activity is linked to the proportion of receptors in the active receptor state (Parra and Bond, 2007), previously unidentified constitutive activity may also become evident in settings where receptor overexpression occurs as a direct consequence of disease pathology. This is particularly relevant for cancerous cells within solid tumors, where the hypoxic microenvironment promotes the upregulation of many receptors and proteins under the influence of hypoxia inducible factor 1-α (HIF-1α) control (Semenza, 2000; Subarsky and Hill, 2003).

The adenosine A_{2B} receptor ($A_{2B}AR$) is one of four structurally similar adenosine GPCRs that display distinct pharmacological profiles via differential coupling to $G_{i/o}$ (A_1AR and A_3AR) or G_s ($A_{2A}AR$ and $A_{2B}AR$) proteins. The $A_{2B}AR$ is a pleiotropically coupled GPCR, signaling via both G_s and G_q proteins (Linden et al., 1999; Fredholm et al., 2001a), and represents a key example of a highly dynamic GPCR whose expression is modified by disease. The $A_{2B}AR$ is significantly upregulated by HIF-1α in a number of cancers (Li et al., 2005), including human prostate cancer (Wei et al., 2013; Mousavi et al., 2015). Until recently, the $A_{2B}AR$ was presumed to have minor physiologic significance, in part due to its relatively low affinity for the endogenous agonist adenosine (Feoktistov and Biaggioni, 1997; Fredholm et al., 2001b). However the substantial increase in extracellular adenosine concentration (Sommerschild and Kirkebøen, 2000) and the upregulation of $A_{2B}AR$ expression under pathologic conditions such as hypoxia (Kong et al., 2006) and inflammation (Ham and Rees, 2008) suggest a possible maladaptive role of the $A_{2B}AR$. Consequently, $A_{2B}AR$ antagonists are currently being explored as a novel therapeutic strategy for the treatment of inflammation (Ham and Rees, 2008), asthma and chronic obstructive pulmonary disease (Polosa and Blackburn, 2009), and diabetic nephropathy (Cárdenas et al., 2013). Importantly, recent studies have revealed a pivotal role for $A_{2B}AR$ signaling in cancer cell proliferation and progression of solid tumors of the bladder, breast, colon, and prostate (Ma et al., 2010; Cekic et al., 2012; Wei et al., 2013). However, no study to date has determined whether the dynamic regulation of the $A_{2B}AR$ results in constitutive activity, and whether this process, in addition to or independently of the influence of adenosine tone, contributes to any observed pathophysiology.

Thus, the current study profiled $A_{2B}AR$ constitutive activity within both a heterologous system and a native 22Rv1 human prostate cancer cell line, probing elevated basal activity with inverse agonists. To investigate the potential significance of constitutive activity on cancer pathophysiology, we subsequently examined the effect of the $A_{2B}AR$ inverse agonist 8-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl)-1-propylxanthine (PSB-603) on the proliferation of two different prostate cancer cell lines in an environment depleted of extracellular endogenous adenosine. Our findings suggest that not only is the $A_{2B}AR$ constitutively active, but this ligand-independent activity is sufficient to drive prostate cancer cell proliferation. Thus, we have identified a novel mechanism by which the $A_{2B}AR$ contributes to disease pathology and supports the development of inverse agonists, rather than neutral antagonists, as potential $A_{2B}AR$ therapeutics.

Materials and Methods

Materials. The AlphaScreen cAMP and SureFire phosphorylated extracellular signal-regulated kinase 1 and 2 (pERK1/2) kits, Ultima Gold scintillation cocktail, and cyclopentyl-1, 3-dipropylxanthine, 8-[dipropyl-2, 3-^{3}H(N)] ([^{3}H]DPCPX) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). The IP-One homogeneous time-resolved fluorescence (HTRF) kit was obtained from Cisbio Bioassays (Codolet, France). 22Rv1 and DU145 cells were purchased from American Type Culture Collection (ATCC; Manassa, VA). Hygromycin B and adenosine deaminase (ADA), derived from calf intestine, were obtained from Roche Diagnostics (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), RPMI 1640 (ATCC 30-2001 modification) medium, high-capacity cDNA transcription kit, Annexin V–Alexa Fluor 488, penicillin/streptomycin, and trypsin were purchased from Life Technologies (Carlsbad, CA). The TaqMan gene expression assay kit was obtained from Applied Biosystems (Carlsbad, CA). Adenosine receptor antagonists PSB-603, 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH 442416), and 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) were all purchased from Tocris Biosciences (Bristol, UK). The RNeasy plus mini kit was obtained from Qiagen (Valencia, CA), and the Pierce BCA protein assay kit, from Thermo Scientific (Rockford, IL). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical quality.

Cell Culture and Membrane Preparation. FlpIn Chinese hamster ovary (FlpInCHO) cells, stably transfected with either the human A_1, A_{2A}, A_{2B}, or A_3 adenosine receptors (A_1AR-FlpInCHO, $A_{2A}AR$-FlpInCHO, $A_{2B}AR$-FlpInCHO and A_3AR-FlpInCHO, respectively) were grown in DMEM supplemented with 10% FBS and hygromycin B (500 μg/ml). The 22Rv1 human-derived prostate cancer cell line was maintained in RPMI 1640–ATCC 30-2001 medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 25 mM glucose, and 18 mM sodium bicarbonate supplemented with 10% FBS. The DU145 human-derived prostate cancer cell line was grown in RPMI medium supplemented with 10% FBS. All cells were maintained at 37°C in a 5% CO_2 humidified incubator, grown to confluence, and then seeded into 96-well culture plates at assay-specific densities. For membrane preparation, $A_{2B}AR$-FlpInCHO cells were grown to 90% confluence before being harvested with detaching buffer (10 mM HEPES, 7 mM EDTA, 150 mM NaCl, pH 7.4) and centrifuged (250 $\times$ *g*, 5 minutes). The cell pellet was resuspended in HEPES homogenization buffer (10 mM HEPES and 10 mM EDTA, pH 7.4) and homogenized using a hand-held homogenizer (Polytron; Kinematica, Littau-Lucerne, Switzerland) for four 5-second intervals interspersed with 30-second cooling on ice. The homogenate was centrifuged (40,000 $\times$ *g*, 30 minutes, 4°C). The cell pellet was resuspended in HEPES homogenization buffer, and homogenization and centrifugation were repeated. The cell pellet was then resuspended in HEPES assay buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4), and the protein content was determined using a Pierce BCA protein assay kit according to the manufacturer's instructions.

Radioligand Binding. Membrane homogenates of $A_{2B}AR$-FlpInCHO cells (100 μg) were incubated in a 500-μl total volume of HEPES-buffered saline solution (25 mM HEPES, 10 mM glucose, 146 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM sodium pyruvate, and 1.3 mM $CaCl_2$, pH 7.4) and 1 U/ml ADA at 37°C for 60 minutes. Homologous competition binding at the $A_{2B}AR$-FlpInCHO was achieved by incubating membranes with [^{3}H]DPCPX (3 or 10 nM) in the absence or presence of 0.3 nM to 10 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Nonspecific binding was determined using 100 μM 5′-*N*-ethylcarboxamidoadenosine (NECA). Incubation was terminated by rapid filtration through 0.5% polyethylenimine

presoaked Whatman GF/B filters using a membrane harvester (Brandel, Gaithersburg, MD). Filters were washed four times with 2 ml of ice-cold 0.9% NaCl, dried before the addition of 4 ml of scintillation cocktail (Ultima Gold; PerkinElmer Life and Analytical Sciences), and allowed to stand for 1 hour before radioactivity was determined by scintillation counting.

cAMP Accumulation. FlpInCHO cells and 22Rv1 cells were seeded into 96-well plates at a density of 20,000 or 40,000 cells/well, respectively, and incubated in complete cell medium for 6 hours at 37°C in a humidified incubator. 22Rv1 cells were then placed in a sealed hypoxic chamber (Hypoxia Subchamber; BioSpherix, Lacona, NY) containing 2% O_2 and 5% CO_2 and incubated at 37°C for 24 hours. FlpInCHO and 22RV1 cell medium was removed and replaced with 200 μl/well culture medium containing 100 U/ml penicillin and 100 μg/ml streptomycin in the absence or presence of inverse agonist (0.1 nM to 10 μM) and/or 1 U/ml ADA, and cells were incubated for 16 hours at 37°C in a 5% CO_2 humidified incubator. Medium was then removed, and cells were incubated with stimulation buffer (140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.2 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM D-glucose, 1 mg/ml bovine serum albumin, 10 μM rolipram, and 5 mM HEPES, pH 7.4) in the absence and presence of 1 U/ml ADA and/or adenosine receptor ligand (concentrations ranging from 10 pM to 10 μM) for 30 minutes at 37°C. Stimulation was terminated by the removal of medium and the addition of 50 μl/well ice-cold ethanol. Detection of cAMP was performed using the AlphaScreen cAMP kit as described previously (Koole et al., 2010). Data were analyzed against a cAMP standard curve performed in parallel and expressed as cAMP concentration per well as a fold over basal.

Phosphorylation of Extracellular Signal-Regulated Kinase 1 and 2. $A_{2B}AR$-FlpInCHO or 22Rv1 cells were seeded into 96-well plates (40,000 cells/well) and allowed to adhere for 6 hours. 22Rv1 cells were placed in a sealed hypoxic chamber containing 2% O_2 and 5% CO_2 and incubated at 37°C for 24 hours. Cells were washed with phosphate-buffered saline and maintained in serum-free DMEM for 16 hours in the absence or presence of inverse agonists (concentrations ranging from 0.1 nM to 10 μM). Cells were then exposed to serum-free medium in the absence or presence of agonist for 5 minutes (concentrations ranging from 10 pM to 100 μM) followed by the removal of medium and addition of 100 μl/well SureFire lysis buffer to each well. Detection of pERK1/2 was performed using the AlphaScreen pERK1/2 SureFire kit as described previously (May et al., 2007). Data were normalized to the response elicited upon the exposure of cells to 10% FBS for 5 minutes.

Inositol Monophosphate Accumulation. $A_{2B}AR$-FlpInCHO cells in phenol red–free medium were seeded into sterilized 384-well ProxiPlates (PerkinElmer Life and Analytical Sciences) (12,000 cells/10 μl) and incubated for 16 hours in the absence or presence of inverse agonists. 22Rv1 cells were seeded into 96-well plates (20,000 cells/well) and placed in a sealed hypoxic chamber containing 2% O_2 and 5% CO_2 and incubated at 37°C for 24 hours. Where appropriate, cells were exposed to agonist for 1 hour prior to inositol monophosphate (IP_1) being measured using the IP-One HTRF accumulation kit (Cisbio Bioassays), according to the manufacturer's instructions, and detected with an EnVision microplate reader (PerkinElmer Life and Analytical Sciences) using standard HTRF settings (665-/630-nm ratio). Results were analyzed as an inverse ratio, with IP_1 concentrations extrapolated from the IP_1 standard curve performed in parallel.

Expression of Adenosine Receptor mRNA. 22Rv1 cells were harvested using 0.05% trypsin/0.53 mM EDTA solution. RNA was extracted using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration was measured at 260 nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (2 μg) was then used for cDNA synthesis using the High Capacity cDNA Transcription Kit (LifeTechnologies, Carlsbad, CA) according to the manufacturer's instructions. Reverse-transcription polymerase chain reaction (RT-PCR) validation was performed using TaqMan Gene Expression assays to detect and quantitate gene transcripts of human A_1AR (TaqMan probe: Hs00379752_m1), $A_{2A}AR$ (TaqMan probe: Hs00169123_m1), $A_{2B}AR$ (TaqMan probe: Hs00386497_m1), and A_3AR (TaqMan probe: Hs00252933_m1). In brief, cDNA samples were diluted 1:4 in nuclease-free water and mixed with TaqMan Fast Advanced Master Mix (Applied Biosystems). RT-PCR was performed using the Mastercycler ep realplex system (Eppendorf, Hamburg, Germany). Samples were analyzed in duplicate. The fluorescence threshold values were obtained, and calculation of relative change in mRNA was performed using the comparative delta delta cycle threshold method as described previously (Livak and Schmittgen, 2001) with normalization for the endogenous control β-actin (TaqMan probe: Hs01060665_g1).

Cell Proliferation and Cell Viability. 22Rv1 and DU145 cells were seeded in 96-well plates at 3000 cells/well in 200 μl of complete medium and cultured for 16 hours at 37°C. Cells were then exposed to complete medium in the absence or presence of inverse agonists PSB-603 (1 μM) or SCH 442416 (100 nM; 22Rv1 cells only) and/or 1 U/ml ADA for 24 or 48 hours. Cells were maintained in a hypoxic chamber containing 2% O_2 and 5% CO_2 at 37°C for the duration of the experiment. Medium, inverse agonists, and ADA were refreshed every 24 hours. Cell proliferation was determined after labeling with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Absorbance of the converted dye was determined by subtraction of the background signal at 690 nm from the measured absorbance at 570 nm on a FlexStationIII plate reader (Molecular Devices, Sunnyvale, CA). 22Rv1 cell viability was determined with annexin V and propidium iodide (PI) staining. 22Rv1 cells, grown in petri dishes and exposed to the same conditions described for cell proliferation assays, were harvested in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) and stained with Annexin V–Alexa Fluor 488 (5/100 μl) and PI (5 μg/ml) for 15 minutes at room temperature. Samples were immediately analyzed using the BD fluorescence-activated cell sorter (FACS) CantoII flow cytometer (BD Biosciences, San Jose, CA).

Data Analysis. All data were analyzed using Prism 6.0 (GraphPad Software Inc., San Diego, CA). Statistical significance was defined as $P < 0.05$ as determined by one-way or two-way analysis of variance (ANOVA) with Tukey's or Bonferroni's multiple comparisons post-hoc analysis or t test, as indicated within the results.

Results

The $A_{2B}AR$-FlpInCHO Cell Line Has Elevated Basal cAMP. Parental and adenosine receptor FlpInCHO cell lines, assessed in parallel, demonstrated significant differences in baseline cAMP accumulation. Specifically, the $A_{2B}AR$-FlpInCHO cell line had significantly higher baseline cAMP

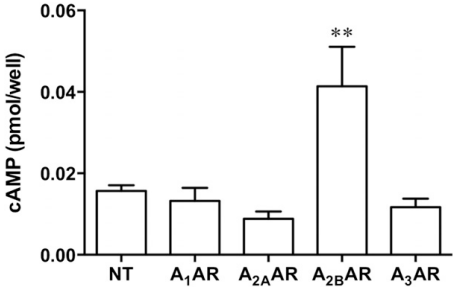

Fig. 1. Human $A_{2B}AR$-FlpInCHO cells have a higher level of baseline cAMP than the parental or other AR-FlpInCHO cells. Basal levels of cAMP accumulation in nontransfected (NT), A_1AR-, $A_{2A}AR$-, $A_{2B}AR$-, and A_3AR-FlpInCHO cells. $^{**}P < 0.01$, one-way ANOVA; Tukey's multiple comparison test. Data represent the mean + S.E.M. from five independent experiments performed in triplicate.

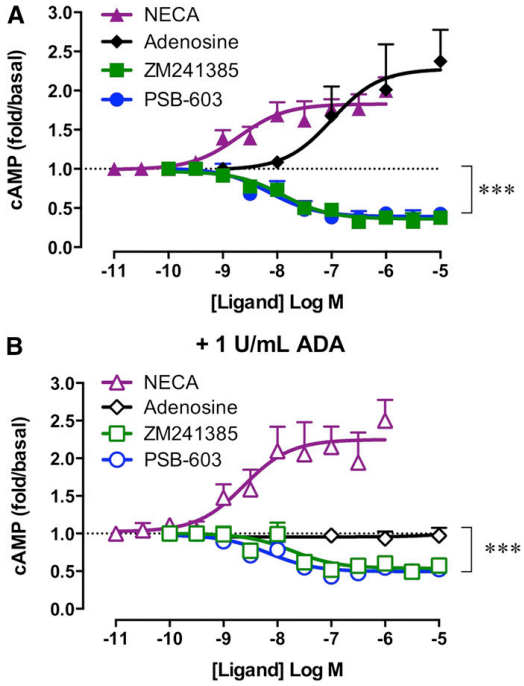

Fig. 2. Inverse agonism of cAMP accumulation reveals $A_{2B}AR$ constitutive activity. (A) In the absence of ADA, exposure of $A_{2B}AR$-FlpInCHO cells to the prototypical adenosine receptor agonists NECA or adenosine mediates robust increases in cAMP, whereas the $A_{2B}AR$ inverse agonists ZM241385 or PSB-603 significantly decrease baseline cAMP levels. (B) In the presence of 1 U/ml ADA, NECA mediates a robust increase in cAMP accumulation, the $A_{2B}AR$ inverse agonists ZM241385 or PSB-603 significantly decrease baseline cAMP levels, whereas the response to adenosine is abolished. ***$P < 0.001$; paired t test. Data represent the mean + S.E.M. from four to seven independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

when compared with the parental, A_1AR-, $A_{2A}AR$-, and A_3AR-FlpInCHO cell lines (Fig. 1; $P < 0.01$, one-way ANOVA Tukey's multiple comparison test, $n = 5$). Receptor number can influence basal cAMP accumulation through constitutive activation of adenylyl cyclase (Nakahara et al., 2004); as such, we sought to determine the level of adenosine receptor expression in the stably transfected A_{2B}-FlpInCHO cell line. [^{3}H]DPCPX homologous competition binding on membrane homogenates of $A_{2B}AR$-FlpInCHO cell membranes yielded an affinity estimate (pK_d of 6.87 ± 0.10; Supplemental Fig. 1) that was similar to published values (Weyler et al., 2006). Importantly, the $A_{2B}AR$ was not grossly overexpressed, with a B_{max} value of 3.13 ± 0.61 pmol mg protein^{-1}, which is comparable to other GPCRs expressed in the FlpInCHO cell system (Yan et al., 2009; Gregory et al., 2010).

Concentration-Response Relationships for Adenosine Receptor Agonists and Inverse Agonists Reveal $A_{2B}AR$ Constitutive Activity for G_s-Coupled cAMP Accumulation. In $A_{2B}AR$-FlpInCHO cells, a robust and concentration-dependent stimulation of cAMP accumulation was observed for the non-selective adenosine receptor agonists, NECA and adenosine (Fig. 2A), with NECA 80-fold more potent than adenosine with respect to cAMP stimulation (Table 1). $A_{2B}AR$ constitutive activity was assessed by exposure of $A_{2B}AR$-FlpInCHO cells to the $A_{2B}AR$ inverse agonists PSB-603 and ZM241385. PSB-603 and ZM241385 both mediated a significant and concentration-dependent reduction in baseline cAMP accumulation (Fig. 2A; $P < 0.001$, paired t test, $n = 4–7$). The observed decrease in baseline cAMP accumulation in the presence of inverse agonist is consistent with either inhibition of endogenous agonist activity or a reduction in constitutive activity. To investigate the influence of endogenous adenosine, concentration-response curves to agonists and inverse agonists were repeated in the presence of 1 U/ml ADA. ADA had no significant effect on the potency (pEC_{50}) or maximal effect (E_{max}) of NECA, but abolished the cAMP accumulation mediated by exogenous adenosine up to 10 μM (Fig. 2B; Table 1; $P > 0.05$, unpaired t test, no statistical significance for pEC_{50} or E_{max} of NECA ± ADA, $n = 4–7$). The concentration-dependent decrease in cAMP accumulation observed upon exposure of $A_{2B}AR$-FlpInCHO cells to PSB-603 or ZM241385 was maintained in the presence of ADA (Fig. 2B; $P < 0.001$, paired t test, $n = 4–7$). Furthermore, the potency and maximal effect of ZM241385 or PSB-603 were not significantly different from that observed in the absence of ADA (Table 1; $P > 0.05$, unpaired t test, $n = 4–7$).

$A_{2B}AR$ Constitutive Activity Cannot Be Detected at Alternative Signal Transduction Pathways, ERK1/2 Phosphorylation, and IP_1 Accumulation. The $A_{2B}AR$ is a pleiotropically coupled GPCR, signaling via both G_s and G_q proteins (Linden et al., 1999; Fredholm et al., 2001a). To investigate the constitutive activation of alternate $A_{2B}AR$ signaling pathways, the influence of agonists and inverse agonists on baseline pERK1/2 (Fig. 3A) and IP_1 accumulation (Fig. 3B) was assessed. Concentration-dependent increases in pERK1/2 and IP_1 accumulation in the $A_{2B}AR$-FlpInCHO cells were observed in the presence of NECA or adenosine, albeit at lower potencies than those observed in the cAMP accumulation assay (Fig. 3). Specifically, the potency (pEC_{50}) of NECA-mediated pERK1/2 and IP_1 accumulation was 6.69 ± 0.07 and 5.74 ± 0.20, respectively, whereas the pEC_{50} for adenosine-mediated pERK1/2 was 5.55 ± 0.07 and could not be determined for IP_1 accumulation. The inverse agonists, PSB-603 or ZM241385, had no effect on baseline pERK1/2 or IP_1 accumulation at any concentration assessed (0.1 nM to 100 μM) (Fig. 3). Concentration-dependent increases in pERK1/2 and IP_1 accumulation in the presence of NECA and

TABLE 1

Potency (pEC_{50}) and maximal response (E_{max}) of $A_{2B}AR$-mediated changes in cAMP accumulation in the presence and absence of ADA in $A_{2B}AR$-FlpInCHO cells

Data are the mean ± S.E.M. from four or more independent experiments performed in triplicate.

	pEC_{50}		E_{max}	
	0 U/ml ADA	1 U/ml ADA	0 U/ml ADA	1 U/ml ADA
NECA	8.42 ± 0.25	8.49 ± 0.19	1.90 ± 0.13	2.36 ± 0.28
Adenosine	6.29 ± 0.37	ND	2.40 ± 0.23	ND
ZM241385	7.80 ± 0.25	8.00 ± 0.26	0.36 ± 0.06	0.51 ± 0.06
PSB-603	8.26 ± 0.22	8.33 ± 0.38	0.40 ± 0.05	0.48 ± 0.04

E_{max}, maximal response elicited by the ligand expressed as cAMP concentration as fold over basal; ND, value could not be determined; pEC_{50}, negative logarithm of the agonist or inverse agonist concentration required to elicit half the maximal response.

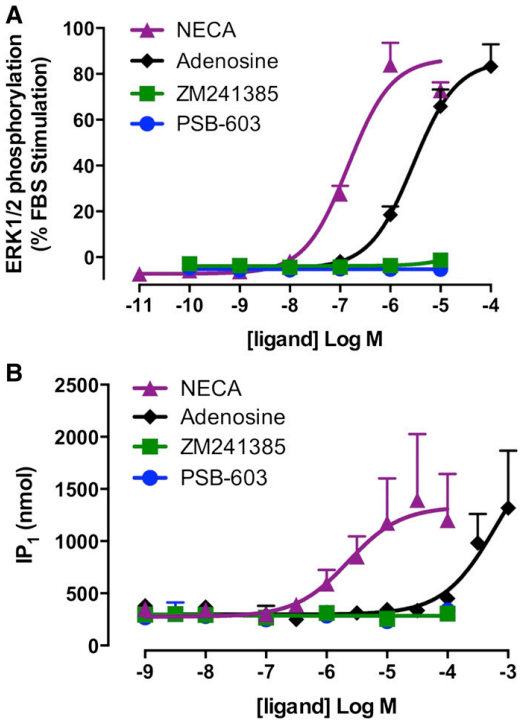

Fig. 3. Inverse agonism was not observed for $A_{2B}AR$-mediated ERK1/2 phosphorylation (A) or IP_1 accumulation (B) in $A_{2B}AR$-FlpInCHO cells. Data represent the mean + S.E.M. from four independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

adenosine could not be detected in 22Rv1 cells (Supplemental Fig. 2).

Hypoxia-Inducible $A_{2B}ARs$ in the 22Rv1 Human Prostate Cancer Cell Line Are Constitutively Active. RT-PCR analysis of the 22Rv1 prostate cancer cells detected a high level of $A_{2A}AR$ mRNA, a moderate level of $A_{2B}AR$ mRNA, and a low level of A_1AR mRNA (Fig. 4A). A_3AR mRNA was below the level of detection. As demonstrated previously in other human carcinoma cell lines (Kong et al., 2006; Ma et al., 2010), expression of $A_{2B}AR$ mRNA was significantly upregulated after 8 hours of hypoxia (2% O_2/5% CO_2; $P < 0.01$, two-way ANOVA Bonferroni's multiple comparison test, $n = 3$). In contrast, no significant differences were observed in the level of A_1AR and $A_{2A}AR$ mRNA detected under normoxic conditions or after 8 hours of hypoxia (Fig. 4B).

Constitutive $A_{2B}AR$ activity in 22Rv1 prostate cancer cells was investigated after exposure of cells to hypoxia, the condition for which increased $A_{2B}AR$ expression is likely to be observed. Specifically, the influence on cAMP accumulation in 22Rv1 prostate cancer cells was quantified for the nonselective adenosine receptor agonists NECA or adenosine, the $A_{2B}AR$ inverse agonists PSB-603 or ZM241385, and the $A_{2A}AR$-selective antagonist SCH 442416 after 24 hours of hypoxia (2% O_2/5% CO_2). The $A_{2A}AR$-selective antagonist, SCH 442416, was used to evaluate the influence the highly expressed $A_{2A}AR$ had on baseline cAMP. Robust concentration-dependent increases in cAMP were detected in both the absence and presence of 1 U/ml ADA for NECA and in the absence of ADA for adenosine (Fig. 5, A and B). The $A_{2A}AR$-selective inverse agonist SCH 442416 had no effect on baseline cAMP under either condition tested (Fig. 5, C and D). In the absence of ADA, ZM241385 or PSB-603 mediated concentration-dependent decreases in basal cAMP with similar potencies (Fig. 5C). Furthermore, the small but significant window of inverse agonism was maintained in the presence of ADA (Fig. 5D; $P < 0.05$, paired t test, $n = 3$–4). The ability of ZM241385 or PSB-603 to inhibit ligand-independent cAMP accumulation in the 22Rv1 cells with similar potency to that observed in the $A_{2B}AR$-FlpInCHO cells (ZM241385 pEC_{50}: 8.44 ± 0.45 and 8.00 ± 0.26, respectively; PSB-603 pEC_{50}: 7.67 ± 0.30 and 8.33 ± 0.38, respectively) reveals that $A_{2B}AR$ constitutive activity can be observed in both model heterologous expression systems and endogenous expression systems.

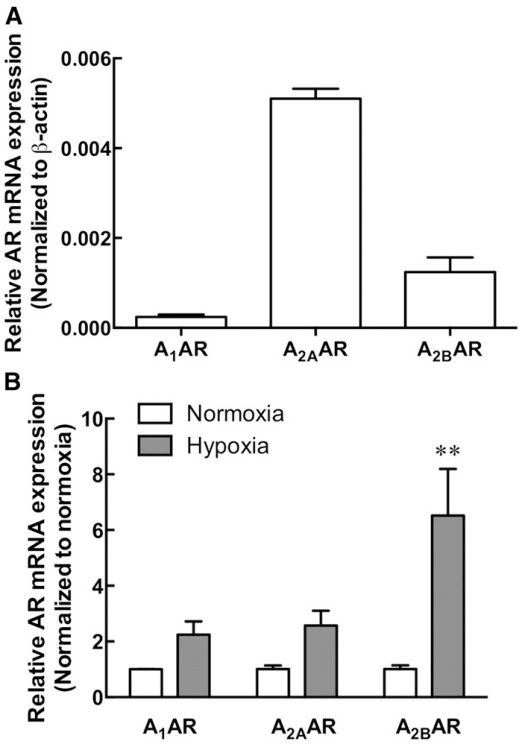

Fig. 4. $A_{2B}AR$ mRNA expression is upregulated under hypoxic conditions in the 22Rv1 prostate cancer cell line. (A) The relative expression of human A_1AR, $A_{2A}AR$, and $A_{2B}AR$ mRNA in 22Rv1 prostate cancer cells under normoxic conditions. A_3AR mRNA was below the level of detection. (B) A_1AR, $A_{2A}AR$, and $A_{2B}AR$ mRNA expression after exposure of 22Rv1 prostate cancer cells to 8-hour hypoxia (2% O_2/5% CO_2) normalized to respective normoxic control. $^{**}P < 0.01$, two-way ANOVA, Bonferroni's multiple comparison test. Data represent the mean + S.E.M. from three independent experiments.

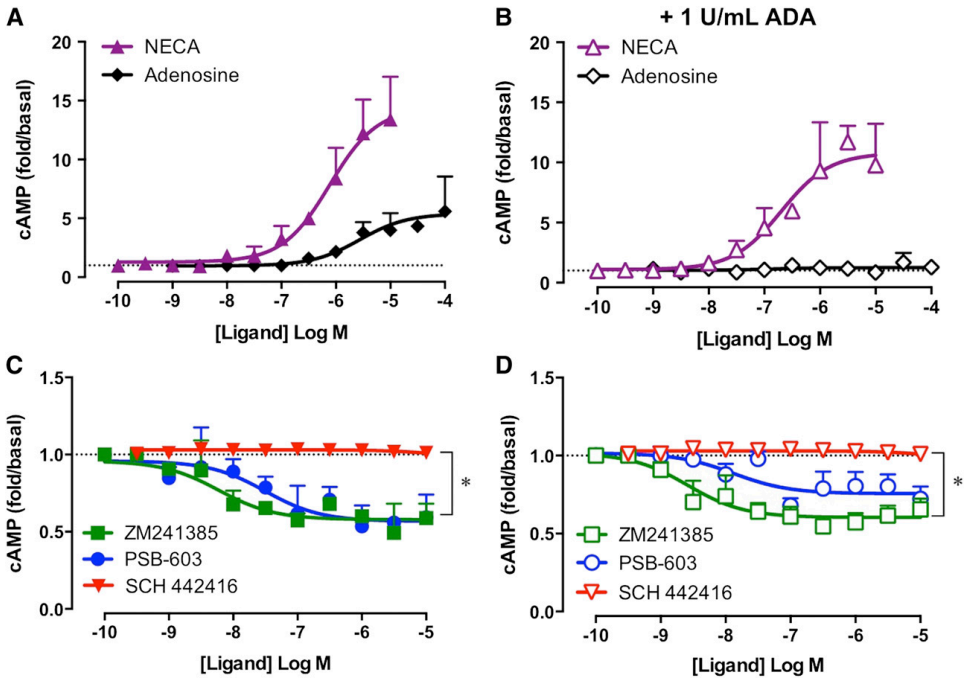

Fig. 5. A_{2B}ARs endogenously expressed in 22Rv1 prostate cancer cells are constitutively active. (A) In the absence of ADA, the prototypical A_{2B}AR agonists NECA or adenosine mediate robust increases in cAMP after 30-minute stimulation. (B) In the presence of ADA, NECA mediates a robust increase in cAMP accumulation, whereas the response to adenosine is abolished. (C and D) The A_{2A}AR antagonist SCH 442416 had no effect on baseline cAMP accumulation. The A_{2B}AR inverse agonists ZM241385 and PSB-603 mediate small but significant decreases in baseline cAMP in the absence (C) and presence (D) of 1 U/ml ADA. 22Rv1 cells were exposed to 24-hour hypoxia (2% O_2/5% CO_2) immediately prior to detection of cAMP accumulation. *$P < 0.05$, paired t test. Data represent the mean + S.E.M. from three to four independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

$A_{2B}AR$ Constitutive Activity Promotes Proliferation of 22Rv1 Human Prostate Cancer Cells. The influence of A_{2B}AR constitutive activity on the proliferation and survival of the 22Rv1 human prostate cancer cell line was investigated under hypoxic conditions (2% O_2/5% CO_2). As described previously, 1 U/ml ADA (refreshed every 24 hours) was used to investigate the influence of endogenous adenosine. Importantly, the activity of ADA after 24 hours at 37°C was confirmed in signal transduction assays (Supplemental Fig. 3). Over a 48-hour period, the A_{2B}AR inverse agonist, PSB-603 (1 μM), significantly reduced 22Rv1 cell proliferation when compared with the respective buffer control, both in the absence and presence of ADA, determined using an MTT proliferation assay (Fig. 6A; $P < 0.01$, two-way ANOVA Tukey's multiple comparison test, $n = 5$). Similar results were also observed in the DU145 prostate cancer cell line, whereby PSB-603 (1 μM) significantly reduced basal cell proliferation at 48 hours (Supplemental Fig. 4; $P < 0.05$, two-way ANOVA Tukey's multiple comparison test, $n = 4$). A limitation of the MTT assay is that it does not differentiate between cell quiescence and increased cell death (Carmichael et al., 1987). Therefore, the 22Rv1 cells were also subjected to annexin V and PI staining and analyzed via FACS to assess whether PSB-603 had a significant influence on cell viability. The inverse agonist, PSB-603 (1 μM), had no significant effect on the percentage of nonviable annexin V or PI-positive cells after 48 hours (Fig. 6B; $P > 0.05$, two-way ANOVA Tukey's multiple comparison test, $n = 3$). Thus, the reduced absorbance observed in the MTT assay is likely to be due to PSB-603 causing a decrease in proliferation as opposed to having a cytotoxic effect. The level of A_{2A}AR mRNA was relatively high in 22Rv1 cells (Fig. 4A). To investigate whether inhibition of the A_{2A}AR could also influence the proliferation of 22Rv1 cells, cells were exposed to the highly selective A_{2A}AR antagonist, SCH 442416 (100 nM), and assessed in the MTT assay as described earlier. Over a 48-hour period, SCH 442416 (100 nM) had no significant effect on 22Rv1 cell proliferation (Fig. 6C; $P > 0.05$, two-way ANOVA Tukey's multiple comparison test, $n = 5$).

Discussion

This study is the first to characterize the constitutive activity of the human wild-type A_{2B}AR within both a heterologous FlpInCHO system and the native 22Rv1 prostate cancer cell line. Constitutive activity of the A_{2B}AR was revealed through the detection of inverse agonism of cAMP accumulation under conditions that removed extracellular endogenous

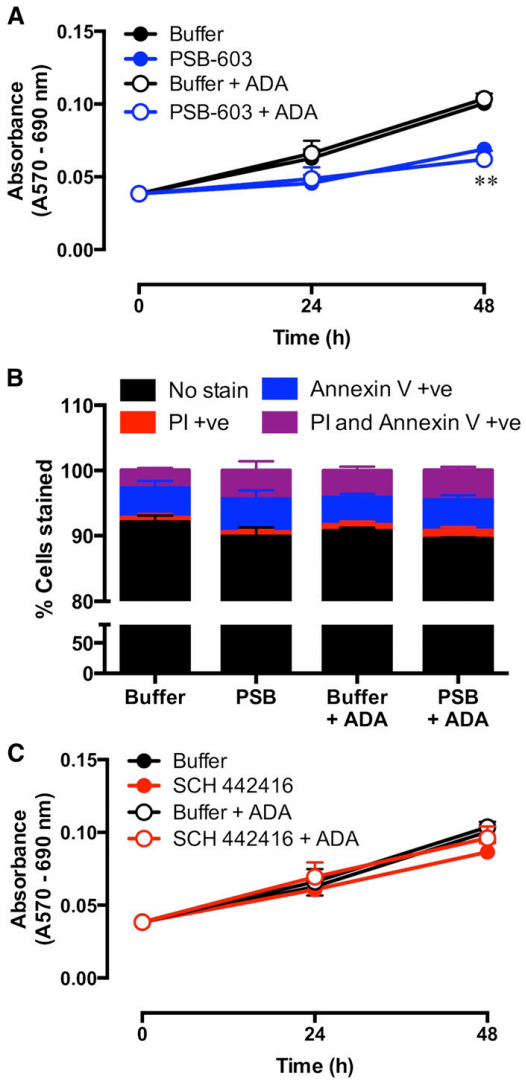

Fig. 6. The $A_{2B}AR$ inverse agonist PSB-603 decreased proliferation of 22Rv1 prostate cancer cells over 48 hours. (A) PSB-603 (1 μM) significantly decreased the proliferation of 22Rv1 cells in the absence (closed symbols) and presence (open symbols) of 1 U/ml ADA as determined by MTT absorbance. $^{**}P < 0.01$ compared with respective buffer control; two-way ANOVA, Tukey's multiple comparison test. (B) The $A_{2B}AR$ inverse agonist PSB-603 (1 μM) has no effect on cell viability after 48 hours, as assessed by the proportion of annexin V, PI, or double positive cells, in the absence and presence of 1 U/ml ADA. (C) The $A_{2A}AR$-selective antagonist SCH 442416 (100 nM) had no effect on the proliferation of 22Rv1 cells in the absence (closed symbols) or presence (open symbols) of 1 U/ml ADA. 22Rv1 cells seeded at 3000 cells/well and maintained under hypoxia (2% O_2/5% CO_2), with medium, drugs, and ADA refreshed every 24 hours. Data represent the mean + S.E.M. from five (A and C) or three (B) independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol. +ve, positive.

agonist. Furthermore, we identified a pathophysiological consequence for the elevated ligand-independent activity. That is, $A_{2B}AR$ constitutive activity stimulated proliferation of the 22Rv1 cell line, a finding that was confirmed in the DU145 prostate cancer cell line. As such, our studies indicate that future drug discovery efforts should differentiate $A_{2B}AR$ inverse agonists from neutral antagonists, as the former may prove a better potential therapeutic approach to slow prostate tumor progression. In addition, our studies suggest that ligand-independent constitutive activity should be taken into consideration when investigating the role of $A_{2B}ARs$ in other disease pathologies.

Experimental observations for $A_{2B}AR$ ligand-mediated changes in cAMP accumulation are in accordance with a constitutively active system described by the two-state model of receptor activation (Leff, 1995; Bennett et al., 2013). In this model, the intrinsic efficacy of a ligand is governed by its relative affinity for the R verses R* state (Burstein et al., 1997). As such, an increase in agonist potency but a decrease in inverse agonist potency is predicted for a receptor system that has a high proportion of receptors existing in the R* state—that is, a constitutively active receptor system. In agreement with the two-state model, the NECA potency in the cAMP accumulation assay is approximately 100-fold higher than its affinity at the R state as estimated using antagonist 125I-3-(4-amino-3-iodobenzyl)-8-(phenyl-4-oxyacetate)-1-propylxanthine ($[^{125}I]$ABOPX) binding (Linden et al., 1999). Conversely the functional potency of the inverse agonist PSB-603 is 10-fold lower than the R-state affinity as estimated using homologous $[^3H]$PSB-603 competition binding (Borrmann et al., 2009). Therefore, the experimentally observed shift in agonist and inverse agonist potencies in the present study is in accordance with the two-state model and supports the suggestion that a significant proportion of $A_{2B}ARs$ exist in the R* state.

Constitutive activity has previously been demonstrated for GPCRs coupling to each of the different heterotrimeric G proteins (Barker et al., 1994; Neilan et al., 1999; Hopkinson et al., 2000). Because $A_{2B}AR$ constitutive activity was only observed in the cAMP accumulation assay, this may reflect preferential ligand-independent stabilization of a G_s-coupled active receptor conformation. This contention is supported by studies of other pleiotropically coupled GPCRs, for which constitutive activity manifests exclusively in the G_s-coupled pathway, such as the gain–of-function mutation in the luteinizing hormone receptor responsible for precocious puberty and the hyperthyroidism-causing mutation of the thyroid-stimulating hormone receptor (Kopp et al., 1995; Liu et al., 1999; Seifert and Wenzel-Seifert, 2002). However, it is important to note that NECA and adenosine have a 20- to 2000-fold higher potency for cAMP when compared with IP_1 and pERK1/2 in the $A_{2B}AR$-FlpInCHO cell background and no detectable G_q-mediated activity in the 22Rv1 cells, suggesting that the $A_{2B}AR$ has a higher coupling efficiency for G_s- rather than G_q-coupled signaling. Therefore, it is possible that the cAMP accumulation assay is the only method sensitive enough to detect small changes below a baseline level (Seifert and Wenzel-Seifert, 2002). Regardless of mechanism, it is apparent that the elevated baseline cAMP is attributable to constitutive coupling to G_s proteins and supports the possibility for $A_{2B}AR$ constitutive activity pathway bias.

To determine whether the $A_{2B}AR$ constitutive activity observed in the heterologous system was relevant in an endogenous setting, we first used the 22Rv1, xenograft-derived human prostate carcinoma epithelial cell line. This cell line was selected based on previous reports of endogenous $A_{2B}AR$ expression within prostate cancer cells (Wei et al., 2013). Under hypoxic conditions, such as those observed within a tumor microenvironment (Subarsky and Hill, 2003; Ryzhov et al., 2008), $A_{2B}AR$ expression is increased by HIF-1α (Kong et al., 2006; Eckle et al., 2014). Adenosine receptor–mediated cAMP accumulation was assessed after 24 hours hypoxia (2% O_2/5% CO_2) to allow for $A_{2B}AR$ up-regulation, thus enhancing the ability to detect constitutive activity (Nakahara et al., 2004). The nonselective agonists, NECA or adenosine, produced substantial increases in cAMP, likely due to the nonselective stimulation of $A_{2B}ARs$ and $A_{2A}ARs$, both of which preferentially couple to G_s proteins. The $A_{2B}AR$ inverse agonists ZM241385 and PSB-603 produced a concentration-dependent decrease in baseline activity in the presence and absence of ADA, whereas the $A_{2A}AR$-selective antagonist SCH 442416 had no effect on baseline cAMP levels. Given adenosine has a higher affinity for the $A_{2A}AR$ than the $A_{2B}AR$ (Liang and Haltiwanger, 1995; Fredholm et al., 2001b), we would anticipate that, if ZM241385 and PSB-603 were simply inhibiting the effects of residual endogenous adenosine, then a comparable or larger effect on baseline cAMP should be observed in the presence of an $A_{2A}AR$-selective competitive antagonist. As such, the absence of an effect in the presence of $A_{2A}AR$ blockade argues against the possibility of a contaminating influence of endogenous adenosine and instead further supports appreciable $A_{2B}AR$ constitutive activity in the 22Rv1 prostate cancer cell line.

$A_{2B}AR$ expression at both the mRNA and protein level is higher in malignant prostate cancer tissue from human patients when compared with normal control prostate tissue (Mousavi et al., 2015). In addition, the $A_{2B}AR$ has been implicated in cell proliferation and angiogenesis, accounting for its apparent role in the pathogenesis of a number of solid tumors (Li et al., 2005; Ryzhov et al., 2008; Ma et al., 2010; Cekic et al., 2012; Iannone et al., 2013; Wei et al., 2013). The involvement of the $A_{2B}AR$ in the proliferation of human oral squamous cell carcinoma–derived cells was confirmed with small hairpin RNA (Kasama et al., 2015). Furthermore, previous studies have demonstrated the inhibitory effects of an $A_{2B}AR$ inverse agonist on the cell proliferation of human colon carcinomas (Ma et al., 2010) and prostate cancer cells (Wei et al., 2013); however, these studies were performed in the presence of endogenous adenosine and thus were unable to differentiate between the influence of agonist tone and any potential ligand-independent receptor activity. We sought to directly determine whether $A_{2B}AR$ constitutive activity contributed to the basal level of cell growth of prostate cancer cells. Our results revealed that, even when endogenous agonist had been removed, the $A_{2B}AR$ inverse agonist PSB-603 significantly suppressed cell growth of malignant 22Rv1 and DU145 prostate cells over the 48-hour period. The $A_{2A}AR$-selective antagonist, SCH 442416, did not replicate the inhibition of cell proliferation in 22Rv1 cells, supporting the supposition that $A_{2B}AR$ constitutive activity, and not endogenous adenosine, significantly contributes to pathologic prostate cancer cell proliferation in vitro. Differentiating the effect of constitutive activity from the effect of the endogenous ligand in an in vivo setting is challenging (Parra and Bond, 2007). However, targeting pathologic $A_{2B}AR$ overexpression and concurrent constitutive activity with inverse agonists is readily achievable and presents an exciting future prospect in cancer treatment.

In conclusion, ligand-independent $A_{2B}AR$ constitutive activity of the G_s-coupled cAMP pathway can be detected in both a heterologous and native cell line. The effect of an $A_{2B}AR$ inverse agonist to reduce the basal level of cell growth of two different prostate cancer cell lines demonstrates the potential therapeutic benefit in targeting $A_{2B}AR$ constitutive activity as a pharmacological adjuvant in prostate cancer treatment. Furthermore, this study highlights the requirement to differentiate potential inverse agonist effects from neutral antagonist effects of $A_{2B}AR$ compounds in the drug discovery pipeline to identify optimal therapeutically efficacious ligands for this receptor. Although our study demonstrates the pathologic relevance of $A_{2B}AR$ constitutive activity within the context of prostate cancer, it may also have wider implications in both physiologic and pathologic conditions where the $A_{2B}AR$ was previously considered to have minimal influence.

Acknowledgments

The authors thank Dr. Michael Crouch and Dr. Ron Osmond for providing the pERK1/2 assay kit, Dr. Gail Risbridger for assistance with the DU145 prostate cancer cells, and Sandy Fung for assistance with FACS analysis.

Authorship Contributions

Participated in research design: Vecchio, White, May.
Conducted experiments: Vecchio, Tan.
Performed data analysis: Vecchio, White, May.
Wrote or contributed to the writing of the manuscript: Vecchio, Gregory, Christopoulos, White, May.

References

Barker EL, Westphal RS, Schmidt D, and Sanders-Bush E (1994) Constitutively active 5-hydroxytryptamine2C receptors reveal novel inverse agonist activity of receptor ligands. *J Biol Chem* **269**:11687–11690.
Bennett KA, Tehan B, Lebon G, Tate CG, Weir M, Marshall FH, and Langmead CJ (2013) Pharmacology and structure of isolated conformations of the adenosine A2A receptor define ligand efficacy. *Mol Pharmacol* **83**:949–958.
Bond RA and IJzerman AP (2006) Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol Sci* **27**:92–96.
Borrmann T, Hinz S, Bertarelli DCG, Li W, Florin NC, Scheiff AB, and Müller CE (2009) 1-alkyl-8-(piperazine-1-sulfonyl)phenylxanthines: development and characterization of adenosine A2B receptor antagonists and a new radioligand with subnanomolar affinity and subtype specificity. *J Med Chem* **52**:3994–4006.
Burstein ES, Spalding TA, and Brann MR (1997) Pharmacology of muscarinic receptor subtypes constitutively activated by G proteins. *Mol Pharmacol* **51**:312–319.
Cárdenas A, Toledo C, Oyarzún C, Sepúlveda A, Quezada C, Guillén-Gómez E, Díaz-Encarnación MM, Pastor-Anglada M, and San Martín R (2013) Adenosine A(2B) receptor-mediated VEGF induction promotes diabetic glomerulopathy. *Lab Invest* **93**:135–144.
Carmichael J, DeGraff WG, Gazdar AF, Minna JD, and Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* **47**:936–942.
Cekic C, Sag D, Li Y, Theodorescu D, Strieter RM, and Linden J (2012) Adenosine A2B receptor blockade slows growth of bladder and breast tumors. *J Immunol* **188**:198–205.
Chalmers DT and Behan DP (2002) The use of constitutively active GPCRs in drug discovery and functional genomics. *Nat Rev Drug Discov* **1**:599–608.
Eckle T, Kewley EM, Brodsky KS, Tak E, Bonney S, Gobel M, Anderson D, Glover LE, Riegel AK, and Colgan SP, et al. (2014) Identification of hypoxia-inducible factor HIF-1A as transcriptional regulator of the A2B adenosine receptor during acute lung injury. *J Immunol* **192**:1249–1256.
Feoktistov I and Biaggioni I (1997) Adenosine A2B receptors. *Pharmacol Rev* **49**:381–402.
Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, and Linden J (2001a) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**:527–552.
Fredholm BB, Irenius E, Kull B, and Schulte G (2001b) Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem Pharmacol* **61**:443–448.

Gregory KJ, Hall NE, Tobin AB, Sexton PM, and Christopoulos A (2010) Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. *J Biol Chem* **285**: 7459–7474.
Ham J and Rees DA (2008) The adenosine a2b receptor: its role in inflammation. *Endocr Metab Immune Disord Drug Targets* **8**:244–254.
Hopkinson HE, Latif ML, and Hill SJ (2000) Non-competitive antagonism of β(2)-agonist-mediated cyclic AMP accumulation by ICI 118551 in BC3H1 cells endogenously expressing constitutively active β(2)-adrenoceptors. *Br J Pharmacol* **131**: 124–130.
Iannone R, Miele L, Maiolino P, Pinto A, and Morello S (2013) Blockade of A2b adenosine receptor reduces tumor growth and immune suppression mediated by myeloid-derived suppressor cells in a mouse model of melanoma. *Neoplasia* **15**: 1400–1409.
Kasama H, Sakamoto Y, Kasamatsu A, Okamoto A, Koyama T, Minakawa Y, Ogawara K, Yokoe H, Shiiba M, and Tanzawa H, et al. (2015) Adenosine A2b receptor promotes progression of human oral cancer. *BMC Cancer* **15**:563.
Kong T, Westerman KA, Faigle M, Eltzschig HK, and Colgan SP (2006) HIF-dependent induction of adenosine A2B receptor in hypoxia. *FASEB J* **20**: 2242–2250.
Koole C, Wootten D, Simms J, Valant C, Sridhar R, Woodman OL, Miller LJ, Summers RJ, Christopoulos A, and Sexton PM (2010) Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. *Mol Pharmacol* **78**:456–465.
Kopp P, van Sande J, Parma J, Duprez L, Gerber H, Joss E, Jameson JL, Dumont JE, and Vassart G (1995) Brief report: congenital hyperthyroidism caused by a mutation in the thyrotropin-receptor gene. *N Engl J Med* **332**:150–154.
Leff P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* **16**: 89–97.
Lefkowitz RJ, Cotecchia S, Samama P, and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14**:303–307.
Li S, Huang S, and Peng S-B (2005) Overexpression of G protein-coupled receptors in cancer cells: involvement in tumor progression. *Int J Oncol* **27**:1329–1339.
Liang BT and Haltiwanger B (1995) Adenosine A2a and A2b receptors in cultured fetal chick heart cells. High- and low-affinity coupling to stimulation of myocyte contractility and cAMP accumulation. *Circ Res* **76**:242–251.
Linden J, Thai T, Figler H, Jin X, and Robeva AS (1999) Characterization of human A (2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells. *Mol Pharmacol* **56**:705–713.
Liu G, Duranteau L, Carel J-C, Monroe J, Doyle DA, and Shenker A (1999) Leydig-cell tumors caused by an activating mutation of the gene encoding the luteinizing hormone receptor. *N Engl J Med* **341**:1731–1736.
Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402–408.
Ma D-F, Kondo T, Nakazawa T, Niu DF, Mochizuki K, Kawasaki T, Yamane T, and Katoh R (2010) Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells. *Hum Pathol* **41**:1550–1557.
May LT, Avlani VA, Langmead CJ, Herdon HJ, Wood MD, Sexton PM, and Christopoulos A (2007) Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors. *Mol Pharmacol* **72**:463–476.
Milligan G (2003) Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol* **64**:1271–1276.
Milligan G and IJzerman AP (2000) Stochastic multidimensional hypercubes and inverse agonism. *Trends Pharmacol Sci* **21**:362–363.
Mousavi S, Panjehpour M, Izadpanahi MH, and Aghaei M (2015) Expression of adenosine receptor subclasses in malignant and adjacent normal human prostate tissues. *Prostate* **75**:735–747.
Nakahara T, Maruko T, Sakamoto K, and Ishii K (2004) Influence of receptor number on the cAMP response to forskolin in Chinese hamster ovary cells transfected with human beta2-adrenoceptor. *Biol Pharm Bull* **27**:239–241.
Neilan CL, Akil H, Woods JH, and Traynor JR (1999) Constitutive activity of the δ-opioid receptor expressed in C6 glioma cells: identification of non-peptide δ-inverse agonists. *Br J Pharmacol* **128**:556–562.
Parra S and Bond RA (2007) Inverse agonism: from curiosity to accepted dogma, but is it clinically relevant? *Curr Opin Pharmacol* **7**:146–150.
Polosa R and Blackburn MR (2009) Adenosine receptors as targets for therapeutic intervention in asthma and chronic obstructive pulmonary disease. *Trends Pharmacol Sci* **30**:528–535.
Ryzhov S, Novitskiy SV, Zaynagetdinov R, Goldstein AE, Carbone DP, Biaggioni I, Dikov MM, and Feoktistov I (2008) Host A(2B) adenosine receptors promote carcinoma growth. *Neoplasia* **10**:987–995.
Seifert R and Wenzel-Seifert K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn Schmiedebergs Arch Pharmacol* **366**:381–416.
Semenza GL (2000) Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol* **35**:71–103.
Sommerschild HT and Kirkebøen KA (2000) Adenosine and cardioprotection during ischaemia and reperfusion–an overview. *Acta Anaesthesiol Scand* **44**: 1038–1055.
Strange PG (2002) Mechanisms of inverse agonism at G-protein-coupled receptors. *Trends Pharmacol Sci* **23**:89–95.
Subarsky P and Hill RP (2003) The hypoxic tumour microenvironment and metastatic progression. *Clin Exp Metastasis* **20**:237–250.
Wei Q, Costanzi S, Balasubramanian R, Gao Z-G, and Jacobson KA (2013) A2B adenosine receptor blockade inhibits growth of prostate cancer cells. *Purinergic Signal* **9**:271–280.
Weyler S, Fülle F, Diekmann M, Schumacher B, Hinz S, Klotz K-N, and Müller CE (2006) Improving potency, selectivity, and water solubility of adenosine A1 receptor antagonists: xanthines modified at position 3 and related pyrimido[1,2,3-cd] purinediones. *ChemMedChem* **1**:891–902.
Yan F, Bikbulatov RV, Mocanu V, Dicheva N, Parker CE, Wetsel WC, Mosier PD, Westkaemper RB, Allen JA, and Zjawiony JK, et al. (2009) Structure-based design, synthesis, and biochemical and pharmacological characterization of novel salvinorin A analogues as active state probes of the κ-opioid receptor. *Biochemistry* **48**: 6898–6908.

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JPET Manuscript# 230003

Supplemental Data

Ligand-independent adenosine A_{2B} receptor constitutive activity as a promoter of prostate cancer cell proliferation

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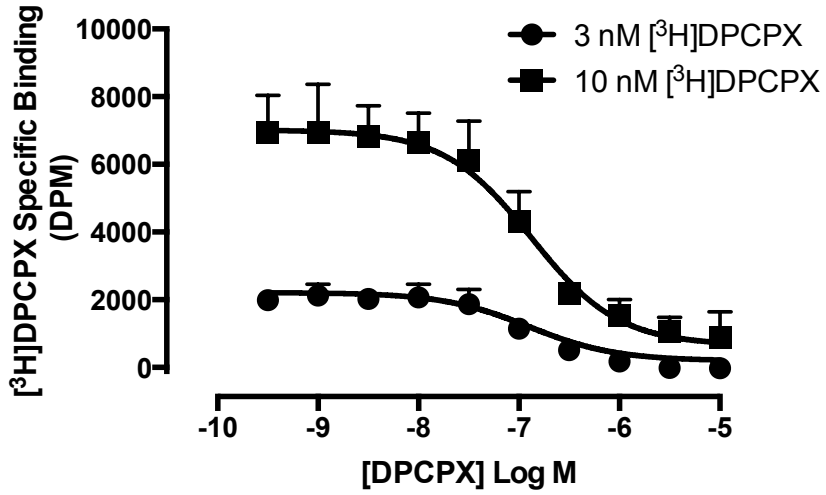
Journal of Pharmacology and Experimental Therapeutics

JPET Manuscript# 230003



Supplemental Figure 1: Homologous [^{3}H]DPCPX competition binding on membrane homogenates from A_{2B}AR-FlpInCHO cell membranes. DPCPX pK_d: 6.87 ± 0.10 and B_{max}: 3.13 ± 0.61 pmol mg protein^{-1}. Data represent the mean + SEM from 4 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

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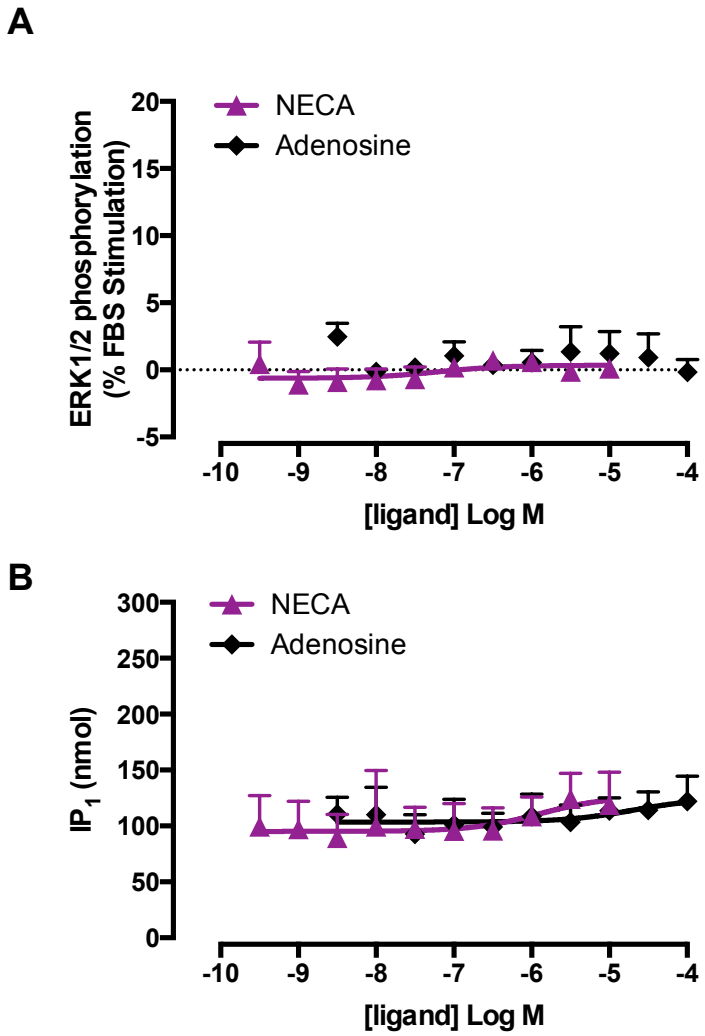

Supplemental Figure 2: G_q-mediated signaling in the 22Rv1 cells was not observed in response to stimulation with A_{2B}AR agonists NECA or adenosine in ERK1/2 phosphorylation (*A*) or IP_1 accumulation (*B*). Data represent the mean + SEM from 2-4 independent experiments performed in duplicate.

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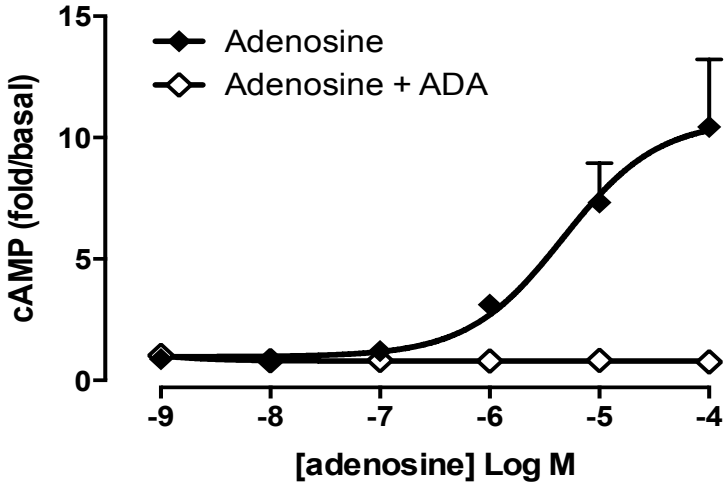

Supplemental Figure 3: ADA-incubated with 22Rv1 cells for 24 h can abolish adenosine-stimulated cAMP accumulation. After 24 h incubation in the absence or presence of 1 U/mL ADA, 22Rv1 cells were exposed to adenosine for 30 min. Data represent the mean + SEM from 3 independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

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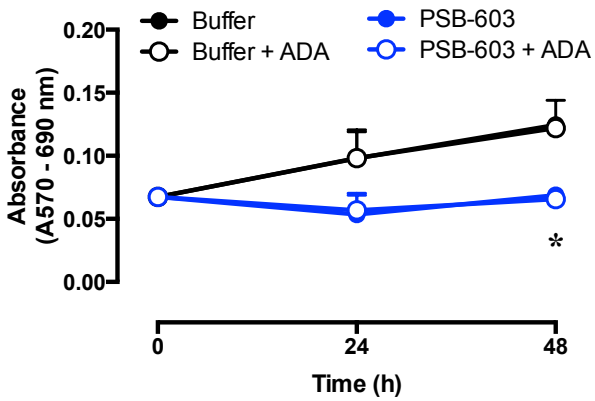

Supplemental Figure 4: The A_{2B}AR inverse agonist PSB-603 decreased proliferation of DU145 prostate cancer cells over 48 h. PSB-603 (1 μM) significantly decreased the proliferation of DU145 cells in the absence (closed symbols) and presence (open symbols) of 1 U/mL ADA as determined by MTT absorbance. **P*<0.05 compared to respective buffer control; two-way ANOVA, Tukey's multiple comparison test. Data represent the mean + SEM from 3 independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

Chapter 3:

Modulation of Adenosine A_1 Receptor-Mediated Cardioprotection by Adenosine A_{2B} Receptors

3.1 Introduction

An acute myocardial infarction or heart attack is defined by regional ischaemia and local tissue death resulting from an imbalance of myocardial blood supply and demand, most commonly from a coronary artery occlusion (Boateng and Sanborn, 2013). Despite significant recent advances in prevention and early intervention with thrombolytics and percutaneous coronary intervention, there remains a large unmet therapeutic need for cardioprotective therapies that limit reperfusion injury which can account for as much as 50% of the final infarct size (Frohlich et al., 2013; Hausenloy and Yellon, 2013; Quintana et al., 2004). The purine nucleoside adenosine represents the most robust and well-characterised modulator of cell survival in the heart, however the translation of adenosine derivatives into the clinic for myocardial ischaemia-reperfusion injury (IRI) is yet to be realised (Headrick and Lasley, 2009; Headrick et al., 2003; McIntosh and Lasley, 2012). This is in part due to the dose-limiting haemodynamic effects on heart rate and blood pressure (Kloner et al., 2006; Kopecky et al., 2003; Ross et al., 2005), but also due to the complexity of adenosine receptor signalling in relation to timing of stimulation and the receptor subtype required (McIntosh and Lasley, 2012; Peart and Headrick, 2007). Adenosine receptors belong to the Class A G protein-coupled receptor (GPCR) family and are comprised of four subtypes, the A_1AR, A_{2A}AR, A_{2B}AR and A_3AR, all of which have been shown to be cardioprotective (Fredholm et al., 2001a; McIntosh and Lasley, 2012).

The A_1AR was the first adenosine receptor subtype to be implicated in the cardioprotective actions of adenosine (Lasley et al., 1990) and it has now been well established that stimulation of the A_1AR prior to ischaemia protects the myocardium from IRI in a variety of animal models (Ashton et al., 2003; Regan et al., 2003; Reichelt et al., 2009; Yang et al., 2002). However, the role of A_1AR activation during reperfusion remains controversial and has prompted the investigation of other adenosine receptor subtypes for their efficacy in limiting the more

clinically relevant reperfusion phase of injury. The A_{2B}AR has been identified as an important modulator of myocardial cell survival and cardioprotection, demonstrated by the ability of an A_{2B}AR selective antagonist to attenuate the infarct-sparing effects of the non-selective agonist, 5'-(N-ethylcarboxamido)adenosine (NECA), both in isolated rat hearts (Xi et al., 2009) and in situ rabbit hearts (Philipp et al., 2006). Importantly, the protection is afforded when the A_{2B}AR is activated immediately prior to, or at the onset of reperfusion (Grube et al., 2011; Kuno et al., 2007; Methner et al., 2010). Interestingly, this A_{2B}AR-mediated reduction in infarct size was often associated with concomitant stimulation of the A_{2A}AR (Lasley et al., 2006; Methner et al., 2010; Xi et al., 2009). Similarly, recent evidence has defined the absolute requirement for coincident activation of the A_2ARs for full A_1AR-mediated cardioprotection (Urmaliya et al., 2009; 2010b; Zhan et al., 2011). The underlying mechanism for such interactions remains to be clarified, potentially reflecting downstream signalling crosstalk or alternatively, the formation of a heteromer or higher order oligomer at the cell surface.

Therefore, the aim of the current study was to investigate functional and binding cooperativity between adenosine receptors in an attempt to understand the molecular basis of how A_{2B}ARs in particular modulate A_1AR-mediated cardioprotection. This was initially performed at the whole organ level using Langendorff-perfused isolated rat hearts, which demonstrated adenosine receptor-mediated reduction of infarct size could be abolished in the presence of either an A_1AR or an A_{2B}AR-selective antagonist. Studies then moved to the cellular level using isolated rat neonatal ventricular cardiomyocytes (NVCMs) and examined the pro-survival kinase pathway of ERK1/2 phosphorylation (pERK1/2) (Hausenloy and Yellon, 2004). The response to the non-selective adenosine receptor agonist NECA could be abolished by an A_1AR-selective antagonist or pertussis toxin, indicating pERK1/2 was downstream of A_1AR-mediated $G_{i/o}$ stimulation. Interestingly, A_1AR-mediated pERK1/2 in cardiomyocytes could be

significantly decreased by the A_{2B}AR antagonist PSB-603, suggestive of functional cooperativity between the A_1AR and A_{2B}AR. Importantly, the [^{3}H]DPCPX dissociation rate from the A_1AR in cardiomyocyte cell membranes was enhanced in the presence of PSB-603, providing robust evidence for allosteric cooperativity across a A_1AR-A_{2B}AR heteromeric interface. To further interrogate the mechanistic basis of these observed interactions, single- and dual-transfected cell lines were generated in the FlpInCHO cell background. Dual-expressing A_1AR-A_{2B}AR cell lines failed to demonstrate any evidence of functional interactions. Therefore, the underlying mechanism for allosteric cooperativity across the A_1AR-A_{2B}AR heteromeric interface could not be fully elucidated.

3.2 Materials & Methods

3.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin, antibiotic-antimycotic, Lipofectamine 2000, pertussis toxin and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). Ultima Gold scintillation cocktail, cyclopentyl-1, 3-dipropylxanthine, 8- [dipropyl-2,3-3H(N)] ([^{3}H]DPCPX), LANCE cAMP and Surefire pERK1/2 kits were purchased from PerkinElmer (Waltham, MA, USA). Type II collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Adenosine receptor ligands, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583) and 8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl))-1-propylxanthine (PSB-603) were from Tocris Bioscience (Bristol, UK). VCP746 was synthesized in-house as described previously (Valant et al., 2014). The QuikChange II site-directed mutagenesis kit was purchased from Agilent (La Jolla, CA) and the Miniprep and Maxiprep DNA kits and MinElute kit were from Qiagen (Hilden, Germany). Primers were purchased from GeneWorks (Hindmarsh, Australia). The SNAP-Surface 488 and

the CLIP-Surface 647 fluorescent dyes were purchased from New England BioLabs (Ipswich, MA, USA). All other reagents were of analytical quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.2 Langendorff-perfused isolated rat hearts

Langendorff perfused isolated rat heart preparation and experimentation was carried out by Chung Hui Chuo (Drug Discovery Biology, Monash University, Melbourne, Australia) as previously described (Urmaliya et al., 2010a). In brief, hearts from adult male Sprague-Dawley rats were quickly excised and perfused with modified Krebs-Henseleit buffer containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM EDTA, 22 mM $NaHCO_3$, 11 mM glucose and 2.5 mM CaCl. The left atrium was removed and a fluid-filled balloon (ADInstruments, Bella Vista, NSW, Australia) connected to a physiological pressure transducer (MLT844, ADInstruments) was placed in the left ventricle. Hearts were allowed to stabilise for 30 min, then subjected to 30 min of no-flow normothermic global ischaemia and 60 min of reperfusion. Compounds were infused at the onset of reperfusion for 15 minutes. Hearts were then frozen, sliced and incubated in 1% 2,3,5-triphenyl-tetrazolium chloride (TTC) solution to distinguish the infarcted regions. Infarct size was expressed as a percentage of the risk area.

3.2.3 Rat neonatal ventricular cardiomyocyte isolation, cell culture and membrane preparation

Isolation of primary neonatal rat ventricular cardiac fibroblasts (NVCFs) from 1-2 day-old Sprague-Dawley rat pups was performed as previously described (Baltos et al., 2017; Laskowski et al., 2006). In brief, hearts were rapidly excised following decapitation and ventricles dissected and placed in Hanks balanced salt solution (HBSS) containing 0.1% trypsin. Ventricles were left for 16 h on a plate shaker at 4°C. Trypsin was deactivated with an

equal volume of FBS and then ventricles were successively digested with type II collagenase (2.5 mg/heart) in HBSS for 10 min in a shaking water bath at 37°C. A total of four digestion steps were performed in order for complete tissue digestion, after which, the supernatants were subsequently pooled and centrifuged for 5 min at 500g. The cell pellet was resuspended in DMEM containing 10% FBS and pre-plated onto 150 mm culture dishes and left for 1 h at 37°C in a 5% CO_2 incubator. Non-adherent cells (neonatal ventricular cardiomyocytes; NVCMs) were removed and transferred to a fresh culture dish for a further 1 h to separate NVCMs from adherent neonatal ventricular cardiac fibroblasts. NVCMs were then collected, counted and plated into 96-well plates for pERK1/2 assays in DMEM containing 10% FBS, 1% antibiotic/antimycotic and 100 μM 5-bromo-2'-deoxyuridine (BrdU), at a density of 3×10^4 cells/well and incubated at 37°C in 5% CO_2 for 24 h. Media was replaced with DMEM containing 10% FBS and 1% antibiotic/antimycotic with experiments performed 72 h later. NVCMs for membrane preparation were grown on 150 mm culture dishes in DMEM with 10% FBS and 1% antibiotic/antimycotic for approximately 72 h at 37°C in a 5% CO_2 incubator until they reached confluency. Cells were harvested and membranes were prepared as previously described (Vecchio et al., 2016b).

3.2.4 Generation and cell culture of SNAP-A_1AR and CLIP-A_{2B}AR cell lines

The SNAP human A_1AR construct and stably expressing FlpInCHO cell line had previously been generated by the laboratory. The human A_{2B}AR was inserted into the pCLIP vector using a double enzyme digest with BamHI and PmeI for N-terminal insertion of the receptor into the vector with the assistance of fellow PhD student Anh T. Nguyen (Drug Discovery Biology, Monash University, Parkville, Australia). Samples were run on a gel, cut out and purified using the Qiagen MinElute gel extraction kit. Ligation of the DNA insert within the vector followed and then was transformed in competent E.Coli cells. Colonies were selected and DNA prepared

with the Wizard Plus SV Miniprep DNA purification kit and ran again on a gel to ensure they contained the insert prior to sequencing. The final step required removal of the start codon at the beginning of the receptor insert using the QuikChange II site-directed mutagenesis kit and primers from GeneWorks. DNA was subsequently prepared using the Qiagen Maxiprep kit. Stable transfection of receptor constructs into FlpInCHO cells was then performed. FlpInCHO cells (either wild type or containing stable expression of the SNAP-A_1AR) were grown in T25cm^2 flasks until 80% confluent. CLIP A_{2B}AR DNA (5 μg) was transfected using Lipofectamine 2000 according to manufacturer's instructions. The following day media was changed to contain the selection agent, hygromycin (500 μg/mL) for cells already containing the SNAP-A_1AR and G418 (750 μg/mL) for the CLIP-A_{2B}AR. Media was changed every 48 h to select for cells containing stable receptor expression. Polyclonal cells were then FACS sorted with sequential labelling of the CLIP (BG-647) and SNAP (BG-488) tagged receptors to select for single cells expressing either the single receptor construct (SNAP-A_1AR-FlpInCHO cells or CLIP-A_{2B}AR-FlpInCHO cells) or dual receptors (SNAP-A_1AR/CLIP-A_{2B}AR-FlpInCHO cell line). Clonal populations were subsequently grown up and functional experiments carried out on these cells.

3.2.5 ERK1/2 phosphorylation (pERK1/2)

NVCMs and transfected FlpInCHO cells were plated at 3×10^4 cells/well and 4×10^4 cells/well, respectively. Prior to assaying NVCMs, cells were incubated for 16 h at 37°C in a 5% CO_2 incubator in serum free media (SFM) containing 50 nM KCl to arrest beating. Transfected FlpInCHO cells were allowed to grow for 6 h before media was changed to SFM in the presence or absence of pertussis toxin (100 ng/mL) and cells incubated for 16 h at 37°C in a 5% CO_2 incubator. Antagonists diluted in SFM were incubated with the cells for 30 min prior to agonist addition. The cells were first exposed to agonists over a 30 min time course to

determine the time corresponding to maximal pERK1/2 (transfected FlpInCHO cells: 5 min for all agonists; NVCMs: 7 min for NECA and MeCCPA, 10 min for BAY60-6583, 5 min for CGS-21680). Detection of pERK1/2 was performed as previously described (Vecchio et al., 2016a; 2016b) and normalised to the response elicited by 10% v/v FBS (5 min exposure).

3.2.6 cAMP accumulation

Inhibition of forskolin stimulated cAMP accumulation in SNAP-A_1AR-FlpInCHO cells and stimulation of cAMP accumulation in CLIP-A_{2B}AR-FlpInCHO cells was performed as previously described (Baltos et al., 2017). Data were analysed against a cAMP standard curve performed in parallel and expressed as cAMP concentration per well.

3.2.7 [^{3}H]DPCPX dissociation kinetics on membrane preparations

Membrane preparations were performed as previously described (Vecchio et al., 2016b). NVCM membranes (150 μg protein/tube) and SNAP-A_1AR-FlpInCHO cell membrane (100 μg protein/tube) was equilibrated for 1 h at 25°C in a 0.5 mL total volume of HEPES-buffered saline solution (25 mM HEPES, 10 mM glucose, 146 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM sodium pyruvate, and 1.3 mM $CaCl_2$, pH 7.4) with 1 U/mL adenosine deaminase (ADA) and 1 nM [^{3}H]DPCPX. A concentration of 1 nM [^{3}H]DPCPX will selectively label A_1ARs, therefore the A_1AR selective antagonist SLV320 (10 μM) was used to define non-specific binding. A final concentration of 1 μM SLV320 in the presence or absence of PSB-603 (100 nM) was added at the indicated time-points (Section 3.3.4 and 3.3.7) using a reverse-time protocol to determine [^{3}H]DPCPX dissociation kinetics.

3.2.8 Data analysis

Statistical analysis and curve fitting were performed using Prism 6 (GraphPad Software, San Diego, CA). Agonist concentration-response data were fitted to a three-parameter Hill equation:

$$Response = Basal + \frac{(E_{max} - Basal) \times [A]}{EC_{50} + [A]} \quad \text{Equation 1}$$

where basal is the baseline response in the absence of agonist, [A] is the concentration of agonist, and EC_{50} is the concentration of agonist required to generate a response halfway between the basal level and maximal effect (E_{max}). Dissociation kinetics of [^{3}H]DPCPX binding was fitted to a one phase exponential decay equation:

$$B_t = B_0 \times e^{-k_{off} \times t} \quad \text{Equation 2}$$

where t denotes incubation time, B_t denotes specific radioligand binding at time t, B_0 denotes the specific radioligand binding at time at equilibrium (time = 0), and k_{off} represents the observed radioligand dissociation rate constant (May et al., 2007a). All results were expressed as the mean ± standard error of the mean (SEM). Statistical significance was defined as $P<0.05$ as determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons post hoc analysis or t-test or global F-test as indicated within the results.

3.3 Results

3.3.1 Adenosine receptor-mediated decrease in infarct size and improvement in cardiac function post ischaemia-reperfusion injury ex vivo requires stimulation of the A_1AR and A_{2B}AR

The first objective of the study was to examine the role of the A_1AR and A_{2B}AR in adenosine receptor-mediated cardioprotection in the whole organ using *ex vivo* Langendorff-perfused isolated rat hearts. This study used the rationally-designed A_1AR/A_{2B}AR agonist; 4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-N-(6-(9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxylmethyl)tetrahydro-furan-2-yl)-9H-purin-6-ylamino)hexyl) benzamide

(VCP746) (Baltos et al., 2016b; Valant et al., 2014; Vecchio et al., 2016a). This is a hybrid molecule consisting of an adenosine moiety linked to an A_1AR allosteric modulator moiety and has previously been shown to mediate cardioprotection in the absence of haemodynamic side effects (Valant et al., 2014). In the current study, VCP746 was shown to significantly decrease infarct size and attenuate the reduction in contractility (dP/dt_{max}) and left ventricular developed pressure (LVDP) observed for vehicle-treated hearts after IRI in the Langendorff-perfused isolated rat heart (Fig. 3.1). Interestingly the beneficial effects of VCP746 could be reversed in the presence of either an A_1AR-selective antagonist (SLV320) or an A_{2B}AR-selective antagonist (PSB-603) suggesting both adenosine receptor subtypes are required for VCP746-mediated cardioprotection (Fig. 3.1).

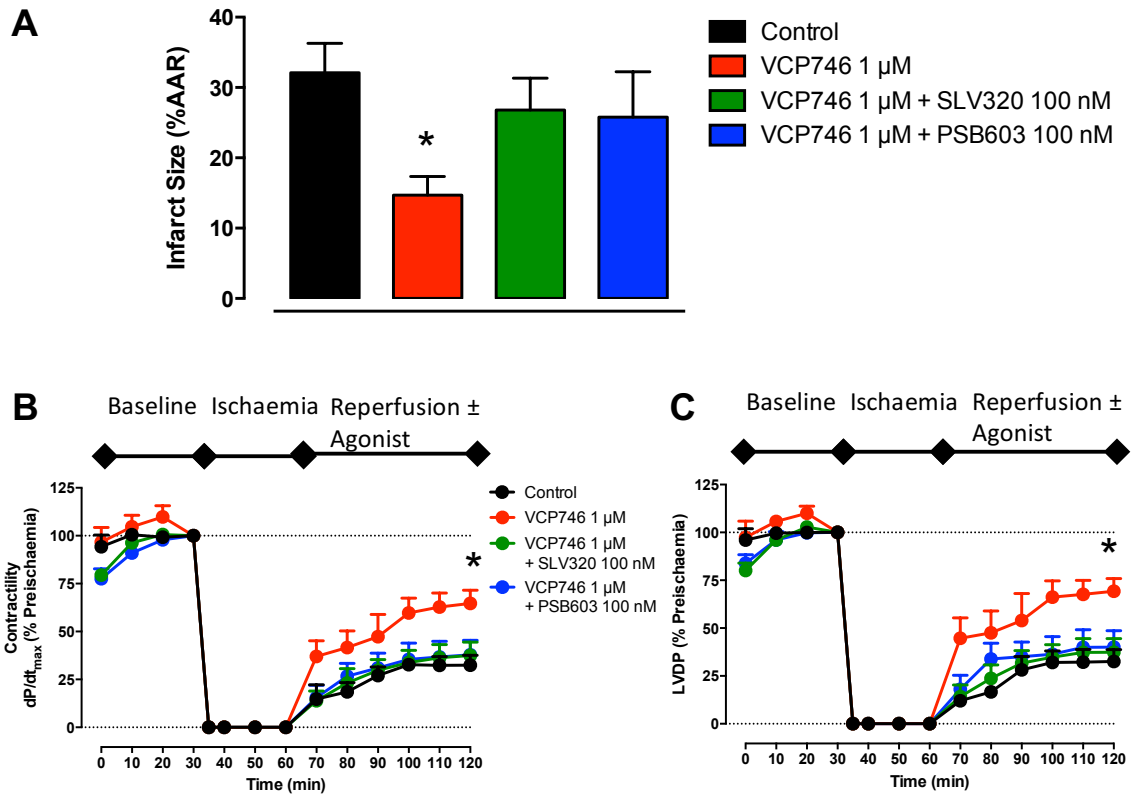

Fig. 3.1. Protective effects of adenosine receptor agonist VCP746 in Langendorff-perfused isolated rat hearts requires coincident activation of A_1AR and A_{2B}ARs. (A) Infarct size was reduced in the isolated rat heart in the presence of the A_1AR/A_{2B}AR agonist, VCP746 (1 µM), at reperfusion compared to the vehicle-treated group; 14.7 ± 2.6% vs 32.1 ±4.2% of area at risk (AAR), respectively. The effect on infarct size reduction could be reversed with either the A_1AR selective antagonist (SLV320 100 nM) or the A_{2B}AR selective antagonist (PSB-630 100 nM). Markers of cardiac function; (B) contractility (dP/dt_{max}) and (C) left ventricular developed pressure (LVDP) after ischaemia-reperfusion were significantly improved with VCP746 treatment compared to vehicle-treated control group. The effect on haemodynamic recovery could be reversed with either the A_1AR selective antagonist (SLV320 100 nM) or the A_{2B}AR selective antagonist (PSB-630 100 nM). *P<0.05; One-way ANOVA with Dunnet's multiple comparisons post-hoc analysis. Data represent the mean + SEM from 5 independent experiments. Experiments carried out by Chung Hui Chuo (Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia).

3.3.2 Functional adenosine receptors can be detected in isolated neonatal ventricular cardiomyocytes

We next examined adenosine receptor-mediated responses at the cellular level in isolated rat NVCMs in the pro-survival kinase pathway of pERK1/2 (Hausenloy and Yellon, 2004). Initially pERK1/2 assays assessed the response to prototypical adenosine receptor agonists in order to establish whether the NVCMs would be a useful model to assess adenosine receptor interactions. A time course was first assessed to determine the time corresponding to maximal pERK1/2 for each agonist (Fig. 3.2A). Agonist-mediated concentration dependent pERK1/2 was detected in the presence of the non-selective adenosine receptor agonist NECA, the A_1AR-selective agonist MeCCPA and the A_{2B}AR-selective ligand BAY60-6583, thus indicating functional A_1AR and A_{2B}ARs are present in NVCMs (Fig. 3.2B). There was no response to the A_{2A}AR-selective agonist CGS-21680 which may be as a result of low A_{2A}AR expression in NVCMs or the poor coupling of the A_{2A}AR to pERK1/2 in this cell background. The response to an A_3AR-selective agonist was not assessed.

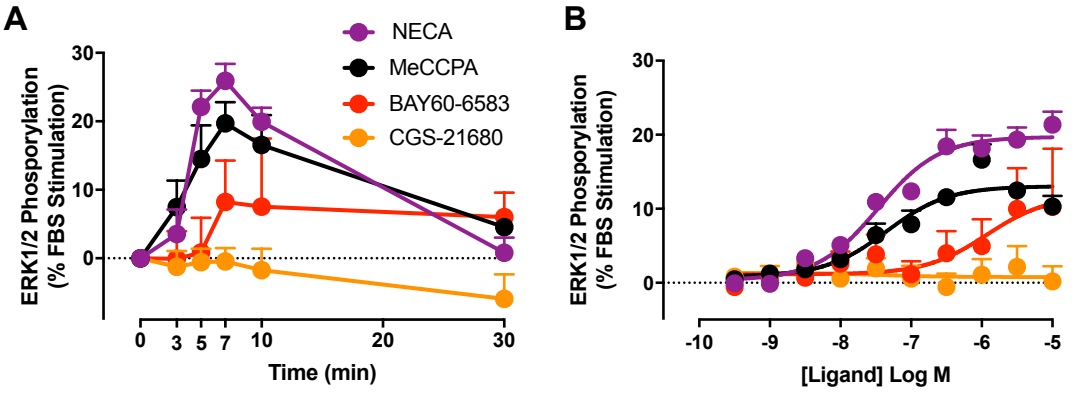

Fig. 3.2. Agonist-mediated ERK1/2 phosphorylation (pERK1/2) demonstrate functional A_1AR and A_{2B}ARs are present in isolated neonatal ventricular cardiomyocytes (NVCMs). (A) Time for peak pERK1/2 determined for each agonist to inform subsequent concentration-response experiments (7 min for NECA and MeCCPA, 10 min for BAY60-6583, 5 min for CGS-21680). (B) The non-selective adenosine receptor agonist NECA, the A_1AR-selective agonist MeCCPA and the A_{2B}AR-selective ligand BAY60-6583 stimulate concentration-dependent pERK1/2 whereas there was no response to the A_{2A}AR agonist CGS-21680. Data represent the mean + SEM from 4-7 independent experiments performed in duplicate.

3.3.3 A_1AR-mediated pERK1/2 in NVCMs is partially inhibited in the presence of the highly selective A_{2B}AR antagonist PSB-603

To investigate potential receptor synergy in the pERK1/2 pathway, interaction studies in NVCMs examined agonist responses in the absence and presence of subtype-selective antagonists (Fig. 3.3). The response to the non-selective agonist NECA could be competitively antagonised by the A_1AR-selective ligand SLV320, indicating virtually full inhibition of A_1AR-mediated signalling. Similarly, the response to NECA could be abolished in the presence of pertussis toxin (Fig. 3.3), confirming adenosine-receptor mediated pERK1/2 is dependent on the stimulation of $G_{i/o}$ proteins downstream of the A_1AR. However, a saturating concentration of the A_{2B}AR antagonist PSB-603 caused a significant 3-fold rightward shift in the NECA-stimulated pERK1/2 (Fig. 3.3; $P < 0.05$; paired t-test on pEC_{50} values). The ability of PSB-603 to modulate A_1AR mediated pERK1/2 is suggestive of functional cooperativity between A_1AR and A_{2B}ARs.

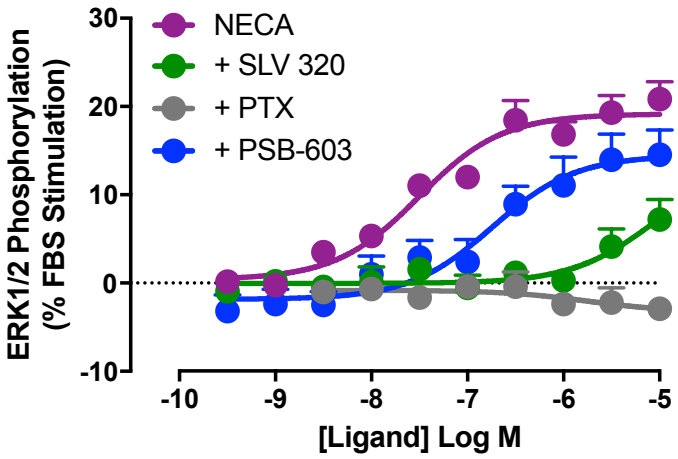

Fig. 3.3. ERK1/2 phosphorylation stimulated in NVCMs upon exposure to the non-selective adenosine receptor agonist NECA, requires A_1AR and A_{2B}AR activation. NECA-mediated pERK1/2 is sensitive to the $G_{i/o}$ inhibitor, pertussis toxin (PTX) (100ng/mL) and virtually abolished in the presence of the A_1AR-selective antagonist, SLV320 (100 nM). NECA-mediated pERK1/2, downstream of A_1AR/$G_{i/o}$ activation, is partially inhibited in the presence of a saturating concentration of the highly selective A_{2B}AR antagonist, PSB-603 (100 nM). Data represent the mean + SEM from 5-6 independent experiments performed in duplicate.

3.3.4 Allosteric cooperativity between A_1AR and A_{2B}ARs in NVCM membranes

The pERK1/2 studies support functional interactions between the A_1AR and A_{2B}AR in ventricular cardiomyocytes. These receptor interactions may be a consequence of downstream signalling crosstalk or from direct interaction of receptors occurring at the binding interface. [^{3}H]DPCPX dissociation kinetics on NVCM membranes were employed to assess the allosteric cooperativity across a potential A_1AR-A_{2B}AR heteromeric interface. The dissociation rate of [^{3}H]DPCPX (k_{off}) from A_1ARs expressed in NVCM membranes was assessed using isotopic dilution to prevent [^{3}H]DPCPX re-association. That is, the addition of a saturating concentration (~1000 times the affinity) of the A_1AR antagonist SLV320 (1 μM) (Fig 3.4). When repeated in the presence of PSB-603 (100 nM), the dissociation rate of [^{3}H]DPCPX was significantly enhanced (Fig. 3.4; [^{3}H]DPCPX k_{off} in the absence (0.12 ± 0.03 min^{-1}) and presence of PSB-603 (0.26 ± 0.07 min^{-1}); P <0.05; F test on globally fitted data). [^{3}H]DPCPX specific binding does not asymptote to 0% which may be due to irreversible binding or may be an artifact of working with a small signal window in primary cells. The change in antagonist dissociation kinetics from the A_1AR in the presence of the highly subtype-selective A_{2B}AR antagonist PSB-603 strongly supports the existence of allosteric cooperativity across an A_1AR-A_{2B}AR heteromer.

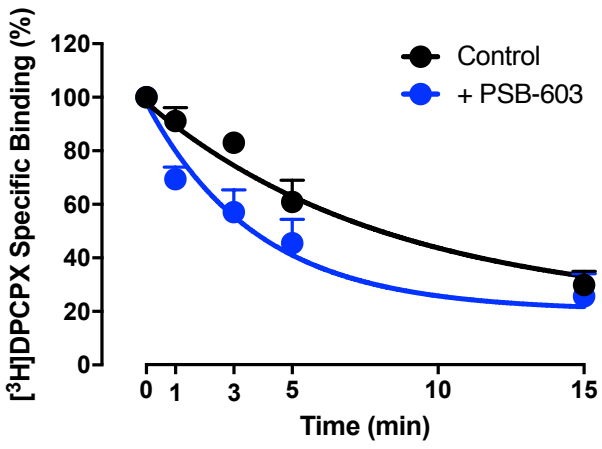

Fig. 3.4. Antagonist dissociation from the A_1AR is allosterically modulated in the presence of a highly selective A_{2B}AR antagonist. Dissociation of the A_1AR antagonist, [^{3}H]DPCPX (1 nM), is significantly enhanced in the presence of the A_{2B}AR antagonist, PSB-603; P<0.05; F test on globally fitted data. Data represent the mean + SEM from 5 independent experiments performed in duplicate.

3.3.5 cAMP accumulation signalling assays confirm the presence of adenosine receptors in generated dual transfected FlpInCHO cells

Our findings using from NVCMs are suggestive of A_1AR-A_{2B}AR heteromerization. To rigorously interrogate the mechanistic basis, stability and ligand dependency of these interactions, a model system was required. We generated dual SNAP tagged A_1AR and CLIP tagged A_{2B}AR stably expressing FlpInCHO cells with the aim of utilising tagged receptors in fluorescent resonance energy transfer (FRET) and other innovative techniques to detect and quantify receptor dimerization. Adenosine receptor signalling was first assessed in the cAMP accumulation assay to profile individual receptors and to confirm the presence of both receptors in the dual-transfected cell lines (Fig. 3.5). Robust inhibition of forskolin stimulated cAMP accumulation was detected in the SNAP-A_1AR-FlpInCHO cells in the presence of NECA or MeCCPA, whereas the A_{2B}AR-selective agonist BAY60-6583 had no effect (Fig. 3.5A). Stimulation of cAMP accumulation in the presence of NECA or BAY60-6583 was detected in the CLIP-A_{2B}AR-FlpInCHO cell line, however agonist responses were relatively low potency, which may suggest lower receptor expression (Fig. 3.5B). In the dual transfected SNAP-A_1AR/CLIP-A_{2B}AR-FlpInCHO cells agonists were assessed both in the presence and absence of forskolin pre-stimulation to test for both $G_{i/o}$ inhibition of cAMP and G_s stimulation of cAMP accumulation. Both A_1AR and A_{2B}AR-mediated responses were observed in the presence of the subtype-selective agonists MeCCPA and BAY60-6583 respectively (Fig. 3.5C). The profile of NECA in the presence of forskolin pre-stimulation demonstrated inhibition of cAMP at low concentrations and stimulation of cAMP accumulation at higher concentrations, suggestive of both $G_{i/o}$ and G_s signalling and the presence of both the A_1AR and A_{2B}AR in the dual transfected cell line (Fig. 3.5C).

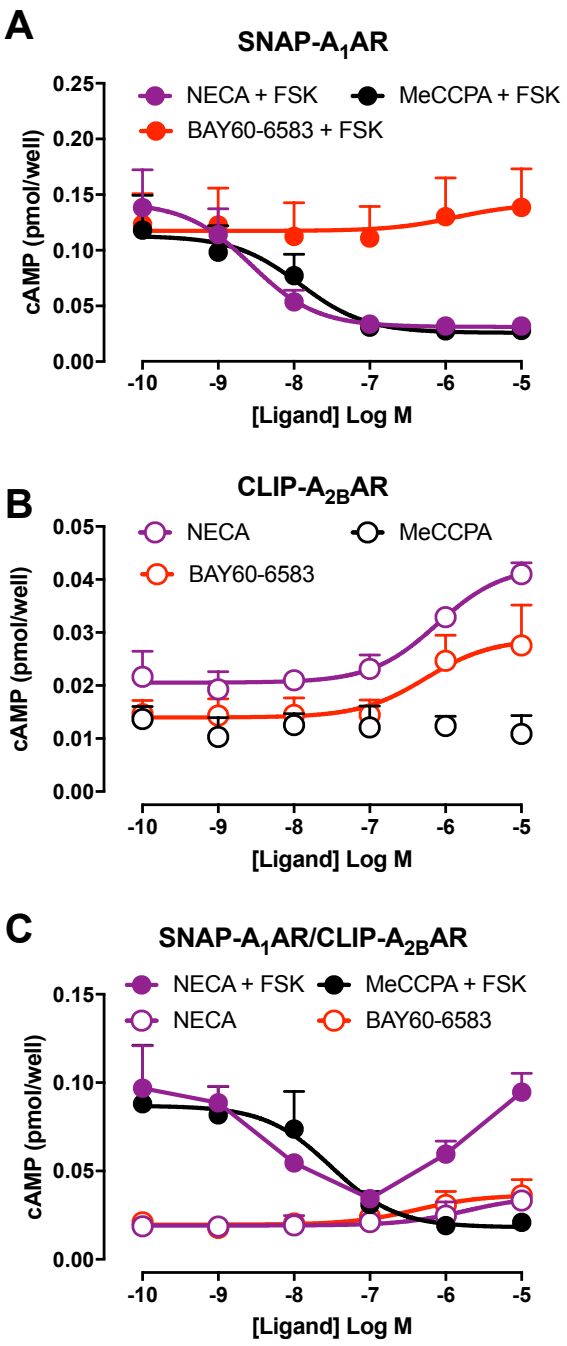

Fig. 3.5. Assessment of cAMP accumulation confirms the presence of functional adenosine receptors in single and dual transfected FlpInCHO cells. (A) Robust inhibition of forskolin (1 μM) stimulated cAMP accumulation was detected in the SNAP-A_1AR cell line after incubation with the non-selective agonist NECA and the A_1AR-selective agonist MeCCPA but not with the A_{2B}AR-selective agonist BAY60-6583. (B) Low levels of cAMP accumulation were observed in the CLIP-A_{2B}AR in response to NECA and BAY60-6583. (C) The SNAP-A_1AR/CLIP-A_{2B}AR cell line generated robust A_1AR-mediated inhibition of cAMP accumulation in response to MeCCPA in the presence of forskolin (FSK; 1 μM) and detectable A_{2B}AR-stimulated cAMP accumulation with BAY60-6583. Both inhibition and stimulation of cAMP accumulation was observed in the presence of the non-selective agonist NECA. Closed symbols represent agonist in the presence of forskolin (FSK; 1 μM) pre-stimulation and open symbols represent agonist alone.

3.3.6 Functional synergy could not be detected at the level of pERK1/2 in model cell line

With the single and dual transfected model cell lines generated, we then assessed whether similar to the NVCMs, functional interactions at the level of pERK1/2 could be observed. As anticipated, the A_{2B}AR antagonist PSB-603 had no effect on NECA stimulated pERK1/2 in the single receptor SNAP-A_1AR cell line and the response to NECA could be completely abolished by the A_1AR antagonist SLV320 (Fig. 3.6A). However, unlike the functional synergy

demonstrated in NVCMs, PSB-603 did not alter the response to NECA stimulation when the A_{2B}AR was present in the dual transfected SNAP-A_1AR/CLIP-A_{2B}AR-FlpInCHO cells (Fig. 3.6B).

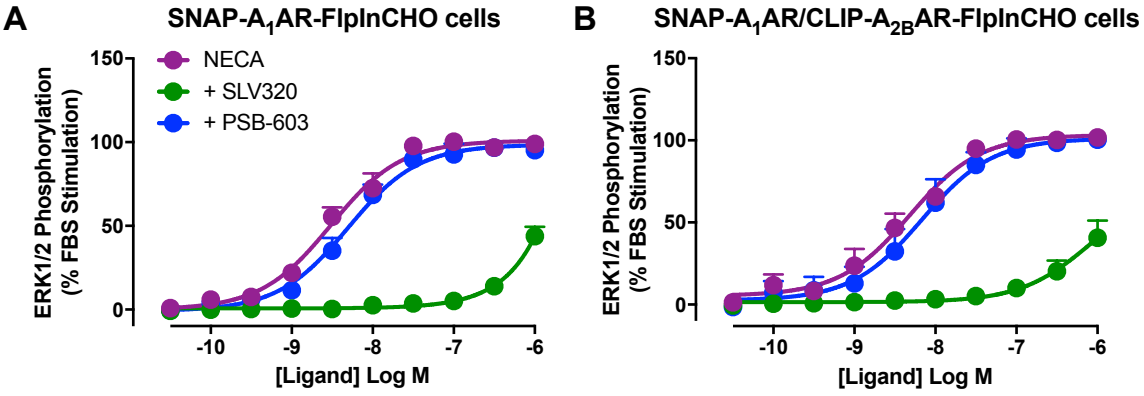

Fig. 3.6. No functional pERK1/2 interactions observed between adenosine receptors in dual transfected FlpInCHO cells. The pERK1/2 response to NECA could be antagonised by SLV320 (100 nM) in the SNAP-A_1AR (A) and SNAP-A_1AR/CLIP-A_{2B}AR (B) cell lines. PSB-603 (100 nM) had no effect on the response to NECA in either cell line. Data represent the mean + SEM from 3-4 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

3.3.7 Allosteric cooperativity between A_1AR and A_{2B}ARs could not be detected in model cell line

Despite not observing functional interactions in pERK1/2, we still wanted to assess if there was any evidence of interactions at the receptor level in the dual transfected cell lines. In contrast to what was observed in the NVCMs, PSB-603 had no effect on the k_{off} of [^{3}H]DPCPX from the A_1AR in the SNAP-A_1AR/CLIP-A_{2B}AR FlpInCHO cell line (Fig. 3.7). As such, there was no evidence of A_1AR-A_{2B}AR cooperativity in the dual transfected FlpInCHO cell line and therefore this model cell line cannot be used to elucidate the mechanism behind functional synergy observed in the whole heart and NVCMs.

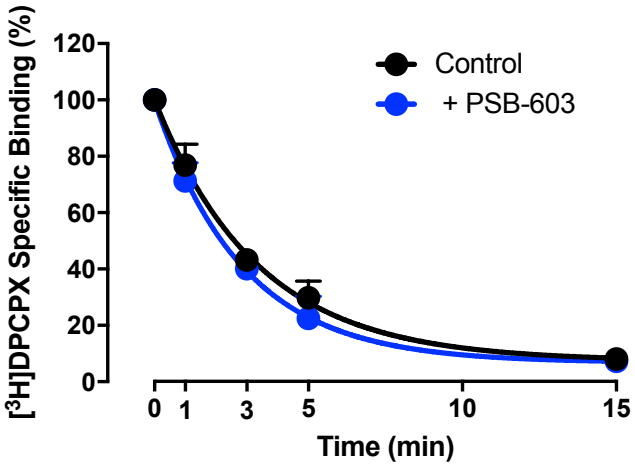

Fig. 3.7. No evidence of adenosine receptor interactions occurring at the receptor level in model cell line. Dissociation kinetics of 1nM [^{3}H]DPCPX is unaltered in the presence of the A_{2B}AR antagonist in the SNAP-A_1AR/CLIP-A_{2B}AR-FlpInCHO cell line. The K_{off} of [^{3}H]DPCPX at the A_1AR (0.30±0.04 min^{-1}) was not significantly different in the presence of PSB-603 (0.36±0.04 min^{-1}). Data represent the mean + SEM from 3 independent experiments performed in duplicate.

3.4 Discussion

This study has investigated the influence of A_1AR-A_{2B}AR interactions on A_1AR-mediated cardioprotection. We have demonstrated in Langendorff-perfused isolated rat hearts that both the A_1AR and A_{2B}AR are required for A_1AR agonist-mediated decreases in infarct size and improvement in cardiac function following IRI. We have also shown at a cellular level, the phosphorylation of the pro-survival kinases ERK1/2 in neonatal ventricular cardiomyocytes (NVCMs) by A_1AR-$G_{i/o}$ stimulation can be modulated by the A_{2B}AR. Collectively these data support recent studies which have suggested activation of the A_2ARs are required for full A_1R-mediated cardioprotection (Urmaliya et al., 2009; 2010b; Zhan et al., 2011). In NVCMs, allosteric cooperativity between A_1AR and A_{2B}AR binding sites was demonstrated by detecting a significant change in the dissociation kinetics of a radiolabelled A_1AR antagonist in the presence of a highly-selective A_{2B}AR antagonist. These finding strongly suggest the ability of A_{2B}AR ligands to modulate A_1AR pharmacology through direct interactions occurring across

an A_1AR-A_{2B}AR heteromeric interface. Unfortunately, our attempts to further interrogate the mechanistic basis of these interactions in a model cell system was unsuccessful.

Physiologically relevant adenosine receptor oligomeric interactions have been identified by evidence gathered largely within the central nervous system. The concept of adenosine receptor interactions has been suggested to be involved in the exquisite control of neurotransmitter activity in the brain and there is growing recognition that adenosine receptors heteromerize (associate in complexes with two or more different receptor subunits) with other GPCRs including the dopamine D_2, the cannabinoid CB_1 and the metabotropic glutamate $mGlu_5$ receptors (Franco et al., 2008; Fredholm et al., 2011). Within the adenosine receptor family, heteromers of the A_1AR and A_{2A}AR have also been implicated in the presynaptic control of glutamatergic neurotransmission and have been detected in recombinant cells with resonance energy techniques and human brain tissue via radioligand binding studies (Casadó et al., 2010; Ciruela et al., 2006b). In the heart, functional interactions of the A_1AR with the δ and κ opioid receptors are proposed to be important in ischaemic preconditioning (Surendra et al., 2013). In addition, heteromeric interactions of the A_1AR with β_1- and β_2-adrenergic receptors leads to complexes with altered receptor pharmacology, functional coupling and intracellular signalling pathways (Chandrasekera et al., 2013). In light of these studies demonstrating the capacity for adenosine receptors to dimerize, it is therefore very plausible that heteromers of the A_1AR and A_2ARs are responsible for the functional synergy observed in adenosine receptor-mediated cardioprotection (Urmaliya et al., 2009; 2010b; Zhan et al., 2011).

One way to examine the presence of heteromers is through changes in dissociation kinetics of a radiolabelled ligand in the presence of subtype-selective antagonists. Studies using the A_{2B}AR antagonist PSB-603, which has virtually no affinity for the human or rat A_1AR (Borrmann et

al., 2009), demonstrated alterations in [^{3}H]DPCPX dissociation kinetics in NVCM membranes. Due to the presence of the sulfonyl-phenyl moiety in PSB-603, which is often contained in many A_1AR allosteric modulators (Fredholm et al., 2011), it would be of interest to examine the effect of other structurally distinct A_{2B}AR antagonists on A_1AR antagonist dissociation kinetics to further interrogate the presence of receptor heteromers. Our data showing alterations in dissociation kinetics in NVCMs would suggest allosteric cooperativity between the two binding sites of the A_1AR and A_{2B}AR and supports a specific functional property for the heteromeric receptor (Pin et al., 2007). In order to confirm these results and further interrogate the mechanism of adenosine receptor interactions, we chose to use heterologous expression of tagged receptors so that activation of the A_1AR in the presence or absence of the A_{2B}AR could be more directly assessed. Unfortunately, in the SNAP-A_1AR/CLIP-A_{2B}AR-FlpInCHO cell line we observed no evidence of adenosine receptor interactions at the level of function or binding as the A_{2B}AR antagonist PSB-603 had no effect on A_1AR-mediated pERK1/2 or [^{3}H]DPCPX dissociation kinetics. A possible explanation for this finding may be that the stoichiometry of A_1ARs to A_{2B}ARs was not reflective of the endogenous levels expressed in NVCMS. The overexpression of recombinant adenosine receptors may also result in changes in G protein coupling which may account for the lack of observed effect in the heterologous cells. Alternatively, the lack of cooperativity may be due to a potential requirement for scaffolding proteins or for the compartmentalisation of adenosine receptors in the lipid rich microdomains of cardiomyocyte caveolae that are not present in FlpInCHO cells (Xiang et al., 2002). Another possibility is that these interactions require more than just the A_1AR and A_{2B}AR to be present. Based on previous studies, full A_1AR-mediated cardioprotection was lost in transgenic animals lacking either the A_{2A}AR or the A_{2B}AR, suggesting all three adenosine receptors are required for cooperative signal transduction (Methner et al., 2010; Urmaliya et al., 2009; Zhan et al., 2011). In fact, experiments demonstrating A_1AR-A_{2A}AR heteromers in a

neurological context have been carried out in brain tissue or transiently transfected HEK cells that are known to also endogenously express the A_{2B}AR (Ciruela et al., 2006a; Cooper et al., 1997). Therefore, it is likely that we have oversimplified the system to such an extent that all interacting partners required for the formation of an oligomeric complex are not present and in order to observe adenosine receptor interactions the system may require, at minimum, the A_1AR, A_{2A}AR and the A_{2B}AR subtypes.

In conclusion, this study has demonstrated that both A_1AR and A_{2B}AR activation is required for A_1AR-agonist mediated reductions in infarct size in *ex vivo* hearts and phosphorylation of pro-survival kinases ERK1/2 in cardiomyocytes *in vitro*. We propose adenosine receptor functional cooperativity may be explained by allosteric interactions occurring across an A_1AR-A_{2B}AR heteromer, which was demonstrated by assessing A_1AR radioligand dissociation kinetics in cardiomyocyte membranes.

Chapter 4:

Capadenoson, a Clinically Trialed Partial Adenosine A_1 Receptor Agonist, can Stimulate Adenosine A_{2B} Receptor Biased Agonism

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Biochemical Pharmacology **2017**, In Press

ARTICLE IN PRESS

Biochemical Pharmacology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Capadenoson, a clinically trialed partial adenosine A_1 receptor agonist, can stimulate adenosine A_{2B} receptor biased agonism

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ARTICLE INFO

Article history:
Received 8 February 2017
Accepted 21 March 2017
Available online xxxx

Chemical compounds studies in the article:
NECA (PubChem CID: 448222)
BAY60-6583 (PubChem CID: 11717831)
Capadenoson (PubChem CID: 9936489)
[^{3}H]DPCPX (PubChem CID: 53319341)
PSB-603 (PubChem CID: 44185871)
SLV320 (PubChem CID: 9953065)
SCH442416 (PubChem CID: 10668061)

Keywords:
Capadenoson
Adenosine A_{2B} receptor
Biased agonist
Cardiomyocytes
Cardiac fibroblasts

ABSTRACT

The adenosine A_{2B} receptor (A_{2B}AR) has been identified as an important therapeutic target in cardiovascular disease, however *in vitro* and *in vivo* targeting has been limited by the paucity of pharmacological tools, particularly potent agonists. Interestingly, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy) phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583), a potent and subtype-selective A_{2B}AR agonist, has the same core structure as 2-amino-6-[[2-(4-chlorophenyl)-1,3-thiazol-4-yl]methylsulfanyl]-4-[4-(2-hydroxyethoxy)phenyl]pyridine-3,5-dicarbonitril (capadenoson). Capadenoson, currently classified as an adenosine A_1 receptor (A_1AR) partial agonist, has undergone two Phase IIa clinical trials, initially in patients with atrial fibrillation and subsequently in patients with stable angina. Capadenoson has also been shown to decrease cardiac remodeling in an animal model of advanced heart failure and a capadenoson derivative, neladenoson bialanate, recently entered clinical development for the treatment of chronic heart failure. The therapeutic effects of capadenoson are currently thought to be mediated through the A_1AR. However, the ability of capadenoson to stimulate additional adenosine receptor subtypes, in particular the A_{2B}AR, has not been rigorously assessed. In this study, we demonstrate that capadenoson does indeed have significant A_{2B}AR activity in physiologically relevant cells, cardiac fibroblasts and cardiomyocytes, which endogenously express the A_{2B}AR. Relative to the non-selective adenosine receptor agonist NECA, capadenoson was a biased A_{2B}AR agonist with a preference for cAMP signal transduction over other downstream mediators in cells with recombinant and endogenous A_{2B}AR expression. These findings suggest the reclassification of capadenoson as a dual A_1AR/A_{2B}AR agonist. Furthermore, a potential A_{2B}AR contribution should be an important consideration for the future clinical development of capadenoson-like therapeutics, as the A_{2B}AR can promote cardioprotection and modulate cardiac fibrosis in heart disease.

Abbreviations: A_1AR, adenosine A_1 receptor; A_{2A}AR, adenosine A_{2A} receptor; A_{2B}AR, adenosine A_{2B} receptor; A_3AR, adenosine A_3 receptor; ADA, adenosine deaminase; AR, adenosine receptor; ANOVA, analysis of variance; BAY60-6583, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide; BrdU, 5-Bromo-2′-deoxyuridine; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; [^{3}H]DPCPX, cyclopentyl-1, 3-dipropylxanthine, 8-[dipropyl-2, 3-^{3}H(N)]; ERK1/2, extracellular signal-regulated kinases 1 and 2; FBS, fetal bovine serum; FlpIn-CHO, Chinese hamster ovary cells with FlpIn^{TM} vector; GPCR, G protein-coupled receptor; HIF-1α, hypoxia-inducible factor 1-alpha; HTRF, homogeneous time resolved fluorescence; IP_1, inositol monophosphate; NECA, 5′-N-ethylcarboxamidoadenosine; NT, non-transfected; NVCF, neonatal ventricular cardiac fibroblast; NVCM, neonatal ventricular cardiomyocyte; pERK1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; PLC, phospholipase C; PSB-603, 8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl)-1-propylxanthine; RTCA, real-time cell analyzer; SCH 442416, 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; SEM, standard error of the mean; VCP746, 4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-*N*-(6-(9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxylmethyl)tetrahydro-furan-2-yl)-9*H*-purin-6-ylamino)hexyl)benzamide.

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http://dx.doi.org/10.1016/j.bcp.2017.03.014
0006-2952/

Please cite this article in press as: J.-A. Baltos et al., Capadenoson, a clinically trialed partial adenosine A_1 receptor agonist, can stimulate adenosine A_{2B} receptor biased agonism, Biochem. Pharmacol. (2017), http://dx.doi.org/10.1016/j.bcp.2017.03.014

1. Introduction

The adenosine A_{2B} receptor (A_{2B}AR) belongs to the adenosine family of G protein-coupled receptors (GPCRs), which also includes the adenosine A_1, A_{2A} and A_3 receptors (A_1AR, A_{2A}AR, A_3AR) [1]. Pleiotropically coupled, activating both G_s and G_q proteins upon ligand stimulation [1,2], the A_{2B}AR is up-regulated by hypoxia-inducible factor-1α (HIF-1α) [3] and implicated in numerous pathologies, including ischemia-reperfusion injury [4–6], fibrosis [7], diabetes [8] and cancer [9–11]. Despite the A_{2B}AR representing a novel therapeutic target for a variety of disease states, there remains a paucity of potent and selective A_{2B}AR agonists. Currently, agonists for the A_{2B}AR can be classified according to the presence or absence of a nucleoside-like core. Adenosine modifications at the N^6 position and C^2 position have generated agonists with increased potency for the A_{2B}AR [12–14]. For example, the N^6 modified adenosine derivative, 5′-N-ethylcarboxamidoadenosine (NECA; Fig. 1), has commonly been used as one of the highest potency A_{2B}AR agonists, despite being non-selective across the different adenosine receptor (AR) subtypes [13,15]. In an effort to enhance subtype selectivity, the focus has shifted to the development of non-nucleoside agonists. Centered around compounds that contain a 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine core, this approach has successfully identified a potent A_{2B}AR agonist, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583; Fig. 1) [16]. Importantly, and in contrast to adenosine-like compounds, the non-nucleoside partial agonist BAY60-6583 shows greater than 3000-fold selectivity for the A_{2B}AR over other AR subtypes [17,18].

In addition to optimizing ligand affinity, efficacy and subtype selectivity, it is becoming increasingly recognized that refining the signaling repertoire stimulated upon receptor activation will likely enhance clinical translation [19,20]. Biased agonism describes the ability of an agonist to stabilize a unique subset of GPCR conformations and thereby modulate intracellular signaling in a manner that is distinct to that of the endogenous ligand [21]. Compounds that explore distinct chemical space, such as the non-nucleoside A_{2B}AR ligands, are likely to stabilize a distinct spectrum of GPCR conformations and subsequently engender biased agonism. At the A_1AR, in contrast to prototypical adenosine-like agonists, structurally distinct agonists such as the bitopic agonist 4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-*N*-(6-(9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxylmethyl)tetrahydro-furan-2-yl)-9*H*-purin-6-ylamino)hexyl) benzamide (VCP746) and the non-nucleoside agonist 2-amino-6-[[2-(4-chlorophenyl)-1,3-thiazol-4-yl]methylsulfanyl]-4-[4-(2-hydroxyethoxy)phenyl]pyridine-3,5-dicarbonitril (capadenoson; Fig. 1) have been shown to engender biased agonism [22,23]. Furthermore, A_{2B}AR homology modeling, based on a high resolution A_{2A}AR crystal structure, suggested that non-nucleoside and nucleoside-containing agonists exhibit different docking conformations [24], which could contribute to biased signaling. In agreement with this suggestion, BAY60-6583 was recently reported to display biased agonism relative to NECA at the A_{2B}AR [25].

Capadenoson, which previously entered into two Phase IIa clinical trials as an A_1AR partial agonist in patients with atrial fibrillation and stable angina, has been purported to be a selective A_1AR agonist with beneficial cardiovascular effects [26–31]. Strikingly, capadenoson shares the same 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine core structure to that of the non-nucleoside A_{2B}AR agonist BAY60-6583 (Fig. 1). The structural similarity with BAY60-6583 raises the possibility that capadenoson may have previously unappreciated A_{2B}AR activity. Furthermore, similar to that observed at the A_1AR, capadenoson may stimulate A_{2B}AR biased agonism and therefore represent a new pharmacological tool to interrogate A_{2B}AR signal transduction.

Therefore, the aim of the current study was to rigorously characterize the pharmacology of capadenoson at the A_{2B}AR. This was initially performed in FlpIn-CHO cells stably transfected with the human A_{2B}AR and then in HEK293T and primary cardiac cells that endogenously express the A_{2B}AR. In addition, the A_{2B}AR signaling profile for capadenoson was investigated across multiple pathways and compared to the non-selective adenosine-like agonist, NECA and the subtype selective non-nucleoside partial agonist, BAY60-6583. Capadenoson was found to promote A_{2B}AR signal transduction with a unique bias profile. Furthermore, capadenoson retained the ability to mediate significant A_{2B}AR activity in primary isolated neonatal rat cardiac myocytes and fibroblasts. Therefore, the ability of capadenoson to stimulate the A_{2B}AR, in addition to the A_1AR, may contribute to the desirable cardiovascular effects previously observed *in vivo*.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), Fluo-4, trypsin, antibiotic/antimycotic and penicillin-streptomycin were purchased

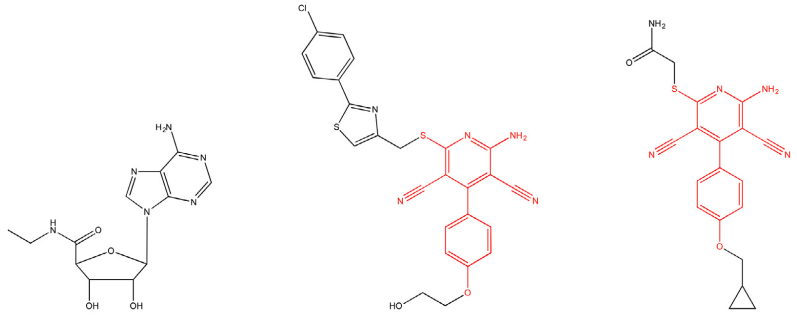

Fig. 1. Structures of the prototypical adenosine-like agonist NECA and the non-nucleoside derivatives capadenoson and BAY60-6583. Capadenoson and BAY60-6583 share the same 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine core (highlighted in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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from Invitrogen (Carlsbad, CA). Adenosine deaminase (ADA) and hygromycin-B were purchased from Roche (Basel, Switzerland). Fetal bovine serum (FBS) was purchased from ThermoTrace (Melbourne, Australia). The IP-One homogeneous time resolved fluorescence (HTRF) kit was obtained from Cisbio Bioassays (Codolet, France). Type II collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). xCELLigence 96-well plates were purchased from ACEA Biosciences (San Diego, CA). AlphaScreen SureFire extracellular signal-regulated kinases 1 and 2 (ERK1/2) kits, AlphaScreen cAMP kits and AlphaScreen LANCE cAMP kits, Ultima Gold scintillation cocktail and cyclopentyl-1, 3-dipropylxanthine, 8-[dipropyl-2, 3-^{3}H(N)] ([^{3}H]DPCPX) were purchased from PerkinElmer (Waltham, MA). Adenosine receptor ligands 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy) phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583), 8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl)-1-propylxanthine (PSB-603) and 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH 442416) were from Tocris Bioscience (Bristol, UK) and capadenoson was purchased from MedChemExpress (Shanghai, China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical quality.

2.2. Cell culture and membrane preparation

Recombinant FlpIn-CHO cells stably expressing the human A_1AR, A_{2A}AR, A_{2B}AR or A_3AR were generated as previously described [32]. HEK293T cells endogenously expressing the A_{2B}AR were purchased from Invitrogen (Carlsbad, CA). All cell lines were grown in DMEM supplemented with 10% FBS and maintained at 37°C in a humidified incubator containing 5% CO_2. In FlpIn-CHO cells, stable AR expression was maintained by the addition of the selection antibiotic hygromycin-B (500 μg/mL). For ERK1/2 phosphorylation, calcium mobilization and IP1 accumulation assays, cells were seeded into 96-well culture plates at a density of 4×10^4 cells/well. After 6 h, cells were washed with serum free DMEM and maintained in serum free DMEM for 12–18 h at 37°C in 5% CO_2 before assaying. For cAMP accumulation assays, cells were seeded into 96-well culture plates at a density of 2×10^4 cells/well and incubated overnight at 37°C in 5% CO_2 prior to assaying. For all assays, HEK293T cells were seeded onto plates pre-coated with poly-D-lysine and HEK293T cell assays were performed in the presence of SCH 442416 (100 nM) to antagonize endogenously expressed A_{2A}AR [33]. A_{2B}AR-FlpIn-CHO cell membranes were prepared as previously described [34].

2.3. Rat neonatal cardiomyocyte and fibroblast isolation

Animal experiments were conducted in accordance with Monash Institute of Pharmacy and Pharmaceutical Sciences animal ethics committee-approved protocols (MIPS.2015.23) and conformed to the requirements of the National Health and Medical Research Council of Australia *Code of Practice for the Care and Use of Animals for Scientific Purposes*. Isolation of primary neonatal rat ventricular cardiomyocytes (NVCMs) and primary neonatal rat ventricular cardiac fibroblasts (NVCFs) was performed as previously described [35]. Briefly, hearts were rapidly excised from 1 to 2 day old Sprague-Dawley rat pups and ventricles dissected and placed in Hanks balanced salt solution containing 0.1% trypsin overnight at 4°C. Following trypsin deactivation, ventricles were successively digested with type II collagenase (2.5 mg/heart) in Hanks balanced salt solution in a shaking water bath at 37°C for 10 min. After the fourth digestion, supernatants were pooled and centrifuged for 5 min at 485 g. The pellet was then resuspended in DMEM containing 10% FBS and pre-plated onto 150-mm culture dishes for 1 h at 37°C in 5% CO_2 to separate neonatal ventricular cardiomyocytes (NVCMs) from neonatal ventricular cardiac fibroblasts (NVCFs). Non-adherent cells (NVCMs) were removed and transferred to a fresh culture dish for a further 1 h of pre-plating prior to counting with a haemocytometer. NVCMs were subsequently plated into 96-well plates in DMEM containing 10% FBS, 1% antibiotic/antimycotic and 100 μM 5-bromo-2′-deoxyuridine (BrdU), at a density of 4×10^4 cells/well and incubated at 37°C in 5% CO_2 for 24 h. Media was replaced with DMEM containing 10% FBS and 1% antibiotic/antimycotic and experiments were performed 72 h later.

Culture dishes containing adherent NVCFs were maintained in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2 humidified incubator and grown to confluence. Cells were passaged after trypsin/versene detachment and subsequently seeded into 96-well plates at a density of 2×10^4 cells/well. For all experiments, NVCFs were used at passage 2 and serum starved for 24 h prior to assaying.

2.4. Radioligand equilibrium competition binding

A_{2B}AR-FlpIn-CHO cell membrane homogenates (100 μg) were incubated in HEPES-buffered saline solution (10 mM HEPES, 146 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1 mM $MgSO_4$, 1.3 mM $CaCl_2$, and 1.5 mM $NaHCO_3$, pH 7.4) containing 1 U/mL ADA and 10 nM [^{3}H]DPCPX in the absence and presence of increasing concentrations of capadenoson for 1 h at room temperature. Non-specific binding was determined in the presence of 100 μM NECA. Incubation was terminated by rapid filtration through 0.9% NaCl pre-soaked Whatman GF/B filters using a membrane harvester (Brandel, Gaithersburg, MD). Filters were washed five times with 2 mL of ice-cold 0.9% NaCl and dried, prior to addition of Ultima Gold scintillation cocktail and determination of radioactivity as described previously [36].

2.5. ERK1/2 phosphorylation

ERK1/2 phosphorylation (pERK1/2) assays were performed as described in detail previously [22]. Briefly, pERK1/2 concentration response curves were generated for A_{2B}AR-FlpIn-CHO and HEK293T cells at 37°C upon exposure of cells to A_{2B}AR agonists for the time corresponding to peak pERK1/2 (NECA: 5 min, BAY60-6583: 5 min, Capadenoson: 7 min). Agonist stimulation was terminated by addition of 50 μL SureFire lysis buffer to each well, followed by AlphaScreen detection as previously described [22]. Agonist concentration-response curves were normalized to the response mediated by the positive control, 10% v/v FBS (5 min exposure).

2.6. Calcium mobilization

Calcium mobilization assays were performed as described in detail previously [22]. Briefly, A_{2B}AR-FlpIn-CHO, pre-incubated for 1 h with 1 μM Fluo4, were stimulated with agonist in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA) and fluorescence measurements were obtained. Agonist concentration-response curves constructed for calcium mobilization were normalized to the response mediated by the positive control, adenosine triphosphate (ATP; 100 μM).

2.7. IP_1 accumulation

Media was removed from 96-well plates and replaced with IP_1 stimulation buffer (10 mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM D-glucose, 50 mM LiCl, pH 7.4) and maintained at 37°C for 30 min. Cells were then exposed to IP_1 stimulation buffer in the absence or presence of agonist

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and incubated for an additional 1 h at 37°C. Agonist stimulation was terminated by the removal of buffer and the addition of 25 μL/well IP-One HTRF lysis buffer. Detection of IP_1 accumulation was performed as described previously [34]. In brief, lysates (14 μL) were transferred to a 384-well proxiplate and a 1:1:40 (v/v/v) dilution of IP_1-d2 conjugate:anti-IP_1 Lumi4™-Tb cryptate conjugate:IP-One HTRF lysis buffer (6 μL) was added to each well. Plates were incubated for 1 h at room temperature before fluorescence (calculated as 665 nm/620 nm ratio) was measured on an EnVision plate reader (PerkinElmer; Waltham, MA, USA). Data were normalized to the maximal response elicited by 10 μM NECA.

2.8. cAMP accumulation

Inhibition of forskolin stimulated cAMP accumulation in A_1AR- and A_3AR-FlpIn-CHO cells was performed as described previously [22,37]. Stimulation of cAMP accumulation in A_{2A}AR- and A_{2B}AR-FlpIn-CHO, and HEK293T cells involved the removal of media and addition of cAMP stimulation buffer (140 mM NaCl, 5 mM KCl, 0.8 μM $MgSO_4$, 0.2 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM D-glucose, 5 mM HEPES, 0.1% BSA, 1 U/mL ADA and 10 μM rolipram, pH 7.4) in the absence or presence of PSB-603 (1 μM), followed by a 30 min incubation at 37°C. Cells were then exposed to agonist for 30 min at 37°C. For NVCFs, media was replaced with stimulation buffer and incubated for 30 min at 37°C, followed by addition of agonist and forskolin (1 μM) for an additional 20 min at 37°C. For NVCMs, media was replaced with stimulation buffer and the A_1AR antagonist, SLV320 (1 μM) and incubated for 1 h at 37°C. Agonist and forskolin (1 μM) were then added for an additional 30 min at 37°C. Stimulation was terminated by removal of cAMP stimulation buffer and addition of 50 μL/well ice-cold 100% ethanol. Subsequent to evaporation of the ethanol, 50 μL cAMP lysis buffer (0.1% BSA, 0.3% Tween-20 and 5 mM HEPES, pH 7.4) was added to each well and plates were agitated for 10 min. Detection of cAMP for FlpIn-CHO cells was performed using AlphaScreen methods as described previously [22]. Detection of cAMP for HEK293T, NVCM and NVCF cells was performed using a LANCE cAMP kit. LANCE cAMP detection involved the addition of 1:200:400 (v/v/v) of Alexa Fluor647-anti cAMP antibody:LANCE cAMP detection buffer:lysate in a 384-well optiplate in a total volume of 15 μL, followed by incubation at room temperature in the dark for 30 min. Subsequently, 10 μL/well of a pre-equilibrated 1:3.5:5000 (v/v/v) dilution of LANCE Eu-W8044 labeled streptavidin beads:biotinylated cAMP:LANCE cAMP detection buffer was added. Plates were further incubated for 1 h at room temperature and fluorescence read on an EnVision plate reader (PerkinElmer; Waltham, MA) using standard LANCE settings. All agonist concentration-response curves were normalized to the response mediated by the positive control, forskolin (FSK; 1, 3 or 10 μM as specified in the results).

2.9. Cellular impedance assay

Agonist-mediated changes in cellular impedance, defined as the cell index, were measured by the xCELLigence real-time cell analyzer (RTCA) single-plate reader (Roche Applied Science and ACEA Biosciences, San Diego, CA). To assess agonist-mediated changes in cellular impedance in NVCMs and NVCFs, media in E-plates was replaced with DMEM containing 10% FBS and placed into the RTCA at 37°C in 5% CO_2 and baseline impedance measured at 10 min intervals. Antagonist or vehicle was then added and cell index values obtained at 15 s intervals for 1 h. Cells were subsequently exposed to agonist or vehicle and cell index values obtained at 15 s intervals for a further 2.5 h. For both NVCMs and NVCFs, data were normalized to the cell index values from the time point immediately prior to agonist or vehicle addition. Data were visualized by plotting the normalized cell index data against time. Agonist-mediated effects on cell index were compared at a single time point, t = 30 min post agonist addition, which corresponded to the approximate time of maximal change in the normalized cell index.

2.10. Data analysis

Statistical analysis and curve fitting were performed using Prism 6 (GraphPad Software, San Diego, CA). Capadenoson inhibition binding data were fit to a one-site inhibition mass action curve:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(X - LogIC_{50})}} \quad (1)$$

where Top is the specific binding of the radioligand in the absence of unlabeled ligand (X), Bottom is the specific radioligand binding equivalent to non-specific binding and IC_{50} is the concentration of unlabeled ligand (X) that reduced the radioligand specific binding by half. The resulting IC_{50} values were converted to dissociation constants, $\log(K_i)$ values, using the Cheng & Prusoff equation [38]. Agonist concentration-response data were fit to a three-parameter Hill equation:

$$Response = Basal + \frac{(E_{max} - Basal) \times [A]}{EC_{50} + [A]} \quad (2)$$

where Basal is the magnitude of the response in the absence of agonist (A) and EC_{50} is the concentration of agonist required to generate a response halfway between Basal and the maximal effect (E_{max}). Signaling bias was quantified by fitting concentration-response curves to a re-parameterization of the operational model of agonism as described previously [22].

All results were expressed as the mean ± standard error of the mean (SEM). Statistical significance was defined as $P < 0.05$ as determined by one-way analysis of variance (ANOVA) with Tukey's or Dunnett's multiple comparisons post hoc analysis or *t*-test as indicated within the results.

3. Results

3.1. Capadenoson has a similar affinity as NECA and BAY60-6583 for the human A_{2B}AR

Given the structural similarity of capadenoson to the prototypical A_{2B}AR agonist BAY60-6583 (Fig. 1), the first objective of the current study was to address whether capadenoson had affinity for the human A_{2B}AR. The affinity of capadenoson, estimated from [^{3}H]DPCPX (10 nM) competition binding on A_{2B}AR-FlpIn-CHO cell membrane homogenates, was in the low micromolar range with a pK_i of 5.49 ± 0.24 (Fig. 2A). The affinity of capadenoson for the human A_{2B}AR was similar to previously published A_{2B}AR affinity values determined under the same conditions for the non-selective adenosine receptor agonist NECA and the A_{2B}AR-selective agonist BAY60-6583 [39], which are conventionally used as the highest affinity A_{2B}AR agonists [18,40].

3.2. Capadenoson stimulates potent A_{2B}AR-mediated cAMP accumulation

As capadenoson had appreciable affinity for the A_{2B}AR, the ability of capadenoson to stimulate A_{2B}AR-mediated signal transduction was assessed. In A_{2B}AR-FlpInCHO cells, NECA, BAY60-6583 and capadenoson stimulated robust concentration-dependent cAMP accumulation, with all ligands being full agonists and equipotent for this well-coupled pathway (Table 1; Fig. 2B).

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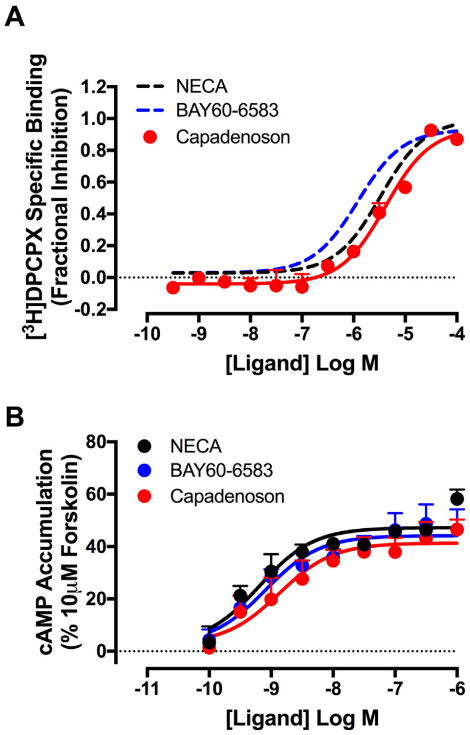

Fig. 2. Capadenoson has affinity and efficacy at the human $A_{2B}AR$. (A) Capadenoson has a similar micromolar affinity as NECA or BAY60-6583 for the $A_{2B}AR$ as determined by [^{3}H]DPCPX radioligand binding on $A_{2B}AR$-FlpInCHO cell membranes. Dashed line represents the non-linear regression of the specific binding for NECA and BAY60-6583, taken from previous publication [39]. (B) Capadenoson stimulation of cAMP accumulation was equipotent and equieffective when compared to the prototypical $A_{2B}AR$ agonists NECA and BAY60-6583 in FlpIn-CHO cells stably expressing the human $A_{2B}AR$. Data represent the mean + SEM from 3 to 5 independent experiments performed in duplicate.

Capadenoson has previously been shown to mediate robust inhibition of cAMP accumulation in A_1AR-FlpInCHO cells. The AR subtype selectivity of capadenoson was investigated by comparing agonist concentration-response curves at the $A_{2A}AR$ and A_3AR to that at the A_1AR and $A_{2B}AR$. $A_{2A}AR$-mediated stimulation and A_3AR-mediated inhibition of cAMP accumulation were assessed in FlpIn-CHO cells stably transfected with the human $A_{2A}AR$ or A_3AR, respectively. In contrast to the robust activity observed at A_1AR and $A_{2B}AR$, capadenoson was a weak to modest activator of the $A_{2A}AR$ and A_3AR (Table 2). As observed previously, BAY60-6583 had strong subtype selectivity for the $A_{2B}AR$, mediating minimal stimulation of the A_1AR, $A_{2A}AR$ and A_3AR (Table 2). Importantly, capadenoson-mediated stimulation of cAMP accumulation was not detected in non-transfected FlpIn-CHO cells (data not shown), confirming the effects observed in the transfected FlpIn-CHO cells were mediated by the $A_{2B}AR$.

3.3. Agonist-mediated signal transduction and biased agonism in $A_{2B}AR$-FlpIn-CHO cells

The $A_{2B}AR$ is pleiotropically coupled and therefore, in addition to cAMP accumulation, a number of additional pathways stimulated by the $A_{2B}AR$ were also investigated [1,2]. These included inositol monophosphate (IP_1) accumulation, intracellular calcium mobilization and stimulation of ERK1/2 phosphorylation (pERK1/2). As previous, these experiments were conducted in $A_{2B}AR$-FlpIn-CHO cells, which allowed for robust detection of signal transduction and quantification of ligand bias.

In contrast to cAMP accumulation, where capadenoson and NECA were equipotent, capadenoson had lower potency than NECA at the other pathways assessed. The rank order of potency for IP_1 accumulation was NECA > BAY60-6583 ≈ capadenoson (Fig. 3A). BAY60-6583 exhibited a partial response for IP_1 accumulation relative to the full agonist NECA, which is in agreement with previous findings that BAY60-6583 behaves as a partial agonist (Table 1) [18,25]. The rank order of potency for both pERK1/2 and calcium mobilization was NECA ≈ BAY60-6583 > capadenoson, with both BAY60-6583 and capadenoson being partial agonists at these pathways (Fig. 3B and C; Table 1).

3.4. Quantification of capadenoson biased agonism in $A_{2B}AR$-FlpIn-CHO cells

It is evident that the rank order of agonist potency at the $A_{2B}AR$ was not maintained across all pathways measured, a hallmark of biased agonism [41,42]. Quantification of biased agonism used a derivation of the Black-Leff operational model of agonism. This equation allows for the estimation of a transduction coefficient, $\text{Log}(\tau/K_A)$, which encompasses both the affinity and efficacy of an agonist for a given pathway. Normalization to a reference ligand, NECA in this study, generates the $\Delta\text{Log}(\tau/K_A)$, which indicates the relative efficiency of the agonist to stimulate a given pathway. Subsequent normalization to a reference pathway, pERK1/2 in this study, generates the $\Delta\Delta\text{Log}(\tau/K_A)$ or Log(Bias Factor), which provides a quantification of the relative bias for each agonist at each pathway assessed (Fig. 4; Table 3).

Relative to pERK1/2, IP_1 accumulation and calcium mobilization, capadenoson had a significant 8– to 20-fold bias towards cAMP accumulation, when normalized to the reference ligand,

Table 1
The potency (pEC_{50}) and maximal effect (E_{MAX}) of AR agonists for different signaling outputs in $A_{2B}AR$-FlpIn-CHO cells. Data represent the mean ± SEM of 3–5 experiments conducted in duplicate or triplicate.

Ligand/Subtype	cAMP pEC_{50} (E_{MAX})	pERK1/2 pEC_{50} (E_{MAX})	IP_1 pEC_{50} (E_{MAX})	iCa^{2+} pEC_{50} (E_{MAX})
NECA	8.61 ± 0.47 (46.27 ± 2.56)	6.50 ± 0.24 (39.37 ± 4.36)	5.76 ± 0.08 (113.20 ± 7.54)	7.12 ± 0.05 (44.48 ± 10.18)
BAY60-6583	8.74 ± 0.42 (44.43 ± 5.91)	6.87 ± 0.26 (24.30 ± 1.66)**	5.05 ± 0.17 (77.73 ± 4.27)*	7.38 ± 0.11 (24.71 ± 5.28)
Capadenoson	8.94 ± 0.33 (43.08 ± 3.93)	6.12 ± 0.46 (8.03 ± 0.89)***	5.03 ± 0.22 (N.D)	6.20 ± 0.22 (14.66 ± 4.37)*

*Significantly different, *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA, Dunnet's post hoc analysis or unpaired *t* test), when compared to the E_{MAX} value of NECA at each respective pathway. N.D. Not determined.

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Table 2
Subtype selectivity of AR agonists (pEC_{50} and E_{MAX} values) in human AR expressing FlpIn-CHO cells. Data were quantified from cAMP concentration-response curves and represent the mean ± SEM of 3–9 experiments conducted in duplicate or triplicate.

Ligand/Subtype	A_1AR pEC_{50} (E_{MAX})	$A_{2A}AR$ pEC_{50} (E_{MAX})	$A_{2B}AR$ pEC_{50} (E_{MAX})	A_3AR pEC_{50} (E_{MAX})
NECA	8.84 ± 0.08* (89.77 ± 2.9)*	7.79 ± 0.10 (84.26 ± 2.71)	8.61 ± 0.47 (46.27 ± 2.56)	8.32 ± 0.15# (80.00 ± 4.07)#
BAY60-6583	6.95 ± 0.87 (7.00 ± 7.54)	N.D N.D	8.74 ± 0.42 (44.43 ± 5.91)	6.32 ± 0.76 (15.77 ± 13.21)
Capadenoson	9.18 ± 0.07* (99.28 ± 3.17)*	5.85 ± 0.08 (74.26 ± 4.02)	8.94 ± 0.33 (43.08 ± 3.93)	6.62 ± 0.53 (43.32 ± 15.10)

N.D. Not determined.
* Denotes data taken from [22].
# Denotes data taken from [37].

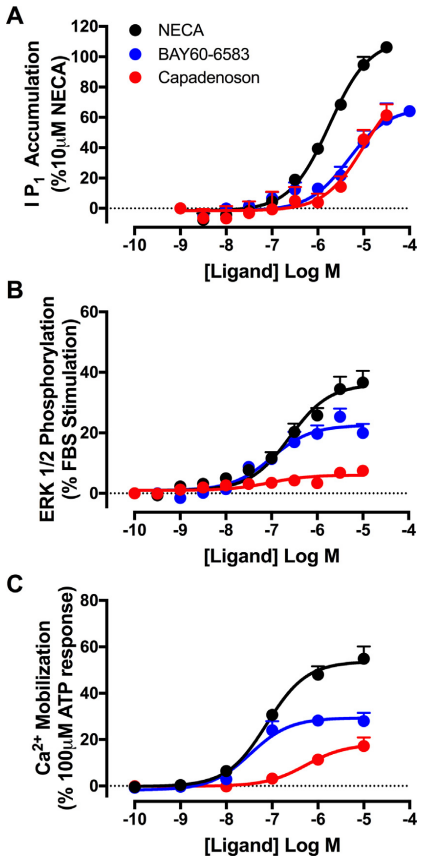

Fig. 3. Capadenoson has relatively low $A_{2B}AR$ potency for (A) IP_1 accumulation, (B) ERK1/2 phosphorylation and (C) intracellular Ca^{2+} mobilization in FlpIn-CHO cells stably expressing the human $A_{2B}AR$. Data represent the mean + SEM from 3 to 5 independent experiments performed in duplicate or triplicate.

NECA (Table 3; $P < 0.05$; One-way ANOVA Tukey's post hoc analysis on the $\Delta Log(\tau/K_A)$). Interestingly, compared to reference agonist NECA, BAY60-6583 showed a significant 5– to 12-fold bias away from IP_1 accumulation relative to the other signaling pathways assessed (Table 3; $P < 0.05$; One-way ANOVA Tukey's post hoc analysis on the $\Delta Log(\tau/K_A)$).

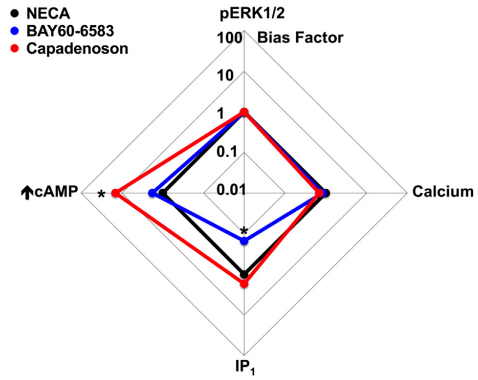

Fig. 4. Capadenoson and BAY60-6583 are $A_{2B}AR$ biased agonists, relative to the reference ligand NECA. The 'web of bias' plots bias factors ($\Delta\Delta(\tau/K_A)$ values) which are calculated by normalizing the transduction ratio (τ/K_A) of each agonist to a reference agonist (NECA) and a reference pathway (pERK1/2). *$P < 0.05$; One-way ANOVA Tukey's post hoc analysis on the $\Delta Log(\tau/K_A)$ (Table 3). Data represent the mean from 3 to 5 independent experiments performed in duplicate or triplicate.

3.5. Agonist-mediated signal transduction in HEK293T cells endogenously expressing the $A_{2B}AR$

A contemporary issue in the field of GPCR biased agonism is how biased agonism changes across different cellular backgrounds and whether biased agonism can be detected and quantified in endogenous expression systems [43]. Although the $A_{2B}AR$-FlpIn-CHO cell line was useful for rapid screening of biased agonism, subsequent studies investigated the translation of bias from a heterologous expression system into cells endogenously expressing the human $A_{2B}AR$. These studies used HEK293T cells, a human embryonic kidney cell line which endogenously expresses the $A_{2B}AR$ and $A_{2A}AR$ [33]. Exposure of HEK293T cells to NECA, BAY60-6583 or capadenoson stimulated robust cAMP accumulation, with all ligands behaving as full agonists (Fig. 5A; Table 4). The cAMP accumulation was $A_{2B}AR$-mediated as the response was virtually abolished in the presence of PSB-603 (1 μM), a highly selective $A_{2B}AR$ antagonist (Fig. 5A). Similar to that observed in $A_{2B}AR$-FlpIn-CHO cells, BAY60-6583 and capadenoson stimulated a partial pERK1/2 response relative to NECA, with capadenoson having relatively low potency (Fig. 5B; Table 4). $A_{2B}AR$-mediated accumulation of IP_1 and calcium mobilization could not be detected in HEK293T cells (data not shown), which may be due to a lower $A_{2B}AR$ expression in these cells compared to the heterologous $A_{2B}AR$-FlpIn-CHO cell line.

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Table 3
Quantification of biased agonism in A_{2B}AR-FlpIn-CHO cells. An operational model was used to estimate agonist transduction coefficients (Log(τ/K_A)) for each signaling output. Normalized transduction coefficients (ΔLog(τ/K_A)) and Log(bias factors), as described within the methods, were used to quantify biased agonism. Data represent the mean ± SEM of 3–5 separate experiments conducted in duplicate or triplicate.

Ligand	Parameter	cAMP	pERK1/2	IP_1	iCa^{2+}
NECA	Log(τ/K_A)	8.62 ± 0.43	6.50 ± 0.27	5.74 ± 0.08	7.13 ± 0.04
	ΔLog(τ/K_A)	0	0	0	0
	Log(bias factor)	0 (1.0)	0 (1.0)	0 (1.0)	0 (1.0)
BAY60-6583	Log(τ/K_A)	8.87 ± 0.39	6.49 ± 0.36	4.90 ± 0.27	7.12 ± 0.14
	ΔLog(τ/K_A)	0.25 ± 0.08	−0.02 ± 0.09	−0.84 ± 0.19*	−0.02 ± 0.11
	Log(bias factor)	0.26 ± 0.10 (1.82)	0 (1.0)	−0.82 ± 0.19 (0.15)	−0.07 ± 0.11 (0.85)
Capadenoson	Log(τ/K_A)	8.52 ± 0.46	5.24 ± 0.14	4.71 ± 0.12	5.71 ± 0.30
	ΔLog(τ/K_A)	−0.10 ± 0.11*	−1.26 ± 0.14	−1.03 ± 0.05	−1.42 ± 0.27
	Log(bias factor)	1.16 ± 0.11 (14.45)	0 (1.0)	0.23 ± 0.05 (1.70)	−0.16 ± 0.274 (0.69)

Bias factors are shown in parentheses.
* Significantly different, $P < 0.05$ (One-way ANOVA, Tukey's post hoc analysis), compared to the ΔLog(τ/K_A) for the corresponding agonist at all other signaling pathways.

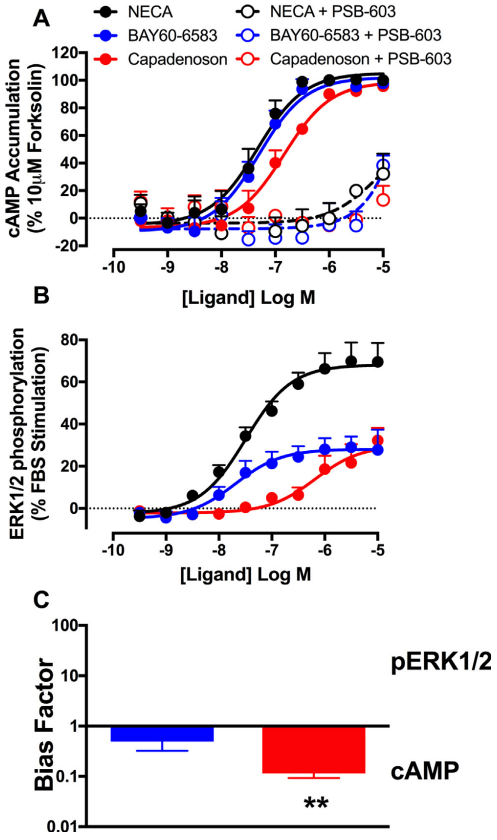

Fig. 5. Capadenoson acts as a biased agonist at A_{2B}ARs endogenously expressed in HEK293T cells. The robust cAMP accumulation stimulated by the adenosine receptor agonists NECA, BAY60-6583 and capadenoson (solid lines and closed symbols) in HEK293T cells (A) was virtually abolished in the presence of the A_{2B}AR antagonist PSB-603 (1 μM) (dashed lines and open symbols). (B) BAY60-6583 and capadenoson stimulated a partial pERK1/2 response relative to NECA. (C) The bias factors of BAY60-6583 and capadenoson, quantified relative to the reference ligand, NECA, and the reference pathway, pERK1/2, demonstrated that capadenoson exhibited a significant bias towards cAMP accumulation over pERK1/2. **$P < 0.01$, unpaired *t*-test on ΔLog(τ/K_A). Data represent the mean + SEM from 3 to 5 independent experiments performed in duplicate.

Table 4
The potency (pEC_{50}) and maximal effect (E_{MAX}) of AR agonists for cAMP accumulation and ERK1/2 phosphorylation in HEK293T cells. Data represent the mean ± SEM of 3–5 experiments conducted in duplicate.

Ligand/Subtype	cAMP pEC_{50} (E_{MAX})	pERK1/2 pEC_{50} (E_{MAX})
NECA	7.34 ± 0.18 (105.9 ± 1.65)	7.52 ± 0.09 (68.08 ± 7.96)
BAY60-6583	7.27 ± 0.14 (103.0 ± 1.06)	7.64 ± 0.18 (27.90 ± 5.38)*
Capadenoson	6.85 ± 0.08 (98.89 ± 1.99)	6.60 ± 0.14 (22.87 ± 8.41)*

* Significantly different, $P < 0.05$ (one-way ANOVA, Dunnet's post hoc analysis), when compared to the E_{MAX} value of NECA at each respective pathway.

Table 5
Quantification of biased agonism in HEK293T cells. An operational model was used to estimate agonist transduction coefficients (Log(τ/K_A)) for each signaling output. Normalized transduction coefficients (ΔLog(τ/K_A)) and Log(bias factors), as described within the methods, were used to quantify biased agonism. Data represent the mean ± SEM of 3–5 separate experiments conducted in duplicate.

Ligand	Parameter	pERK1/2	cAMP
NECA	Log(τ/K_A)	7.49 ± 0.07	7.38 ± 0.17
	ΔLog(τ/K_A)	0	0
	Log(bias factor)	0 (1.0)	0 (1.0)
BAY60-6583	Log(τ/K_A)	7.06 ± 0.22	7.25 ± 0.14
	ΔLog(τ/K_A)	−0.44 ± 0.18	−0.13 ± 0.11
	Log(bias factor)	0 (1.0)	0.31 ± 0.11 (2.04)
Capadenoson	Log(τ/K_A)	6.00 ± 0.13	6.83 ± 0.09
	ΔLog(τ/K_A)	−1.49 ± 0.09*	−0.55 ± 0.11
	Log(bias factor)	0 (1.0)	0.94 ± 0.11 (8.71)

Bias factors are shown in parentheses.
* Significantly different, $P < 0.05$ (*t*-test), when compared to the ΔLog(τ/K_A) estimated for the corresponding agonist for cAMP.

The bias profiles of BAY60-6583 and capadenoson were quantified relative to the reference ligand, NECA, and the reference pathway, pERK1/2, as described in section 3.4. Capadenoson exhibited an approximate 9-fold bias towards cAMP accumulation over pERK1/2 (Fig. 5C; Table 5; $P < 0.01$, unpaired *t*-test on ΔLog(τ/K_A)), which is comparable to the 14-fold bias towards cAMP accumulation observed in A_{2B}AR-FlpIn-CHO cells.

3.6. Capadenoson stimulates A_{2B}AR signaling in cardiomyocytes and cardiac fibroblasts

The therapeutic effects of capadenoson have been investigated in a number of cardiovascular diseases including angina, atrial

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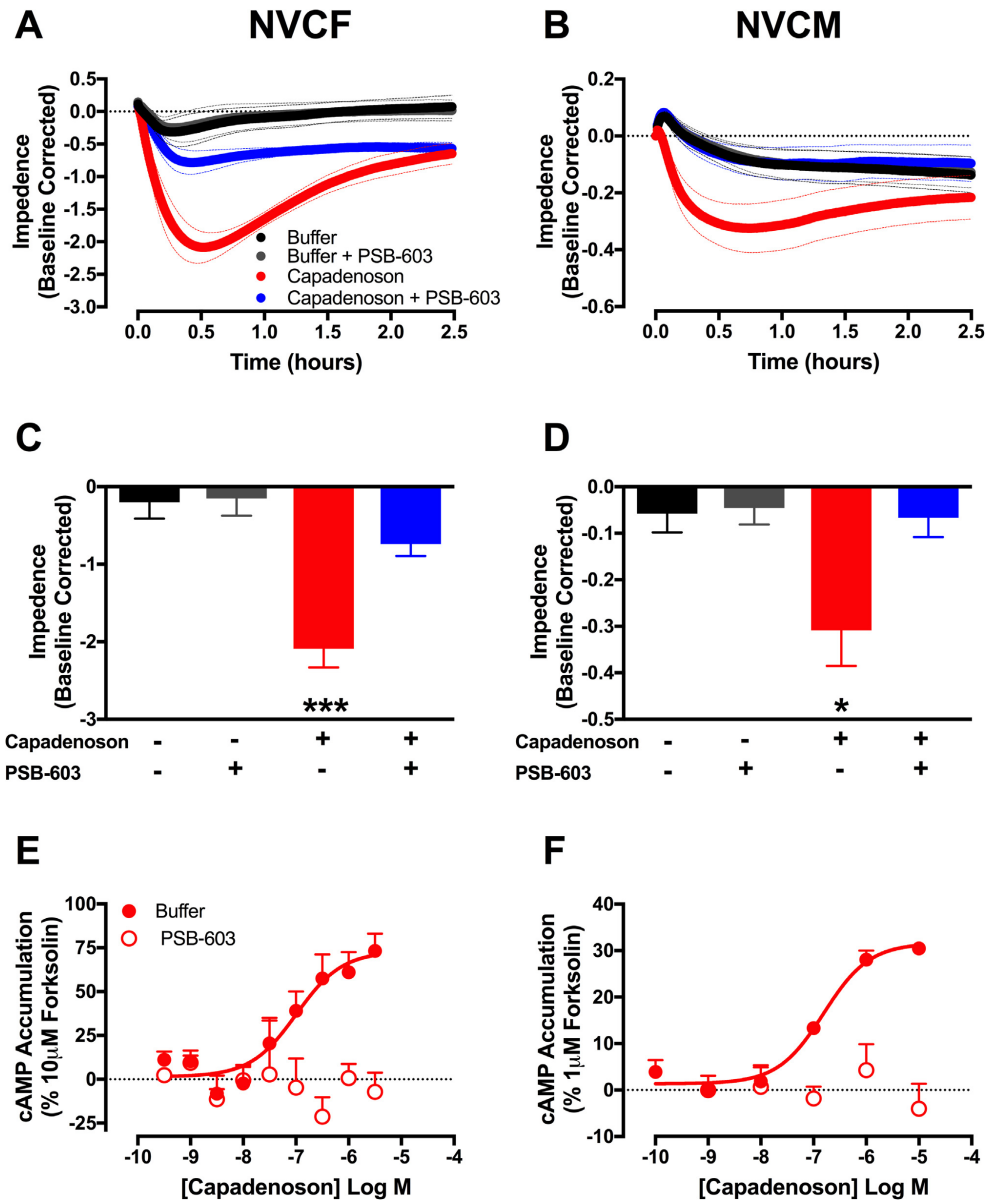

Fig. 6. Capadenoson can stimulate robust $A_{2B}AR$-mediated signal transduction in primary isolated neonatal ventricular cardiac fibroblasts (NVCFs) and cardiomyocytes (NVCMs). Real-time global cellular changes in response to the AR ligands were assessed in an impedance-based assay (xCELLigence assay). Capadenoson (1 μM) stimulated a significant change in impedance relative to the buffer control in both (A and C) NVCFs and (B and D) NVCMs. In (C) NVCFs and (D) NVCMs, the maximal effect of capadenoson, which occurred approximately 30 min after agonist addition was significantly reduced in the presence of the $A_{2B}AR$ antagonist PSB-603 (1 μM). Capadenoson stimulated robust cAMP accumulation in (E) NVCFs and (F) NVCMs that was abolished in the presence of PSB-603 (1 μM). $^{*}P < 0.05$; $^{***}P < 0.001$; One-way ANOVA Dunnett's post hoc analysis. Data represent the mean + SEM from 3 to 5 independent experiments performed in duplicate or triplicate.

fibrillation and heart failure [26,27,29,30]. Within these studies, the disease-modulating capacity of capadenoson was ascribed to signal transduction downstream of the A_1AR. However, given that capadenoson is a biased $A_{2B}AR$ agonist that can stimulate potent cAMP accumulation in $A_{2B}AR$-FlpIn-CHO and HEK293T cells, the question remains as to whether capadenoson-mediated $A_{2B}AR$

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signaling can be observed in cardiac cells that endogenously express the A_{2B}AR. Therefore, capadenoson-mediated A_{2B}AR signal transduction was investigated in isolated neonatal ventricular cardiomyocytes (NVCMs) and neonatal ventricular cardiac fibroblasts (NVCFs). Global cellular changes were assessed using a label-free impedance-based technology, the xCELLigence assay, that assesses changes in the electrical impedance of cells adhered to a microelectrode array. Capadenoson (1 μM) stimulated a significant change in impedance relative to buffer control in both NVCMs and NVCFs (Fig. 6A and B). The significant decrease in cellular impedance stimulated by capadenoson was reduced in the presence of the A_{2B}AR antagonist PSB-603 (1 μM) (Fig. 6C and D; $P < 0.05$; One-way ANOVA Dunnett's post hoc analysis). In addition to the A_{2B}AR-mediated change in cellular impedance, capadenoson also stimulated robust cAMP accumulation in NVCFs and NVCMs (Fig. 6E and F), which was virtually abolished in the presence of PSB-603 (1 μM). These results demonstrate that capadenoson can stimulate appreciable A_{2B}AR-mediated signal transduction in physiologically relevant cells.

4. Discussion

Capadenoson, characterized as an A_1AR partial agonist with cardioprotective pharmacology, has progressed into two Phase IIa clinical trials, the first in patients with atrial fibrillation and the second in patients with stable angina [25,29]. Furthermore, neladenoson bialanate, a capadenoson-like compound, is currently under clinical development as a novel selective A_1AR partial agonist for the treatment of heart failure [53]. The current study reveals, for the first time, that capadenoson is a potent A_{2B}AR agonist and furthermore it has biased agonist activity at the A_{2B}AR. We have demonstrated that capadenoson stimulates A_{2B}AR-mediated signal transduction in both recombinant and endogenously expressing cell lines as well as primary isolated cardiac myocytes and fibroblasts. The signaling profile of capadenoson diverged from that of the non-selective adenosine receptor agonist, NECA, and the A_{2B}AR-selective agonist BAY60-6583. Relative to NECA, capadenoson was significantly biased towards stimulation of cAMP accumulation compared to other second messenger pathways.

The A_{2B}AR is described as a low-affinity receptor due to its modest affinity for the cognate ligand adenosine and other prototypical adenosine receptor agonists [44,45]. The current paucity in pharmacological tools has contributed to a limited understanding of the A_{2B}AR relative to the other AR subtypes. The identification of BAY60-6583, the first subtype-selective A_{2B}AR agonist, enabled a clearer understanding of the physiological role of the A_{2B}AR and identified it as a key pharmacological target for a number of conditions including cardiac ischemia-reperfusion injury and fibrosis [4,16,17]. A similar 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine derivative, capadenoson, was subsequently synthesized by Bayer and classified as an A_1AR-selective agonist [26,29,31]. However, our study using [^{3}H]DPCPX binding, has demonstrated that capadenoson has A_{2B}AR affinity in the low micromolar range, similar to the A_{2B}AR affinity of both NECA and BAY60-6583 [39]. Interestingly, a recent study characterizing a range of novel capadenoson derivatives failed to observe significant inhibition in the binding of the radiolabelled A_{2B}AR antagonist, [^{3}H]PSB-603, in the presence of 1 μM capadenoson [28]. The negligible A_{2B}AR activity would appear to contradict our data demonstrating moderate A_{2B}AR affinity, however, the divergence likely reflects the disparity between the affinity of the different radiolabelled antagonists employed. Specifically, at the A_{2B}AR, the affinity of [^{3}H]PSB-603 is approximately 100-fold higher than that of [^{3}H]DPCPX [46,47]. Therefore, the lack of significant [^{3}H]PSB-603 inhibition may be a consequence of the low affinity of capadenoson relative to the high affinity antagonist [^{3}H]PSB-603 and may explain why capadenoson affinity and/or activity at the A_{2B}AR has not been reported previously.

G_s-stimulated cAMP accumulation is an efficiently-coupled pathway downstream of the A_{2B}AR. Capadenoson stimulated robust cAMP accumulation with a similar potency and maximal effect as the potent A_{2B}AR agonists NECA and BAY60-6583. In addition to the effects on cAMP accumulation, capadenoson also stimulated multiple other signaling pathways in A_{2B}AR-FlpIn-CHO cells, including IP_1 accumulation, ERK1/2 phosphorylation and intracellular calcium mobilization. However, in contrast to the response observed for cAMP accumulation, capadenoson had lower potency than NECA for the other pathways assessed and behaved as a partial agonist in pERK1/2 and intracellular Ca^{2+} mobilization. These divergent effects are reflected in the bias analysis of the A_{2B}AR-FlpIn-CHO concentration response curves, demonstrating that relative to the other pathways assessed, capadenoson was significantly biased towards cAMP accumulation, when normalized to the reference agonist NECA. Importantly, the bias of capadenoson towards cAMP accumulation relative to pERK1/2 was recapitulated in HEK293T cells that endogenously express A_{2B}AR. Interestingly, although structurally similar, the bias profile of BAY60-6583 in A_{2B}AR-FlpIn-CHO cells was different to that observed for capadenoson. Specifically, BAY60-6583 was significantly biased away from IP_1 accumulation relative to all other pathways assessed. In HEK293T cells, no significant IP_1 accumulation could be detected upon stimulation of endogenously expressed A_{2B}ARs and therefore the biased agonism of BAY60-6583 away from IP_1 accumulation could not be further investigated. The absence of significant IP_1 accumulation may reflect a lower A_{2B}AR expression in HEK293T cells compared to A_{2B}AR-FlpIn-CHO cells. Capadenoson and BAY60-6583, which have a 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine non-nucleoside core, both stimulate biased agonism relative to the prototypical adenosine-like NECA, supporting the suggestion that structurally distinct compounds have the potential to stimulate physiological responses that differentiate from that of the endogenous agonist. Indeed, previous structure-activity relationship studies of dicarbonitrile pyridines indicated that differential side-chain substitutions could not only affect adenosine receptor selectivity, but also have significant effects on agonist efficacy [13]. Accordingly, it would be of interest to further interrogate the structure-activity relationship of dicarbonitrile pyridines with respect to biased agonism.

Biased agonists are thought to enrich a unique spectrum of GPCR conformations relative to that of the endogenous agonist. Biased agonism has considerable therapeutic potential as it provides the opportunity to selectively stimulate desired activity whilst eschewing detrimental signaling [20,48]. However, for effective translation, pathways responsible for the therapeutic and adverse effects must be rigorously assessed in the relevant tissue and/or cell type. The A_{2B}AR has been identified as a novel target for the treatment of ischemia-reperfusion injury in many organs, including the heart [4–6]. In addition, A_{2B}AR signaling has an important role in modulating cardiac fibrosis, however, both pro- and anti-fibrotic effects have been observed [7,49,50]. Intriguingly, the divergent fibrotic effects may arise from differential effector coupling to the pleiotropic A_{2B}AR. In cardiac (myo)fibroblasts, cAMP production stimulated by G_s-coupled GPCRs has been shown to inhibit proliferation and collagen synthesis. In contrast, stimulation of $G_{q/11}$-coupled GPCRs, such as the angiotensin II type 1 receptor, promotes fibrosis [51,52]. As such, an A_{2B}AR agonist with significant bias towards G_s-mediated cAMP accumulation relative to G_q-mediated IP_1 accumulation or Ca^{2+} mobilization may selectively stimulate anti-fibrotic signal transduction. This study has demonstrated that in cardiac cells, A_{2B}AR-mediated global cellular changes and cAMP accumulation can be detected in response to

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capadenoson. Furthermore, in a heterologous expression system, capadenoson displays an A_{2B}AR bias profile which may be conducive to a potential anti-fibrotic agent. Future studies will investigate the bias profile of capadenoson in cardiac cells. Interestingly, a recent study demonstrated capadenoson exerted anti-fibrotic effects *in vivo* in dogs with heart failure [29], which were attributed to an A_1AR-mediated effect. However, in light of the A_{2B}AR biased agonism observed for capadenoson in the current study, A_{2B}AR-mediated signaling likely contributes to the observed decrease in fibrosis. Notably, a reclassification of adenosine receptor subtype selectivity as a result of atypical cardiovascular pharmacology has occurred previously for clinically trialed compounds. Specifically, the cardioprotection stimulated by AMP579, originally characterized as an A_1AR/A_{2A}AR agonist, was found to be mediated by the A_{2B}AR, as selective antagonism of A_{2B}AR abolished protective effects in rabbit hearts [53].

In conclusion, we have demonstrated that capadenoson stimulates appreciable signal transduction downstream of A_{2B}ARs endogenously expressed in physiologically relevant cells, including cardiomyocytes and cardiac fibroblasts. Furthermore, this study has also demonstrated that capadenoson has a biased agonist profile relative to the adenosine-like agonist, NECA. Our findings highlight that capadenoson should be reclassified as a dual A_1AR/A_{2B}AR agonist and future studies should investigate whether some of the desirable cardiovascular effects of capadenoson can be attributed to the hitherto unappreciated A_{2B}AR activity. The clinical relevance of this pharmacological reclassification is topical as a capadenoson derivative, neladenoson bialanate, is currently under development as a novel selective A_1AR partial agonist for the treatment of heart failure [54].

Conflict of interest statement

Authors have no conflict of interest to declare.

Acknowledgements

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia Project Grant (ID APP1084487) and the Australian Research Council (ARC; ID DE130100117). AC is an NHMRC of Australia Senior Principal Research Fellow (ID 1102950), RHR is an NHMRC of Australia Senior Research Fellow (ID 1059960), J-AB is a recipient of an Australian Government Research Training Program Scholarship and EAV holds an Australian Government Research Training Program Scholarship and an Australian Cancer Therapeutics scholarship.

References

[1] B.B. Fredholm, A.P. IJzerman, K.A. Jacobson, K.N. Klotz, J. Linden, International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors, Pharmacol. Rev. 53 (2001) 527–552.
[2] J. Linden, T. Thai, H. Figler, X. Jin, A.S. Robeva, Characterization of human A2B adenosine receptors: radioligand binding, western blotting, and coupling to Gq in human embryonic kidney 293 cells and HMC-1 mast cells, Mol. Pharmacol. 56 (1999) 705–713.
[3] T. Kong, K.A. Westerman, M. Faigle, H.K. Eltzschig, S.P. Colgan, HIF-dependent induction of adenosine A2B receptor in hypoxia, FASEB J. 20 (2006) 2242–2250, http://dx.doi.org/10.1096/fj.06-6419com.
[4] H.K. Eltzschig, S.K. Bonney, T. Eckle, Attenuating myocardial ischemia by targeting A2B adenosine receptors, Trends Mol. Med. 19 (2013) 345–354, http://dx.doi.org/10.1016/j.molmed.2013.02.005.
[5] M.A. Zimmerman, A. Grenz, E. Tak, M. Kaplan, D. Ridyard, K.S. Brodsky, et al., Signaling through hepatocellular A2B adenosine receptors dampens ischemia and reperfusion injury of the liver, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 12012–12017, http://dx.doi.org/10.1073/pnas.1221733110.
[6] H.K. Eltzschig, J. Rivera-Nieves, S.P. Colgan, Targeting the A2B adenosine receptor during gastrointestinal ischemia and inflammation, Expert Opin. Ther. Targets 13 (2009) 1267–1277, http://dx.doi.org/10.1517/14728220903241666.
[7] R.K. Dubey, D.G. Gillespie, E.K. Jackson, Adenosine inhibits collagen and protein synthesis in cardiac fibroblasts: role of A2B receptors, Hypertension 31 (1998) 943–948, http://dx.doi.org/10.1161/01.HYP.31.4.943.
[8] S. Merighi, P.A. Borea, S. Gessi, Adenosine receptors and diabetes: focus on the A2B adenosine receptor subtype, Pharmacol. Res. 99 (2015) 229–236, http://dx.doi.org/10.1016/j.phrs.2015.06.015.
[9] D.-F. Ma, T. Kondo, T. Nakazawa, D.-F. Nio, K. Mochizuki, T. Kawasaki, et al., Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells, Hum. Pathol. 41 (2010) 1550–1557, http://dx.doi.org/10.1016/j.humpath.2010.04.008.
[10] Q. Wei, S. Costanzi, R. Balasubramanian, Z.-G. Gao, K.A. Jacobson, A2B adenosine receptor blockade inhibits growth of prostate cancer cells, Purinergic Signal. 9 (2013) 271–280, http://dx.doi.org/10.1007/s11302-012-9350-3.
[11] C. Cekic, D. Sag, Y. Li, D. Theodorescu, R.M. Strieter, J. Linden, Adenosine A2B receptor blockade slows growth of bladder and breast tumors, J. Immunol. 188 (2011) 198–205, http://dx.doi.org/10.4049/jimmunol.1101845.
[12] P.G. Baraldi, D. Preti, M.A. Tabrizi, F. Fruttarolo, R. Romagnoli, M.D. Carrion, et al., Synthesis and biological evaluation of novel 1-deoxy-1-[6-[((hetero) arylcarbonyl)hydrazino]- 9 h-purin-9-yl]- N-ethyl-β- d-ribofuranuronamide derivatives as useful templates for the development of A2B adenosine receptor agonists, J. Med. Chem. 50 (2007) 374–380, http://dx.doi.org/10.1021/jm061170a.
[13] M.W. Beukers, I. Meurs, A.P. IJzerman, Structure-affinity relationships of adenosine A2B receptor ligands, Med. Res. Rev. 26 (2006) 667–698, http://dx.doi.org/10.1002/med.20069.
[14] P.G. Baraldi, R. Romagnoli, D. Preti, F. Fruttarolo, M.D. Carrion, M.A. Tabrizi, Ligands for A2B adenosine receptor subtype, Curr. Med. Chem. 13 (2006) 3467–3482, http://dx.doi.org/10.2174/092986706779010306.
[15] M. de Zwart, R. Link, J.K. von Frijtag Drabbe Kunzela, G. Cristalli, K.A. Jacobson, A. Townsend-Nicholson, et al., A functional screening of adenosine analogues at the adenosine A2B receptor: a search for potent agonists, Nucleosides Nucleotides 17 (1998) 969–985, http://dx.doi.org/10.1080/07328319808004215.
[16] U. Rosentreter, R. Henning, M. Bauser, T. Krämer, A. Vaupel, W. Hübsch, et al., Substituted 2-thio-3,5-dicyano-4-aryl-6-aminopyridines and the use thereof as adenosine receptor ligands, Bayer, Patent WO2001025210A2, 2001.
[17] P.G. Baraldi, M.A. Tabrizi, F. Fruttarolo, R. Romagnoli, D. Preti, Recent improvements in the development of A2B adenosine receptor agonists, Purinergic Signal. 4 (2008) 287–303, http://dx.doi.org/10.1007/s11302-008-9097-z.
[18] S. Hinz, S.K. Lacher, B.F. Seibt, C.E. Muller, BAY60-6583 acts as a partial agonist at adenosine A2B receptors, J. Pharmacol. Exp. Ther. 349 (2014) 427–436, http://dx.doi.org/10.1124/jpet.113.210849.
[19] J. Shonberg, L. Lopez, P.J. Scammells, A. Christopoulos, B. Capuano, J.R. Lane, Biased agonism at G protein-coupled receptors: the promise and the challenges-a medicinal chemistry perspective, Med. Res. Rev. 34 (2014) 1286–1330, http://dx.doi.org/10.1002/med.21318.
[20] T. Kenakin, A. Christopoulos, Signalling bias in new drug discovery: detection, quantification and therapeutic impact, Nat. Rev. Drug Discov. 12 (2013) 205–216, http://dx.doi.org/10.1038/nrd3954.
[21] T. Kenakin, Functional selectivity and biased receptor signaling, J. Pharmacol. Exp. Ther. 336 (2011) 296–302, http://dx.doi.org/10.1124/jpet.110.173948.
[22] J.-A. Baltos, K.J. Gregory, P.J. White, P.M. Sexton, A. Christopoulos, L.T. May, Quantification of adenosine A1 receptor biased agonism: implications for drug discovery, Biochem. Pharmacol. 99 (2016) 101–112, http://dx.doi.org/10.1016/j.bcp.2015.11.013.
[23] C. Valant, L.T. May, L. Aurelio, C.H. Chuo, P.J. White, J.A. Baltos, et al., Separation of on-target efficacy from adverse effects through rational design of a bitopic adenosine receptor agonist, Proc. Natl. Acad. Sci. U.S.A. 111 (2014) 4614–4619, http://dx.doi.org/10.1073/pnas.1320962111.
[24] D.D. Ben, M. Buccioni, C. Lambertucci, A. Thomas, R. Volpini, Simulation and comparative analysis of binding modes of nucleoside and non-nucleoside agonists at the A2B adenosine receptor, Silico Pharmacol. 1 (2013) 1–14, http://dx.doi.org/10.1186/2193-9616-1-24.
[25] Z.-G. Gao, R. Balasubramanian, E. Kiselev, Q. Wei, K.A. Jacobson, Probing biased/partial agonism at the G protein-coupled A2B adenosine receptor, Biochem. Pharmacol. 90 (2014) 297–306, http://dx.doi.org/10.1016/j.bcp.2014.05.008.
[26] M. Tendera, E. Gaszewska-Żurek, Z. Parma, P. Ponikowski, E. Jankowska, K. Kawecka-Jaszcz, et al., The new oral adenosine A1 receptor agonist capadenoson in male patients with stable angina, Clin. Res. Cardiol. 101 (2012) 585–591, http://dx.doi.org/10.1007/s00392-012-0430-8.
[27] B.E. Albrecht-Küpper, K. Leineweber, P.G. Nell, Partial adenosine A1 receptor agonists for cardiovascular therapies, Purinergic Signal. 8 (2011) 91–99, http://dx.doi.org/10.1007/s11302-011-9274-3.
[28] J. Louvel, D. Guo, M. Soethoudt, T.A.M. Mocking, E.B. Lenselink, T. Mulder-Krieger, et al., Structure-kinetics relationships of Capadenoson derivatives as adenosine A1 receptor agonists, Eur. J. Med. Chem. 101 (2015) 681–691, http://dx.doi.org/10.1016/j.ejmech.2015.07.023.
[29] H.N. Sabbah, R.C. Gupta, S. Kohli, M. Wang, S. Rastogi, K. Zhang, et al., Chronic therapy with a partial adenosine A1-receptor agonist improves left ventricular function and remodeling in dogs with advanced heart failure, Circ. Heart Fail. 6 (2013) 563–571, http://dx.doi.org/10.1161/CIRCHEARTFAILURE.112.000208.
[30] Bayer, Study to investigate the effect of the A1 agonist capadenoson on ventricular HR in patients with persistent or permanent atrial fibrillation by administration of capadenoson in a dose of 4 mg once daily for five days. In:

Please cite this article in press as: J.-A. Baltos et al., Capadenoson, a clinically trialed partial adenosine A_1 receptor agonist, can stimulate adenosine A_{2B} receptor biased agonism, Biochem. Pharmacol. (2017), http://dx.doi.org/10.1016/j.bcp.2017.03.014

ClinicalTrial.gov [Internet]. Bethesda (MD): National Library of Medicine (US), https://clinicaltrials.gov/show/NCT00568945 (accessed October 25, 2016).
[31] U. Rosentreter, T. Kramer, M. Shimada, W. Hubsch, N. Diedrichs, T. Krahn, et al., Substituted 2-thio-3,5-dicyano-4-phenyl-6-aminopyridines and the use of the same, Bayer, Patent WO2003053441 A1, 2003.
[32] G.D. Stewart, C. Valant, S.J. Dowell, D. Mijaljica, R.J. Devenish, P.J. Scammells, et al., Determination of adenosine A1 receptor agonist and antagonist pharmacology using Saccharomyces cerevisiae: implications for ligand screening and functional selectivity, J. Pharmacol. Exp. Ther. 331 (2009) 277–286, http://dx.doi.org/10.1124/jpet.109.158667.
[33] B.K. Atwood, J. Lopez, J. Wager-Miller, K. Mackie, A. Straiker, Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis, BMC Genomics 12 (2011) 14, http://dx.doi.org/10.1186/1471-2164-12-14.
[34] E.A. Vecchio, C.Y.R. Tan, K.J. Gregory, A. Christopoulos, P.J. White, L.T. May, Ligand-independent adenosine A2B receptor constitutive activity as a promoter of prostate cancer cell proliferation, J. Pharmacol. Exp. Ther. 357 (2016) 36–44, http://dx.doi.org/10.1124/jpet.115.230003.
[35] A. Laskowski, O. Woodman, A. Cao, G. Drummond, T. Marshall, D. Kaye, et al., Antioxidant actions contribute to the antihypertrophic effects of atrial natriuretic peptide in neonatal rat cardiomyocytes, Cardiovasc. Res. 72 (2006) 112–123, http://dx.doi.org/10.1016/j.cardiores.2006.07.006.
[36] L.T. May, V.A. Avlani, C.J. Langmead, H.J. Herdon, M.D. Wood, P.M. Sexton, et al., Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors, Mol. Pharmacol. 72 (2007) 463–476, http://dx.doi.org/10.1124/mol.107.037630.
[37] J.A. Baltos, S. Paoletta, A.T.N. Nguyen, K.J. Gregory, D.K. Tosh, A. Christopoulos, et al., Structure-activity analysis of biased agonism at the human adenosine A3 receptor, Mol. Pharmacol. 90 (2016) 12–22, http://dx.doi.org/10.1124/mol.116.103283.
[38] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction, Biochem. Pharmacol. 22 (1973) 3099–3108.
[39] E.A. Vecchio, C.H. Chuo, J.-A. Baltos, L. Ford, P.J. Scammells, B.H. Wang, et al., The hybrid molecule, VCP746, is a potent adenosine A2B receptor agonist that stimulates anti-fibrotic signalling, Biochem. Pharmacol. (2016) 1–11, http://dx.doi.org/10.1016/j.bcp.2016.08.007.
[40] M.W. Alnouri, S. Jepards, A. Casari, A.C. Schiedel, S. Hinz, C.E. Müller, Selectivity is species-dependent: characterization of standard agonists and antagonists at human, rat, and mouse adenosine receptors, Purinergic Signal. 11 (2015) 389–407, http://dx.doi.org/10.1007/s11302-015-9460-9.
[41] J.D. Urban, W.P. Clarke, M. von Zastrow, D.E. Nichols, B. Kobilka, H. Weinstein, et al., Functional selectivity and classical concepts of quantitative pharmacology, J. Pharmacol. Exp. Ther. 320 (2006) 1–13, http://dx.doi.org/10.1124/jpet.106.104463.
[42] T. Kenakin, C. Watson, V. Muniz-Medina, A. Christopoulos, S. Novick, A simple method for quantifying functional selectivity and agonist bias, ACS Chem. Neurosci. 3 (2012) 193–203, http://dx.doi.org/10.1021/cn200111m.
[43] T. Kenakin, The effective application of biased signaling to new drug discovery, Mol. Pharmacol. 88 (2015) 1055–1061, http://dx.doi.org/10.1124/mol.115.099770.
[44] M.W. Beukers, H. den Dulk, E.W. van Tilburg, J. Brouwer, A.P. IJzerman, Why are A2B receptors low-affinity adenosine receptors? Mutation of Asn273 to Tyr increases affinity of human A2B receptor for 2-(1-Hexynyl)adenosine, Mol. Pharmacol. 58 (2000) 1349–1356, http://dx.doi.org/10.1124/mol.58.6.1349.
[45] K.N. Klotz, J. Hessling, J. Hegler, C. Owman, B. Kull, B.B. Fredholm, et al., Comparative pharmacology of human adenosine receptor subtypes – characterization of stably transfected receptors in CHO cells, Naunyn-Schmiedebergs Arch. Pharmacol. 357 (1998) 1–9, http://dx.doi.org/10.1007/PL00005131.
[46] T. Borrmann, S. Hinz, D.C.G. Bertarelli, W. Li, N.C. Florin, A.B. Scheiff, et al., 1-Alkyl-8-(piperazine-1-sulfonyl)phenylxanthines: development and characterization of adenosine A2B receptor antagonists and a new radioligand with subnanomolar affinity and subtype specificity, J. Med. Chem. 52 (2009) 3994–4006, http://dx.doi.org/10.1021/jm900413e.
[47] A.S. Robeva, R.L. Woodard, X. Jin, Z. Gao, A.S. Robeva, R.W.X.J.Z. Cao, et al., Molecular characterization of recombinant human adenosine receptors, Drug Dev. Res. 39 (1996) 243–252, http://dx.doi.org/10.1002/(SICI)1098-2299(199611/12)39:3/4*243::AID-DDR3**3.0.CO;2-R.
[48] R.B. Mailman, GPCR functional selectivity has therapeutic impact, Trends Pharmacol. Sci. 28 (2007) 390–396, http://dx.doi.org/10.1016/j.tips.2007.06.002.
[49] M. Wakeno, T. Minamino, O. Seguchi, H. Okazaki, O. Tsukamoto, K.I. Okada, et al., Long-term stimulation of adenosine A2B receptors begun after myocardial infarction prevents cardiac remodeling in rats, Circulation 114 (2006) 1923–1932, http://dx.doi.org/10.1161/CIRCULATIONAHA.106.630087.
[50] H. Zhang, H. Zhong, T.H. Everett, E. Wilson, R. Chang, D. Zeng, et al., Blockade of A2B adenosine receptor reduces left ventricular dysfunction and ventricular arrhythmias 1 week after myocardial infarction in the rat model, Heart Rhythm. 11 (2014) 101–109, http://dx.doi.org/10.1016/j.hrthm.2013.10.023.
[51] D. Lu, N. Aroonsakool, U. Yokoyama, H.H. Patel, P.A. Insel, Increase in cellular cyclic AMP concentrations reverses the profibrogenic phenotype of cardiac myofibroblasts: a novel therapeutic approach for cardiac fibrosis, Mol. Pharmacol. 84 (2013) 787–793, http://dx.doi.org/10.1124/mol.113.087742.
[52] Y. Ikeda, T. Nakamura, H. Takano, H. Kimura, J.-E. Obata, S. Takeda, et al., Angiotensin II–induced cardiomyocyte hypertrophy and cardiac fibrosis in stroke-prone spontaneously hypertensive rats, J. Lab. Clin. Med. 135 (2000) 353–359, http://dx.doi.org/10.1067/mlc.2000.105617.
[53] Y. Liu, X. Yang, X.-M. Yang, S. Walker, K. Förster, M.V. Cohen, et al., AMP579 is revealed to be a potent A2B-adenosine receptor agonist in human 293 cells and rabbit hearts, Basic Res. Cardiol. 105 (2009) 129–137, http://dx.doi.org/10.1007/s00395-009-0056-9.
[54] A.A. Voors, H.-D. Düngen, M. Senni, S. Nodari, P. Agostoni, P. Ponikowski, et al., Safety and tolerability of neladenoson bialanate, a novel oral partial adenosine A1 receptor agonist, in patients with chronic heart failure, J. Clin. Pharmacol. (2016), http://dx.doi.org/10.1002/jcph.828.

Please cite this article in press as: J.-A. Baltos et al., Capadenoson, a clinically trialed partial adenosine A_1 receptor agonist, can stimulate adenosine A_{2B} receptor biased agonism, Biochem. Pharmacol. (2017), http://dx.doi.org/10.1016/j.bcp.2017.03.014

Chapter 5:

The Hybrid Molecule, VCP746, is a Potent Adenosine A_{2B} Receptor Agonist that Stimulates Anti-Fibrotic Signalling

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Biochemical Pharmacology **2016**, 117: 46-56

Biochemical Pharmacology 117 (2016) 46–56

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The hybrid molecule, VCP746, is a potent adenosine A_{2B} receptor agonist that stimulates anti-fibrotic signalling

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ARTICLE INFO

Article history:
Received 31 May 2016
Accepted 8 August 2016
Available online 9 August 2016

Chemical compounds studied in this article:
NECA (PubChem CID: 448222)
BAY60-6583 (PubChem CID: 11717831)
DPCPX (PubChem CID: 1329)
[3H]DPCPX (PubChem CID: 53319341)
PSB-603 (PubChem CID: 44185871)
SLV320 (PubChem CID: 9953065)
SCH442416 (PubChem CID: 10668061)
Angiotensin II (PubChem CID: 172198)
TGF-β1 (PubChem CID: 56842206)

Keywords:
Adenosine
Adenosine A_{2B} receptor
Fibroblasts
Collagen
Bivalent agonist

ABSTRACT

We have recently described the rationally-designed adenosine receptor agonist, 4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-*N*-(6-(9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxylmethyl)tetrahydro-furan-2-yl)-9*H*-purin-6-ylamino)hexyl)benzamide (VCP746), a hybrid molecule consisting of an adenosine moiety linked to an adenosine A_1 receptor (A_1AR) allosteric modulator moiety. At the A_1AR, VCP746 mediated cardioprotection in the absence of haemodynamic side effects such as bradycardia. The current study has now identified VCP746 as an important pharmacological tool for the adenosine A_{2B} receptor (A_{2B}AR). The binding and function of VCP746 at the A_{2B}AR was rigorously characterised in a heterologous expression system, in addition to examination of its anti-fibrotic signalling in cardiac- and renal-derived cells. In FlpInCHO cells stably expressing the human A_{2B}AR, VCP746 was a high affinity, high potency A_{2B}AR agonist that stimulated G_s- and G_q-mediated signal transduction, with an apparent lack of system bias relative to prototypical A_{2B}AR agonists. The distinct agonist profile may result from an atypical binding mode of VCP746 at the A_{2B}AR, which was consistent with a bivalent mechanism of receptor interaction. In isolated neonatal rat cardiac fibroblasts (NCF), VCP746 stimulated potent inhibition of both TGF-β1- and angiotensin II-mediated collagen synthesis. Similar attenuation of TGF-β1-mediated collagen synthesis was observed in renal mesangial cells (RMC). The anti-fibrotic signalling mediated by VCP746 in NCF and RMC was selectively reversed in the presence of an A_{2B}AR antagonist. Thus, we believe, VCP746 represents an important tool to further investigate the role of the A_{2B}AR in cardiac (patho)physiology.

1. Introduction

Myocardial fibrosis, characterised by the excess deposition of extracellular matrix (ECM) proteins, causes adverse ventricular remodelling and is a hallmark feature of heart failure pathophysiology [1,2]. Induced by hypertension and ischaemia-reperfusion injury following myocardial infarction (MI), fibrosis is essential in restoring structural and mechanical stability to damaged myocardium [3]. However, prolonged or overactive fibrosis, particularly in areas remote from the initial infarct, can lead to tissue stiffness and altered transmission of mechanical and electrical forces, ultimately resulting in diastolic and/or systolic dysfunction [3–5]. Fibrosis is principally regulated by cardiac fibroblasts and their differentiated counterpart myofibroblasts (alpha-smooth muscle actin (α-SMA)-expressing contractile cells), which stimulate the accumulation of collagen and production of cytokines [6]. Thus, regulating fibroblast cell proliferation, transformation and excess collagen deposition represents a highly desirable objective for the treatment and/or prevention of heart failure. To date, however, this therapeutic objective remains sub-optimally

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http://dx.doi.org/10.1016/j.bcp.2016.08.007
0006-2952/

targeted by current pharmaceuticals, and the disease burden of heart failure remains high [7].

Cardiac fibroblast activation and synthesis of collagen is promoted by a number of circulating and cardiac-cell derived factors, including angiotensin II (Ang II), transforming growth factor β1 (TGF-β1) and endothelin-1 (ET-1) [8,9]. At present, therapies for the management of heart failure are therefore aimed at inhibiting the actions of these known pro-fibrotic neuro-hormonal agents such as Ang II [8,10]. However, it is increasingly being recognised that endogenous inhibitors of the fibrotic process may have the potential to be exploited therapeutically. These include adenosine, a retaliatory autocoid with key regulatory and cytoprotective actions during times of metabolic imbalance in the heart [11,12]. Among a plethora of physiological actions [13], adenosine inhibits the proliferation and collagen synthesis of cardiac fibroblasts, attenuates myocardial fibrosis and reduces cardiac remodelling [11,14]. These actions are largely mediated by the adenosine A_{2B} receptor (A_{2B}AR), one of four adenosine G protein-coupled receptors (GPCRs) expressed in the heart [15–17]. However, effective targeting of the A_{2B}AR *in vitro* and *in vivo* is currently limited by a lack of pharmacological tools, particularly high affinity agonists [13].

'Bivalent' ligands, single molecules composed of two covalently linked active moieties, are an emerging class of compound that provide a novel therapeutic avenue for the development of high affinity GPCR agonists [18]. A synergistic increase in the binding affinity of a bivalent ligand is predicted upon simultaneous interaction with neighbouring binding sites on its macromolecular target. The observed synergy contrasts from simple "additive" increases in binding affinity predicted if the individual moieties are administered in combination. A special class of bivalent ligand is the "bitopic" molecule, which engages specifically with the endogenous agonist (orthosteric) site and a spatially distinct allosteric modulatory site on one and the same receptor molecule [18–20]. We recently described such a rationally-designed hybrid molecule, 4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-*N*-(6-(9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxylmethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-ylamino)hexyl)benzamide (VCP746; Fig. 1), which combines the cognate orthosteric agonist, adenosine, with an adenosine A_1 receptor (A_1AR)-selective allosteric modulator, (2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl) methanone (VCP171), to form a high affinity A_1AR ligand [21–23]. The signalling profile of VCP746 at the A_1AR was shown to be unique from that of prototypical agonists. That is, in contrast to prototypical A_1AR agonists, VCP746 mediated cardioprotection in rat H9c2 cardiac myoblasts and neonatal cardiac myocytes (NCM) during simulated ischaemia at concentrations that have no effect on heart rate, thus identifying VCP746 as a 'biased' A_1AR agonist that demonstrated a separation of on-target efficacy from adverse effects [23]. Although the selectivity of VCP746 for the A_1AR was confirmed via comparison to its effects on the most closely related adenosine receptor subtype in terms of homology, i.e., the A_3AR [23], its actions at other adenosine receptor subtypes, in particular the A_{2B}AR, which is involved in cardiac fibroblast-mediated fibrosis, remain undetermined.

Thus, the aims of the present study were to rigorously characterise the binding and function of VCP746 at the A_{2B}AR while comparing it to the non-selective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) and the A_{2B}AR-selective agonist 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583) (Fig. 1). NECA was used as potent adenosine analogue that has greater *in vitro* stability, as it is not metabolised by adenosine deaminase, whereas BAY60-6583 was included as the highest affinity A_{2B}AR agonist identified to date [24]. In addition, we examined the anti-fibrotic potential of VCP746 in isolated neonatal cardiac fibroblasts (NCF) and renal mesangial cells (RMC) and demonstrate VCP746, via A_{2B}AR activation, potently inhibits collagen synthesis and fibrogenic gene expression.

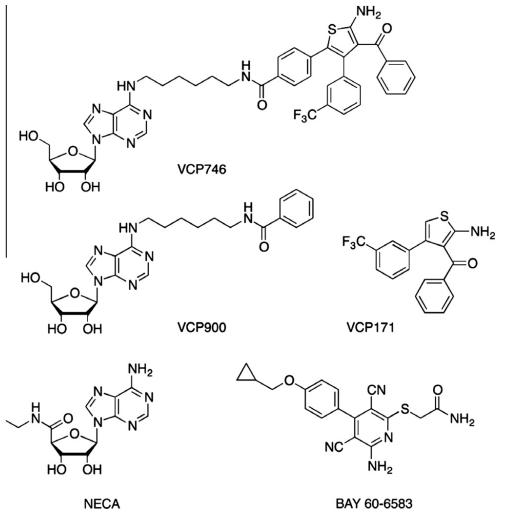

Fig. 1. Structures of the hybrid ligand VCP746, the orthosteric moiety VCP900, the A_1AR allosteric moiety VCP171 and the prototypical A_{2B}AR ligands NECA and BAY60-6583.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), trypsin, penicillin/streptomycin, Fluo-4 AM and antibiotic/antimycotic were from Life Technologies (Carlsbad, CA, USA). Ultima Gold scintillation cocktail, cyclopentyl-1, 3-dipropylxanthine, 8-[dipropyl-2,3-^{3}H(N)] ([^{3}H]DPCPX), [^{3}H]proline, LANCE® cAMP and Surefire™ pERK1/2 kits were purchased from PerkinElmer (Waltham, MA, USA). The IP-One homogeneous time resolved fluorescence (HTRF®) kit was purchased from Cisbio Bioassays (Codolet, France). Adenosine deaminase (ADA) and hygromycin-B were purchased from Roche (Basel, Switzerland). 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583) and 8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl)-1-propylxanthine (PSB-603), 2-(2-furanyl)-7-(3-(4-methoxyphenyl)propyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH442416) and *trans*-4-((2-phenyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)cyclohexanol (SLV320) were from Tocris Bioscience (Bristol, UK). The rat renal mesangial cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The MultiScribe® reverse transcriptase and SYBR™ Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, USA). The RNAquos® isolation kit was from Ambion (Austin, TX, USA). Transforming growth factor-β1 (TGF-β1) was purchased from PeproTech (Rocky Hill, NJ, USA) and angiotensin II (Ang II) was purchased from Auspep (VIC, Australia). The adenosine receptor ligands VCP900, VCP171 and VCP746 were synthesised in-house as described previously [21,23]. All other reagents were of analytical quality and were purchased from Sigma-Aldrich (St. Louis MO, USA).

2.2. A_{2B}AR-FlpInCHO cell culture and membrane preparation

FlpIn™ Chinese hamster ovary (FlpInCHO) cells stably expressing the human A_{2B}AR (A_{2B}AR-FlpInCHO; B_{max} 3.13 ± 0.61 pmol mg protein^{-1} [25]) or the human A_{2A}AR (A_{2A}AR-FlpInCHO) were grown in DMEM supplemented with 10% FBS and hygromycin-B (500 μg/mL) and maintained at 37 °C in a 5% CO_2 humidified incubator as previously described [25]. Cells were seeded in DMEM supplemented with 10% FBS into 96-well plates at a density of 2×10^4 cells/well for cAMP accumulation assays and 4×10^4 cells/well for all other assays (A_{2B}AR-FlpInCHO cells only). After 6 h incubation, plates for ERK1/2 phosphorylation assays were washed twice with 100 μL phosphate buffered saline (PBS) and incubated in serum-free DMEM overnight at 37 °C in 5% CO_2. All plates were maintained at 37 °C in a humidified incubator containing 5% CO_2 for 18–24 h prior to assaying. A_{2B}AR-FlpInCHO cell membranes were prepared as previously described [25].

2.3. Rat neonatal cardiac fibroblast and renal mesangial cell culture

Animal experiments were conducted in accordance with Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee-approved protocols (ethics approval number: E/0981/2010/M) and conformed to the requirements of the National Health and Medical Research Council of Australia *Code of Practice for the Care and Use of Animals for Scientific Purposes*. Neonatal rat cardiac fibroblasts (NCF) were isolated from 1 to 2 day-old Sprague-Dawley rat pups using enzymatic digestion, as described previously [26,27]. NCF were seeded and maintained in high-glucose (25 mM) DMEM in the presence of 1% antibiotic/antimycotic and 10% FBS. For all experiments, NCF were used at passage 2. Rat renal mesangial cells (RMC) were cultured and maintained in low-glucose (1 g/L) DMEM in the presence of 1% antibiotic/antimycotic and 10% FBS. All cells were maintained at 37 °C in a 5% CO_2 humidified incubator.

2.4. Radioligand competition binding and dissociation kinetics assays on A_{2B}AR-FlpInCHO cell membranes

[^{3}H]DPCPX binding assays involved incubation of A_{2B}AR-FlpInCHO cell membrane homogenates (100 μg) in HEPES-buffered saline solution (10 mM HEPES, 146 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1 mM $MgSO_4 \cdot 7H_20$, 1.3 mM $CaCl_2$, and 1.5 mM $NaHCO_3$, pH 7.4) containing 1 U/mL ADA and 10 nM [^{3}H]DPCPX for 1 h at room temperature. For equilibrium competition binding this was done in the absence and presence of increasing concentrations of unlabelled adenosine receptor ligand. For dissociation kinetics binding this was followed by the addition of 100 μM 5′-*N*-ethylcarboxamidoadenosine (NECA) in the absence or presence of 10 μM VCP171 for the specified time. Non-specific binding was determined using 100 μM NECA. Incubation was terminated by rapid filtration through 0.9% NaCl pre-soaked Whatman GF/B filters using a membrane harvester (Brandel, Gaithersburg, MD, USA). Filters were washed five times with 2 mL of ice-cold 0.9% NaCl and dried prior to determination of radioactivity as described previously [28].

2.5. Calcium mobilisation in A_{2B}AR-FlpInCHO cells

Calcium mobilisation was performed as described previously [29]. Briefly, media from 96-well plates was removed and replaced with HEPES-buffered saline solution containing 4 mM probenecid, 0.5% bovine serum albumin (BSA), 1 U/mL ADA and 1 μM Fluo-4 AM. Plates were incubated in the dark at 37 °C for 30 min. Fluorescence (excitation, 485 nm; emission, 520 nm) was measured on a FlexStation plate reader (Molecular Devices; Sunnyvale, CA, USA) after the automated addition of HEPES-buffered saline in the presence or absence of adenosine receptor ligand. Calcium mobilisation was measured as the difference between peak and baseline fluorescence reads and normalised to the adenosine triphosphate (100 μM) response.

2.6. Stimulation of cAMP accumulation in A_{2A}AR- and A_{2B}AR-FlpInCHO cells

Media from 96-well plates was removed and replaced with cAMP stimulation buffer (140 mM NaCl, 5 mM KCl, 0.8 μM $MgSO_4$, 1.3 mM $CaCl_2$, 0.2 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM D-glucose, 0.1% BSA, 10 μM rolipram and 5 mM HEPES, pH 7.4) and incubated at 37 °C for 30 min. Adenosine receptor agonists were then added and plates incubated for a further 30 min at 37 °C. The reaction was terminated by the removal of buffer and the addition of 50 μL/well ice-cold ethanol. Detection of cAMP was performed following the evaporation of ethanol and the addition of 50 μL/well lysis buffer (0.3% Tween-20, 0.1% BSA, 5 mM HEPES, pH 7.4). After 10 min agitation, a 1:200:400 (v/v) dilution of Alexa Fluor® 647-anti cAMP antibody:LANCE® cAMP detection buffer: lysate was transferred into a 384-well optiplate in a total volume of 15 μL and incubated at room temperature in the dark for 30 min. Subsequently, 10 μL/well of a pre-equilibrated 1:3.5:5040 (v/v/v) dilution of LANCE® Eu-W8044 labelled streptavidin beads: biotinylated cAMP:LANCE® cAMP detection buffer was added. Plates were further incubated for 1 h at room temperature and fluorescence read on an EnVision plate reader (PerkinElmer; Waltham, MA, USA) using standard LANCE® settings. Agonist concentration-response curves were normalised to the forskolin (10 μM) response.

2.7. Phosphorylation of ERK1/2 in A_{2B}AR-FlpInCHO cells

Cells maintained in serum-free media at 37 °C in a 5% CO_2 humidified incubator were exposed to serum-free media in the presence or absence of agonist. Agonist concentration-response curves were generated at the time of peak-response (5 min). Interaction assays involved pre-incubation of the adenosine receptor antagonist, DPCPX, for 30 min prior to the addition of agonist. Agonist stimulation was terminated by the rapid removal of media and the addition of 50 μL/well SureFire™ lysis buffer. Detection of ERK1/2 phosphorylation was performed as described previously using the AlphaScreen® *SureFire*® kit and fluorescence was measured with an EnVision® plate reader (PerkinElmer, Boston, MA) using standard AlphaScreen™ settings [28]. Data were normalised to the response elicited upon the exposure of cells to 10% FBS for 5 min.

2.8. Inositol monophosphate (IP_1) accumulation in A_{2B}AR-FlpInCHO cells

Media was removed from 96-well plates and replaced with stimulation buffer (10 mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM D-glucose, 50 mM LiCl, pH 7.4) and maintained at 37 °C for 30 min. Cells were then exposed to stimulation buffer in the absence or presence of agonist and incubated for an additional 1 h at 37 °C. Agonist stimulation was terminated by the removal of buffer and the addition of 25 μL/well IP-One HTRF® lysis buffer. Lysates (14 μL) were transferred to a 384-well proxiplate and a 40:1:1 (v/v/v) dilution of IP-One HTRF® lysis buffer:IP_1-d2 conjugate:anti-IP_1 Lumi4™-Tb cryptate conjugate in a 6 μL total volume was added to each well. Plates were incubated at room temperature for 1 h before fluorescence (calculated as 665 nm/620 nm ratio) was measured on an EnVision plate

reader (PerkinElmer; Waltham, MA, USA). Data were normalised to the maximal response elicited by 10 μM NECA, a full adenosine receptor agonist.

2.9. [^{3}H]proline incorporation in rat neonatal cardiac fibroblasts and renal mesangial cells

NCF and RMC collagen synthesis was determined by [^{3}H]proline incorporation as described previously [27]. NCF were seeded in 12-well plates at 5×10^4 cells/well, incubated (37 °C, 5% CO_2) overnight and then serum-starved in high-glucose medium with 0.5% BSA for 48 h prior to treatment. RMC were seeded at a density of 1×10^4 cells/well in 12-well plates and incubated (37 °C, 5% CO_2) overnight prior to serum-starvation with 0.1% BSA for 48 h. NCF and RMC were pre-treated in the absence or presence of the novel adenosine receptor agonist VCP746 for 1 h. When adenosine receptor antagonists were used, they were added 45 min prior to the addition of VCP746. NCF were then stimulated with TGF-β1 (10 ng/mL) or Ang II (100 nM). RMC were stimulated with TGF-β1 only. [^{3}H]proline (1 μCi) was then added to each well. After 48 h cells were washed with cold PBS and harvested by trichloroacetic acid (10%) precipitation on ice for 30 min before being solubilised with 1 M NaOH overnight at 4 °C. The samples were then neutralised with 1 M HCl and radioactivity determined using scintillation counting. Data were expressed as a percentage of unstimulated control (100%).

2.10. Quantification of mRNA expression in rat neonatal cardiac fibroblasts

NCF were pre-treated in the absence or presence of the novel adenosine receptor agonist VCP746 for 40 min. Adenosine receptor antagonists, where used, were added 20 min prior to the addition of VCP746. NCF were then stimulated with cytokines for 24 h before harvesting. Ang II (100 nM) and TGF-β1 (10 ng/mL) were used to stimulate fibrogenic gene expression in NCF. Total RNA was extracted using an RNA isolation kit (RNAquos®, Ambion) according to manufacturer's instruction. Reverse transcription to cDNA with MultiScribe® reverse transcriptase was then performed whereby reaction mixture was heated to 25 °C for 10 min, 42 °C for 12 min, 95 °C for 5 min, and cooled to 4 °C. Quantitative real time reverse-transcription-polymerase chain reaction (RT-PCR) was used to detect the mRNA levels of collagen I (Col I), connective tissue growth factor (CTGF) and TGF-β1. RT-PCR was performed with SYBR™ Green PCR Master Mix using the QuantStudio 12K Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instruction. 18S ribosomal RNA was used as the endogenous control in all experiments to correct for the relative gene expression.

2.11. Data analysis

Nonlinear regression curve fitting and statistical analysis were performed using Prism 6 (GraphPad Software, San Diego, CA, USA). Agonist inhibition binding data were fitted to a one-site inhibition mass action curve:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(X - Log\,IC_{50})}} \quad (1)$$

where Top is the specific binding of the radioligand in the absence of unlabelled ligand, Bottom is the specific binding of the radioligand equal to non-specific binding, IC_{50} is the concentration of unlabelled ligand that reduces radioligand specific binding by half and X is the logarithm of the concentration of the unlabelled ligand. Resulting IC_{50} values were converted to dissociation constants (K_i values) using the Cheng & Prusoff equation [30].

The 'Loewe additivity' and 'Bliss model of independence' [31] was used to characterise the interaction of the two moieties within the putative bivalent ligand and predict the fractional response upon co-incubation of the individual moieties [31]. Additive interactions between co-incubated individual moieties can be defined by:

$$10^{(A-AB)} + 10^{(B-AB)} = i \quad (2)$$

where A and B are the negative logarithm of the individual moiety dissociation constants, AB is the value for co-incubation of the individual moieties and i is the interaction index. To calculate the bivalent effect, τ, the negative logarithm of the bivalent ligand can be used for AB. The parameter τ is defined as $\tau = 1/i$, where values for $\tau > 1$ represent a synergistic interaction between the moieties and values for $\tau < 1$ represent an antagonistic interaction between the moieties.

Agonist concentration-response curves preferentially fitted to a three-parameter Hill equation (extra sum-of-squares F test):

$$Response = Basal + \frac{(E_{max} - Basal) \times [A]}{EC_{50} + [A]} \quad (3)$$

where Basal is the magnitude of the response in the absence of agonist, [A] is the concentration of agonist, and EC_{50} is the concentration of agonist required to generate a response halfway between the basal level and maximal effect (E_{max}).

Statistical significance was defined as $P < 0.05$ as determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons post hoc analysis or t-test as indicated within the results.

3. Results

3.1. VCP746 is a high affinity, high potency agonist at the $A_{2B}AR$

The $A_{2B}AR$ affinity of the structurally distinct adenosine receptor ligands, NECA, BAY60-6583, VCP746, VCP900 and VCP171, was estimated from [^{3}H]DPCPX competition binding on $A_{2B}AR$-FlpInCHO cell membrane homogenates (Fig. 2A; Table 1). The affinity of the $A_{2B}AR$ agonists, NECA and BAY60-6583 was in the low micromolar range. The individual moieties, VCP900 and VCP171 of the hybrid ligand, VCP746, both had low affinity for the $A_{2B}AR$. In contrast, VCP746 had a relatively high, nanomolar affinity for the $A_{2B}AR$. As such, the affinity of the hybrid ligand VCP746 was approximately 600- to 5000-fold greater than either of the constituent molecules alone. Furthermore, the $A_{2B}AR$ affinity of VCP746 was approximately 30-fold greater than either NECA or BAY60-6583; agonists with the highest reported $A_{2B}AR$ affinity [24,32].

The high affinity of VCP746 suggests that the individual moieties within the molecule can simultaneously engage multiple binding sites within the $A_{2B}AR$, resulting in a higher total free energy of interaction when compared to the sum of the free energies of interaction of the individual molecules [33]. A synergistic interaction for the molecule can clearly be observed when the 'Bliss model of independence' [31,33] was used to predict the additive interaction upon co-incubation of the individual moieties, VCP900 and VCP171 (Fig. 2B). The affinity of VCP746 is significantly higher than the affinity predicted when VCP900 and VCP171 interact in an additive manner, consistent with the hypothesis that VCP746 has a bivalent mechanism of interaction with the $A_{2B}AR$. Quantification of the bivalent effect (τ) identified significant synergy upon covalently linking the two fragments VCP900 and VCP171, with a τ value for VCP746 of

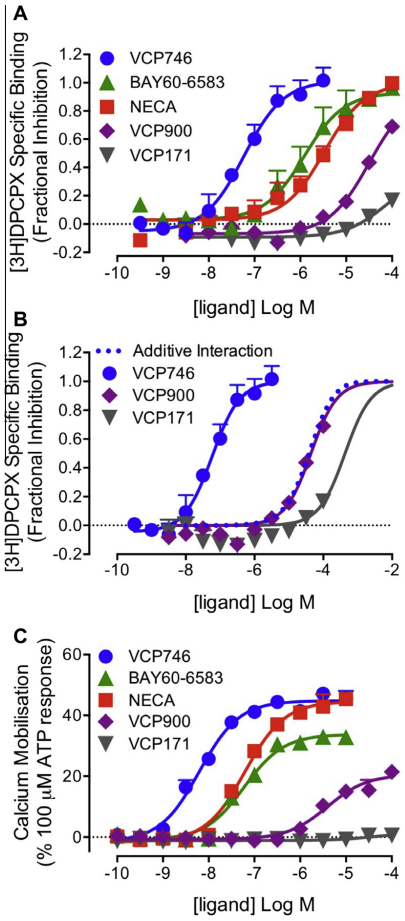

Fig. 2. At the $A_{2B}AR$, VCP746 is a high affinity, high potency agonist that has a profile consistent with a bivalent mode of interaction. (A) VCP746 has a significantly higher $A_{2B}AR$ affinity than either of its constituent molecules VCP900 or VCP171 or the prototypical $A_{2B}AR$ agonists NECA or BAY60-6583 as determined by [^{3}H]DPCPX radioligand binding on $A_{2B}AR$-FlpInCHO cell membranes. (B) Synergy, consistent with a bivalent interaction of VCP746, can be visualised by comparing experimental data (solid blue line) with simulations (dashed blue line) according to the 'Bliss model of independence', which assumes additivity between the two moieties. The affinity of VCP746 was significantly greater than the predicted effect upon co-incubation of the individual moieties, VCP900 and VCP171. (C) VCP746 is a more potent $A_{2B}AR$ agonist than NECA, BAY60-6583, VCP900 or VCP171 in stimulating Ca^{2+} mobilisation in $A_{2B}AR$-FlpInCHO cells. Data represent the mean + SEM from 3 to 4 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

477. The superior $A_{2B}AR$ activity of VCP746 also translated into a whole-cell calcium mobilisation assay (Fig. 2C; Table 1). In this functional assay, VCP746 had a 10-fold higher potency than the prototypical agonists NECA and BAY60-6583. Furthermore, the potency of VCP900 was approximately 1000-fold lower than VCP746, while VCP171 had negligible activity. These data demonstrate VCP746 has higher potency and affinity for the $A_{2B}AR$ compared to the prototypical orthosteric ligands NECA and BAY60-6583, likely a result of the bivalent mechanism of action of VCP746. A similar increase in VCP746 potency was not evident at the $A_{2A}AR$. In cAMP accumulation assays, VCP746 displayed a lower potency relative to the non-selective adenosine receptor agonist, NECA in $A_{2A}AR$-FlpInCHO cells (VCP746 pEC_{50}: 5.97 ± 0.29; NECA pEC_{50}: 8.17 ± 0.24).

3.2. VCP746 has an atypical mode of interaction with the $A_{2B}AR$

The synergy observed suggests VCP746 can interact simultaneously with two neighbouring binding sites on the $A_{2B}AR$. Therefore, to assess whether the mode of receptor engagement for VCP746 was distinct from that of prototypical $A_{2B}AR$ agonists, the functional interaction with the antagonist DPCPX was assessed. In $A_{2B}AR$-FlpInCHO cells, the surmountable inhibition of NECA-mediated ERK1/2 phosphorylation observed in the presence of increasing concentrations of DPCPX conformed to that predicted for a competitive interaction between an agonist and antagonist acting at the same orthosteric site (Fig. 3A). The DPCPX affinity estimate from this interaction, pK_B of 7.87 ± 0.22, corresponds with previously reported DPCPX affinity values at the $A_{2B}AR$ [25,34]. In contrast, the insurmountable inhibition of VCP746-mediated ERK1/2 phosphorylation in the presence of increasing antagonist concentration is suggestive of a more complex mechanism of action (Fig. 3B). Specifically, in the presence of high concentrations of DPCPX, the VCP746 concentration-response curve was associated with reduction in the maximal response (E_{max}) with no additional change in potency (pEC_{50}). A non-competitive collapse in E_{max} can result from allosteric interactions between the orthosteric and allosteric moieties, or other complex modes of activity, such as differential kinetics between agonist, competitor and signalling pathway responsiveness. Of note, and in contrast to the A_1AR [21], we found no evidence of VCP171 acting as an $A_{2B}AR$ allosteric modulator. Specifically, VCP171 was unable to alter the dissociation kinetics of the radiolabelled orthosteric antagonist [^{3}H]DPCPX (Fig. 4A), or demonstrate any cooperativity with the orthosteric agonist NECA in an IP_1 accumulation assay (Fig. 4B). Taken together, these data are consistent with VCP746 having a 'bivalent', but unlikely a 'bitopic' mode of interaction with the $A_{2B}AR$. That is, VCP746 can potentially simultaneously engage two spatially distinct sites on the $A_{2B}AR$, but there is no observable allosteric cooperativity between these two sites. Nonetheless, this bivalent mode of engagement has the potential to engender unique properties to the molecule relative to canonical AR agonists.

3.3. VCP746 mediates potent G_s and G_q protein $A_{2B}AR$ signal transduction

The $A_{2B}AR$ is a pleiotropically coupled GPCR, interacting with both G_s and G_q proteins to modulate a multitude of intracellular second messenger systems [13,35]. The activity of VCP746 in $A_{2B}AR$-FlpInCHO cells was assessed across multiple functional pathways and its agonist profile compared to the prototypical $A_{2B}AR$ agonists NECA and BAY60-6583 (Fig. 5; Table 1). All agonists were equipotent in the well-coupled G_s-mediated cAMP accumulation assay, with high potencies in the nanomolar range (Fig. 5A). NECA and BAY60-6583 had lower potency for the stimulation of ERK1/2 phosphorylation and G_q-mediated IP_1 accumulation with EC_{50} values in the micromolar range; moreover BAY60-6583 was a partial agonist (Fig. 5B and C). In comparison, VCP746 was a potent agonist in all pathways, maintaining nanomolar EC_{50} values for ERK1/2 phosphorylation and IP_1 accumulation (Fig. 5B and C). Therefore, despite the prototypical $A_{2B}AR$ agonists, NECA and BAY60-6583, favouring G_s protein signal transduction, VCP746 does not display this natural system bias and remains instead a

Table 1
Affinity (pK_I) and potency (pEC_{50}) estimates of ligands across multiple signalling pathways in FlpInCHO cells stably expressing the A_{2B}AR. Data are mean ± SEM from three or more independent experiments performed in duplicate.

	pK_I (K_I)	Calcium	pEC_{50} (EC_{50}) cAMP	IP	pERK1/2
NECA	5.61 ± 0.30 (2.5 μM)	7.20 ± 0.01 (63 nM)	8.04 ± 0.27 (9 nM)	6.10 ± 0.39 (794 nM)	7.04 ± 0.12 (91 nM)
BAY60-6583	5.82 ± 0.32 (1.5 μM)	7.22 ± 0.05 (60 nM)	8.03 ± 0.42 (9 nM)	5.37 ± 0.79 (4.0 μM)	7.01 ± 0.06 (98 nM)
VCP746	7.26 ± 0.12 (55 nM)	8.21 ± 0.09 (6 nM)	8.04 ± 0.09 (9 nM)	7.60 ± 0.23 (25 nM)	8.53 ± 0.08 (3 nM)
VCP900	4.46 ± 0.07 (35 μM)	5.28 ± 0.03 (5.2 μM)	N.D.	N.D.	N.D.
VCP171	3.54 ± 0.57 (290 μM)	N.D.	N.D.	N.D.	N.D.

N.D. denotes value could not be determined.
pK_I denotes the negative logarithm of the ligand equilibrium dissociation constant.
pEC_{50} denotes the negative logarithm of the ligand concentration required to elicit half the maximal response.

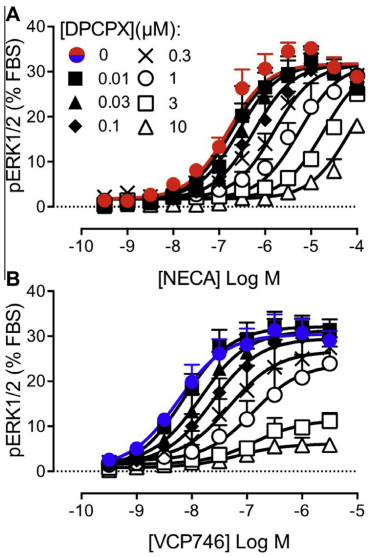

Fig. 3. The functional interaction between VCP746 and DPCPX represents an atypical mode of receptor interaction. The functional interaction of (A) NECA or (B) VCP746 with the orthosteric antagonist (DPCPX), reveals different profiles in pERK1/2. Interactions between NECA and DPCPX conform to a classic competitive model, whereas the interaction between VCP746 and DPCPX is more complex. Data represent the mean + SEM from 3 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

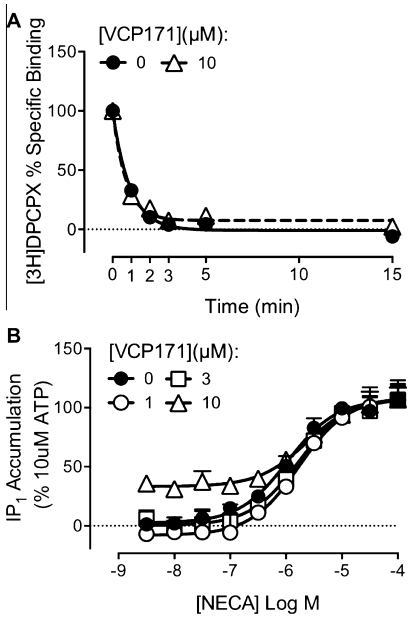

Fig. 4. Binding and functional assays provide no evidence for A_{2B}AR allosteric modulation by VCP171, the A_1AR allosteric moiety of VCP746. (A) [^{3}H]DPCPX dissociation kinetics or (B) agonist-mediated IP_1 accumulation was not altered in the presence of VCP171 in A_{2B}AR-FlpInCHO cells. Data represent the mean + SEM from 3 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

potent activator of both G_s and G_q protein-coupled pathways in a heterologous expression system (Table 1).

3.4. VCP746 has potent anti-fibrotic activity in cardiac fibroblasts and renal mesangial cells

We next tested whether VCP746 possessed A_{2B}AR-mediated anti-fibrotic activity in native A_{2B}AR-expressing NCF or RMC. Proline, along with glycine, are the predominant amino acids in collagen, hence [^{3}H] proline incorporation is routinely used as a marker of collagen synthesis [36]. The pro-fibrotic hormone Ang II and cytokine TGF-β1 significantly stimulated NCF collagen synthesis over control levels as determined by [^{3}H]proline incorporation (Fig. 6; 139.16 ± 9.8% and 193 ± 7.1% of untreated control, respectively; $P < 0.05$, t test). Pre-treatment of NCF with VCP746 (0.1 nM–1 μM) produced a concentration-dependent reduction in TGF-β1 and Ang II stimulated [^{3}H]proline incorporation (pIC_{50} 7.6 ± 0.4 and 7.8 ± 0.4, respectively; Fig. 6A). The addition of a saturating concentration of an A_1AR-selective antagonist, SLV320 (1 μM), or an A_{2A}AR-selective antagonist, SCH442416 (1 μM) had no effect on the VCP746-mediated reduction in NCF collagen synthesis. However, the addition of an A_{2B}AR-selective antagonist, PSB-603 (1 μM), abolished the inhibitory effects of 100 nM VCP746 (Fig. 6B and C). These data indicate that VCP746 decreases collagen synthesis in NCF through activation of the A_{2B}AR. To determine whether these anti-fibrotic effects were confined to cardiac cells we investigated the ability of VCP746 to decrease collagen synthesis in RMC, cells for which A_{2B}AR-mediated inhibition of proliferation has previously been reported [37]. Similar to NCF, pre-treatment of RMC with VCP746 (0.1 nM–1 μM) mediated a concentration-dependent decrease in TGF-β1-stimulated [^{3}H]proline incorporation (pIC_{50} 8.8 ± 0.5; Fig. 7A). The decrease in [^{3}H] proline incorporation was largely A_{2B}AR mediated, as it was

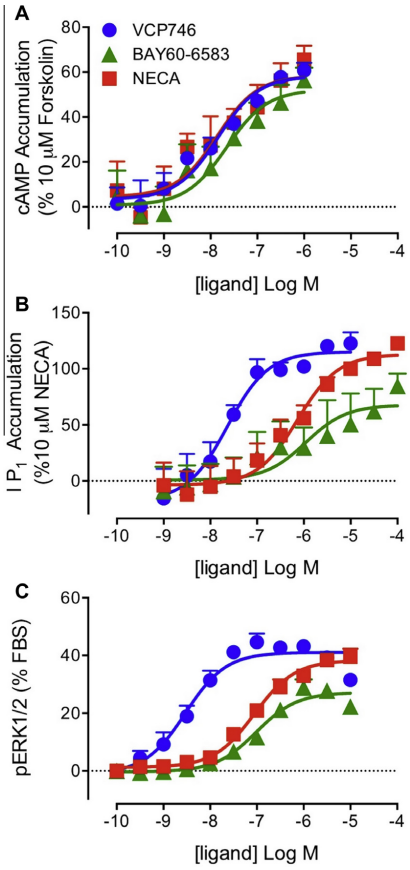

Fig. 5. VCP746 is a potent agonist in each of the signalling pathways assessed. VCP746 mediated potent and robust stimulation of (A) cAMP accumulation, (B) IP_1 accumulation and (C) ERK1/2 phosphorylation in FlpInCHO cells stably expressing the A_{2B}AR. VCP746 is equipotent with NECA and BAY60-6583 in stimulating cAMP accumulation but is significantly more potent in stimulating IP_1 accumulation or pERK1/2. Data represent the mean + SEM from 3 to 4 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

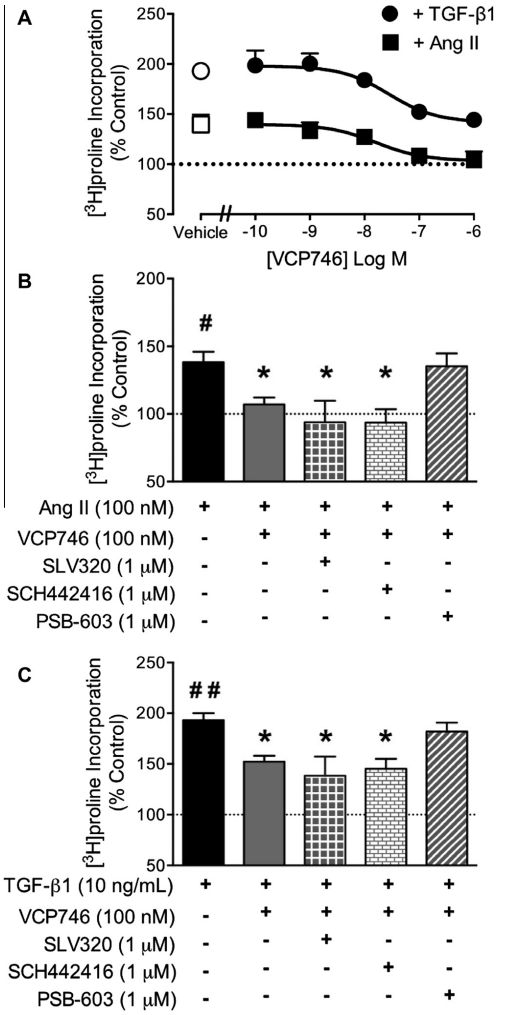

Fig. 6. VCP746 stimulated potent inhibition of collagen synthesis in NCF. (A) VCP746 mediated a concentration-dependent inhibition of Ang II- and TGF-β1-stimulated collagen synthesis in rat NCF as determined by [^{3}H]proline incorporation. The inhibitory effect of VCP746 on (B) Ang II- or (C) TGF-β1-stimulated collagen synthesis was blocked by an A_{2B}AR antagonist (PSB-603) but not by an A_1AR-selective (SLV320) or an A_{2A}AR-selective (SCH442416) antagonist. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. 100%; *t*-test, $^{*}P < 0.05$; vs. TGF-β1 or Ang II; one-way ANOVA; Dunnett's multiple comparisons test. Data represent the mean + SEM from 4 to 5 independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

blocked in the presence of PSB-603, but not with SLV320, and only partially reversed in the presence of SCH442416 (Fig. 7B).

A potential limitation of using [^{3}H]proline incorporation as an index of collagen synthesis is that a reduction in cell viability could also register as a reduction in [^{3}H]proline incorporation, thus leading to a false interpretation of the anti-fibrotic effect of VCP746. We therefore performed an MTT toxicity assay to assess if VCP746 was reducing NCF or RMC viability. Results demonstrate that co-culture with Ang II or TGF-β1 in the absence or presence of VCP746 did not decrease cell viability, nor did the addition of the adenosine receptor antagonists (data not shown). Therefore, the observed reduction in [^{3}H]proline incorporation mediated by VCP746 reflects an anti-fibrotic effect and not a reduction in NCF or RMC viability. Collectively, these data demonstrate that VCP746 potently inhibits collagen synthesis in both cardiac- and renal-derived cells via A_{2B}AR stimulation.

3.5. VCP746 attenuates fibrogenic gene expression via A_{2B}AR activation

The pro-fibrotic agents Ang II and TGF-β1 are known to increase the expression of a number of pro-fibrotic genes in NCF including Collagen I (Col I), connective tissue growth factor (CTGF) and TGF-β1 [2,38,39]. Quantitative real-time PCR analysis of total RNA extracted from NCF stimulated with Ang II or TGF-β1 for

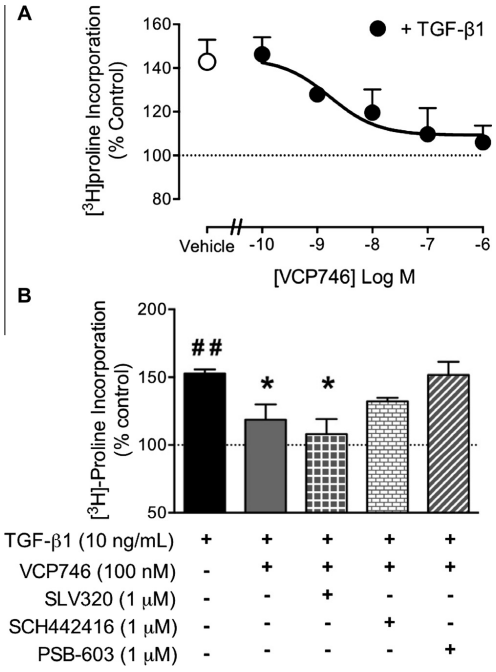

Fig. 7. VCP746 stimulated potent inhibition of RMC collagen synthesis. (A) VCP746 mediated a concentration-dependent inhibition of TGF-β1-stimulated collagen synthesis in rat RMC as determined by [^{3}H]proline incorporation. (B) The inhibitory effect of VCP746 on TGF-β1- stimulated collagen synthesis was blocked by an $A_{2B}AR$ antagonist (PSB-603) but not by an A_1AR-selective antagonist (SLV320) and only partially reversed in the presence of an $A_{2A}AR$-selective antagonist (SCH442416). $^{\#\#}P < 0.01$; vs. 100%; *t*-test, $^{*}P < 0.05$; vs. TGF-β1; one-way ANOVA; Dunnett's multiple comparisons test Data represent the mean + SEM from 3 to 4 independent experiments performed in triplicate. Error bars not shown lie within the dimensions of the symbol.

24 h significantly increased Col I, CTGF and TGF-β1 mRNA expression levels compared to control, with the exception of TGF-β1 stimulation of TGF-β1 expression, which maintained a similar trend but did not reach statistical significance (Fig. 8; $P < 0.05$, one-way ANOVA Dunnett's multiple comparison test). Pre-treatment of NCF with VCP746 resulted in the inhibition of Col I, CTGF and TGF-β1 mRNA expression stimulated by Ang II (Fig. 8A, C and E) or TGF-β1 (Fig. 8B, D and F). The attenuation of pro-fibrotic gene expression by VCP746 was potent; with a concentration of 100 nM VCP746 sufficient to return mRNA expression to levels equivalent to control. The addition of SLV320 or SCH442416 (1 μM) had little effect on VCP746-mediated decreases in fibrogenic gene expression. In contrast, application of PSB-603 (1 μM) abolished the inhibitory effect of VCP746 on Col I, CTGF and TGF-β1 mRNA expression, further supporting VCP746-mediated anti-fibrotic effects are downstream of $A_{2B}AR$ activation.

4. Discussion

The current study reveals, for the first time, the potent $A_{2B}AR$ activity of the hybrid ligand VCP746. The extended structure of VCP746, combining two distinct moieties, appears to engender a unique pharmacological profile with a higher $A_{2B}AR$ affinity and potency than the currently best available $A_{2B}AR$ agonists, NECA and BAY60-6583. In a heterologous FlpInCHO system, we rigorously characterised the binding and function of VCP746, identifying an $A_{2B}AR$ interaction that was consistent with a bivalent mode of receptor engagement. The high potency and efficacy of VCP746 at the $A_{2B}AR$ extended to functional activity *in vitro*. The present study demonstrated that VCP746, in a concentration-dependent manner, inhibited Ang II and TGF-β1 stimulation of NCF and RMC collagen synthesis, as well as potently attenuating NCF pro-fibrotic gene expression. The effects of VCP746 in NCF and RMC were mediated by the $A_{2B}AR$ but not the A_1AR or the $A_{2A}AR$. As such, we believe VCP746 provides a novel tool to further investigate, either *in vitro* or *in vivo*, the role of the $A_{2B}AR$ in (patho)physiology, in particular in attenuating myocardial fibrosis for the treatment of heart failure.

The $A_{2B}AR$ has relatively low affinity for its endogenous agonist, adenosine, and as consequence has low receptor occupancy at physiological concentrations of adenosine [13,40]. However, the upregulation of both $A_{2B}ARs$ and endogenous adenosine under conditions such as hypoxia and inflammation has called into attention the need for high affinity, high efficacy agonists to further probe the role of this adenosine receptor subtype both in physiology and pathophysiology. To date, there is a very limited repertoire of $A_{2B}AR$ agonists, with the most selective $A_{2B}AR$ agonist, BAY60-6583 demonstrating moderate potency and only partially activating some signalling pathways [32,41]. It is now widely appreciated that a number of GPCRs have extracellular allosteric sites that are topographically distinct from the orthosteric site recognised by the endogenous agonist [42,43]. In recent years the possibility of bitopic ligands, which link orthosteric and allosteric moieties to yield ligands with increased affinity and/or selectivity has been explored [18,19]. We recently characterised VCP746, as a rationally designed bitopic A_1AR ligand, comprised of adenosine linked to an A_1AR allosteric enhancer moiety. At the A_1AR, VCP746 had significantly higher affinity when compared to the individual orthosteric and allosteric moieties alone [23]. Similarly, in the current study, we demonstrated that the affinity of VCP746 for the $A_{2B}AR$ was significantly greater than the predicted additive effects of VCP900 and VCP171 according to the 'Bliss model of independence' [31,33]. The strong synergy observed suggests that VCP746 acts as a bivalent ligand at the $A_{2B}AR$ with an atypical mode of receptor engagement, highlighted by the functional interaction of VCP746 with the orthosteric antagonist DPCPX. Future studies, involving mutagenesis and computational modelling, are required to identify the specific molecular interactions involved in VCP746 binding to the $A_{2B}AR$.

In the heterologous system, the $A_{2B}AR$ preferentially coupled to G_s proteins over G_q proteins, similar to previous reports [25]. This observation is based on the higher potency of the prototypical agonists NECA and BAY60-6583, in the cAMP accumulation assay relative to the stimulation of IP_1 accumulation or Ca^{2+} mobilisation. However, unlike prototypical agonists, VCP746 does not conform to this natural system bias, potently activating pathways downstream from both G_s and G_q proteins in $A_{2B}AR$-FlpInCHO cells. This distinct pharmacological profile may result from the hybrid nature of VCP746, which can form additional interactions with the $A_{2B}AR$ and consequently stabilise a unique spectrum of $A_{2B}AR$ active conformations. The differential ability of ligands to selectively activate a subset of signalling pathways over others is termed "functional selectivity" or "biased agonism" [44,45] and is an emerging paradigm that has considerable clinical potential for GPCR drug discovery as it presents the opportunity to specifically shape on-target downstream signal transduction [46]. The desired bias profile of $A_{2B}AR$ agonists will differ depending on the cell system and the nature of the pathways leading to desired and unwanted clinical effects. Nonetheless, we believe, the potent agonism and apparent lack

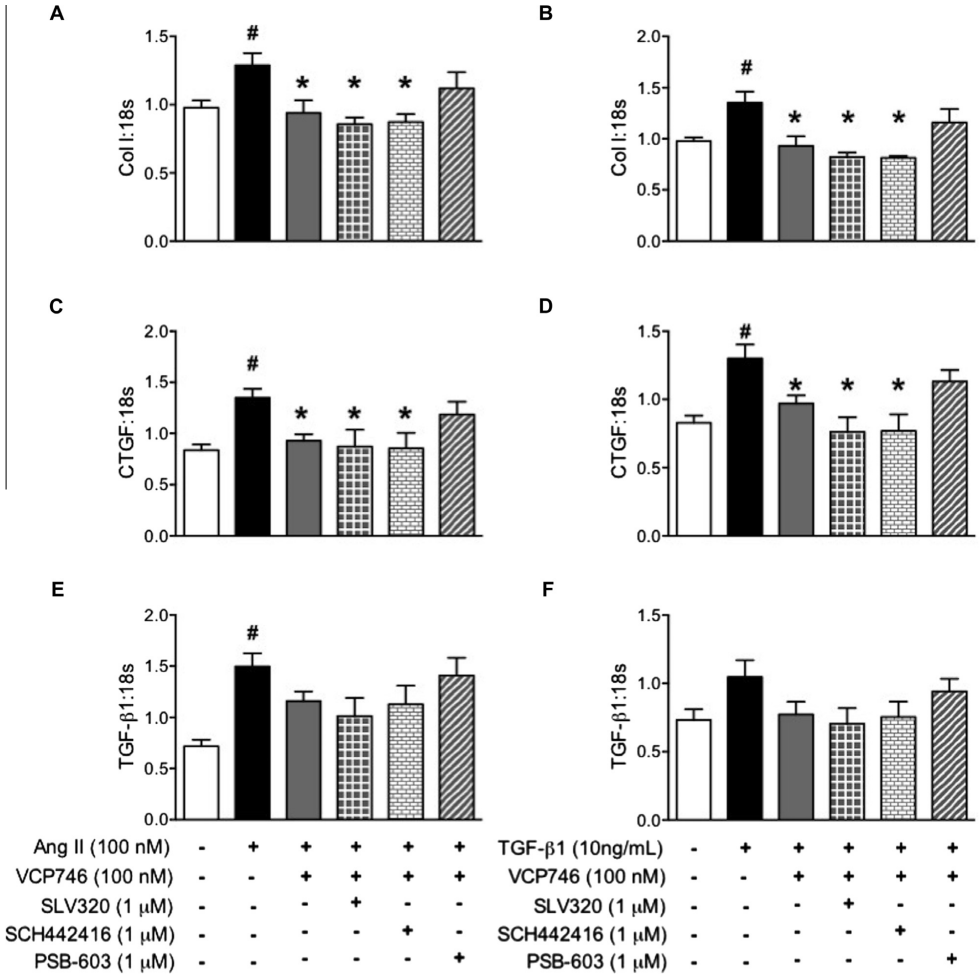

Fig. 8. VCP746 inhibits pro-fibrotic gene expression in cardiac fibroblasts. Ang II and TGF-β1 stimulated (A and B) Col I, (C and D) CTGF and (E and F) TGF-β1 mRNA expression in NCF. Pre-treatment of NCF with VCP746 significantly attenuated (A and C) Ang II- and (B and D) TGF-β1-stimulated Col I and CTGF mRNA expression and markedly, but did not significantly attenuate TGF-β1 mRNA expression stimulated upon exposure to (E) Ang II and (F) TGF-β1. The inhibitory effect of VCP746 was blocked by an A_{2B}AR antagonist (PSB-603) but not by an A_1AR-selective (SLV320) or an A_{2A}AR-selective (SCH442416) antagonist. $^{\#}P < 0.05$; vs. control, $^{*}P < 0.05$; vs. TGF-β1 or Ang II; one-way ANOVA; Dunnett's multiple comparisons test. Data represent the mean + SEM from 3 to 5 independent experiments performed in triplicate.

of VCP746 system bias will be advantageous for rigorous interrogation of the desired and unwanted A_{2B}AR signalling for therapy.

The potential of VCP746 as a high potency A_{2B}AR agonist was examined in an *in vitro* model of cardiac fibrosis, specifically examining the ability of VCP746 to inhibit Ang II and TGF-β1 pro-fibrotic collagen synthesis in NCF. Previous studies have shown that activation of the A_{2B}AR is able to reduce cardiac fibrosis and improve left ventricle (LV) remodelling outcomes [16,17,47]. *In vitro* studies using rat cardiac fibroblasts have found that 2-chloroadenosine and NECA, stable adenosine analogues, were able to inhibit [^{3}H] proline incorporation with approximate EC_{50} values of 10 μM and these effects were dependent upon the activation of the A_{2B}AR [15,16]. The relative importance of the A_{2B}AR over other adenosine receptor subtypes as a key regulator of cardiac fibrosis is perhaps unsurprising as data suggest that the A_{2B}AR is the most abundantly expressed subtype in rat cardiac fibroblasts [48,49]. Our study found VCP746, acting via the A_{2B}AR, potently attenuates Ang II- and TGF-β1-stimulated collagen synthesis with EC_{50} values of less than 30 nM. Collectively, these results suggest the potency of VCP746 is approximately 300-fold higher than 2-chloroadenosine and NECA for stimulating A_{2B}AR-mediated inhibition of collagen synthesis. These data indicate that VCP746, a novel and potent A_{2B}AR agonist, may provide a new mechanism to inhibit myocardial fibrosis by activating anti-fibrotic adenosine receptor signalling.

The ability of VCP746 to confer potent inhibition of Ang II and TGF-β1-stimulated collagen synthesis is pertinent to cardiac pathophysiology as these neuro-hormonal factors act as powerful stimuli for fibrosis in the injured heart. Ang II has been implicated as a potent pro-fibrotic molecule and is involved in the progression of myocardial fibrosis [50]. Increased circulating plasma levels of Ang II are found in patients with cardiovascular diseases characterised by myocardial fibrosis including atherosclerosis and heart failure [51]. The expression of the inflammatory cytokine TGF-β1 is also significantly increased (in part due to Ang II-mediated angiotensin type 1 receptor-activation) in cardiac myocytes and fibroblasts post-MI and during heart failure [2]. TGF-β1 is heavily involved in the development of cardiac fibrosis by promoting cardiac myofibroblast activation, proliferation and increased collagen synthesis [52,53]. Furthermore, Ang II and TGF-β1 increase pro-fibrotic gene expression in fibroblasts in an attempt to repair the damaged heart in response to cardiac injury [38,39]. It is of great importance therefore, that any effective anti-fibrotic therapy should be able to regulate gene expression stimulated by exposure to Ang II and TGF-β1. We found that VCP746, via A_{2B}AR activation, potently inhibited the upregulation of Col I, TGF-β1 and CTGF expression in NCFs, further emphasising the potential of VCP746 as a novel agent to modulate myocardial fibrosis.

Fibrosis of the kidney is commonly associated with cardiac fibrosis and contributes to morbidity and mortality in conditions such as cardiorenal syndrome [54]. Several cytokines, in particular TGF-β1, are major contributors to renal interstitial fibrosis [55]. As such, it is likely that systemic inflammatory responses involved in post-MI cardiac repair and remodelling will not only contribute to the progressive dysfunction of the heart but also the kidney. A recent study found that rats with MI induced by left anterior descending artery ligation had impaired renal function and renal interstitial fibrosis that was associated with an increase in TGF-β1 and phospho-Smad2 protein expression [56]. We were therefore interested to determine if the anti-fibrotic effects of VCP746 were relevant to the kidney and not just confined to cardiac cells. In the present study, we found that VCP746 was able to suppress TGF-β1-stimulated kidney mesangial cell (RMC) collagen synthesis via the activation of the A_{2B}AR. This outcome parallels the results of previous studies that demonstrated adenosine inhibits collagen synthesis in glomerular mesangial cells, an effect that was downstream of A_{2B}AR activation [37,57].

Collectively, this study reveals that the previously characterised novel cardioprotective pharmacology of VCP746 [23], now extends to include potent A_{2B}AR agonist effects. That is, in addition to being a biased agonist that elicits A_1AR-mediated cardioprotection in the absence of bradycardic side effects, we have now demonstrated that VCP746 also stimulates potent A_{2B}AR-mediated anti-fibrotic activity [22,23]. It has been established that the endogenous agonist adenosine attenuates cardiac remodelling and improves LV function in whole animals subjected to cardiac injury, specifically by regulating cardiac myocyte hypertrophy (A_1AR-mediated) and fibrosis (A_{2B}AR-mediated) [47,58]. As such we believe the dual potent A_1/A_{2B}AR agonism of VCP746 and the novel bivalent mechanism of receptor interaction may in fact represent a highly attractive therapeutic approach for modulating both myocardial fibrosis and hypertrophy in the treatment of heart failure.

Funding sources

This work was supported by Project Grants from the National Health and Medical Research Council (NHMRC) of Australia (APP1084487; APP1084246; APP546272) and the Australian Research Council (ARC; ID DE130100117). LTM is an ARC DECRA Postdoctoral Research Fellow (ID DE130100117) and AC is an NHMRC Senior Principal Research Fellow (ID APP1102950). EAV holds an Australian Postgraduate Award and an Australian Cancer Therapeutics scholarship.

References

[1] C.A. Beltrami, N. Finato, M. Rocco, G.A. Feruglio, C. Puricelli, E. Cigola, et al., Structural basis of end-stage failure in ischemic cardiomyopathy in humans, Circulation 89 (1994) 151–163, http://dx.doi.org/10.1161/01.CIR.89.1.151.
[2] M. Bujak, N.G. Frangogiannis, The role of TGF-beta signaling in myocardial infarction and cardiac remodeling, Cardiovasc. Res. 74 (2007) 184–195, http://dx.doi.org/10.1016/j.cardiores.2006.10.002.
[3] A.M. Segura, O.H. Frazier, L.M. Buja, Fibrosis and heart failure, Heart Fail. Rev. 19 (2012) 173–185, http://dx.doi.org/10.1007/s10741-012-9365-4.
[4] E.P. Carroll, J.S. Janicki, R. Pick, K.T. Weber, Myocardial stiffness and reparative fibrosis following coronary embolisation in the rat, Cardiovasc. Res. 23 (1989) 655–661, http://dx.doi.org/10.1093/cvr/23.8.655.
[5] F.A. van Nieuwenhoven, N.A. Turner, The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction, Vascul. Pharmacol. 58 (2013) 182–188, http://dx.doi.org/10.1016/j.vph.2012.07.003.
[6] W. Chen, N.G. Frangogiannis, Fibroblasts in post-infarction inflammation and cardiac repair, Biochem. Biophys. Acta 1833 (2013) 945–953, http://dx.doi.org/10.1016/j.bbamcr.2012.08.023.
[7] V.L. Roger, Epidemiology of heart failure, Circ. Res. 113 (2013) 646–659, http://dx.doi.org/10.1161/CIRCRESAHA.113.300268.
[8] F. See, A. Kompa, J. Martin, D.A. Lewis, H. Krum, Fibrosis as a therapeutic target post-myocardial infarction, Curr. Pharm. Des. 11 (2005) 477–487, http://dx.doi.org/10.2174/1381612053382098.
[9] J.G. Travers, F.A. Kamal, J. Robbins, K.E. Yutzey, B.C. Blaxall, Cardiac fibrosis: the fibroblast awakens, Circ. Res. 118 (2016) 1021–1040, http://dx.doi.org/10.1161/CIRCRESAHA.115.306565.
[10] K.E. Porter, N.A. Turner, Cardiac fibroblasts: at the heart of myocardial remodeling, Pharmacol. Ther. 123 (2009) 255–278, http://dx.doi.org/10.1016/j.pharmthera.2009.05.002.
[11] F. Villarreal, S. Zimmermann, L. Makhsudova, A.C. Montag, M.D. Erion, D.A. Bullough, et al., Modulation of cardiac remodeling by adenosine: in vitro and in vivo effects, Mol. Cell. Biochem. 251 (2003) 17–26, http://dx.doi.org/10.1023/A:1025401026441.
[12] J.P. Headrick, B. Hack, K.J. Ashton, Acute adenosinergic cardioprotection in ischemic-reperfused hearts, Am. J. Physiol. Heart Circ. Physiol. 285 (2003) H1797–H1818, http://dx.doi.org/10.1152/ajpheart.00407.2003.
[13] I. Feoktistov, I. Biaggioni, Adenosine A2B receptors, Pharmacol. Rev. 49 (1997) 381–402.
[14] F. Villarreal, S.A. Epperson, I. Ramirez-Sanchez, K.G. Yamazaki, L.L. Brunton, Regulation of cardiac fibroblast collagen synthesis by adenosine: roles for Epac and PI3K, Am. J. Physiol. Cell Physiol. 296 (2009) C1178–C1184, http://dx.doi.org/10.1152/ajpcell.00291.2008.
[15] Y. Chen, S. Epperson, L. Makhsudova, B. Ito, J. Suarez, W. Dillmann, et al., Functional effects of enhancing or silencing adenosine A2B receptors in cardiac fibroblasts, Am. J. Physiol. Heart Circ. Physiol. 287 (2004) H2478–H2486, http://dx.doi.org/10.1152/ajpheart.00217.2004.
[16] R.K. Dubey, D.G. Gillespie, E.K. Jackson, Adenosine inhibits collagen and protein synthesis in cardiac fibroblasts: role of A2B receptors, Hypertension 31 (1998) 943–948, http://dx.doi.org/10.1161/01.HYP.31.4.943.
[17] R.K. Dubey, D.G. Gillespie, Z. Mi, E.K. Jackson, Exogenous and endogenous adenosine inhibits fetal calf serum–induced growth of rat cardiac fibroblasts: role of A2B receptors, Circulation 96 (1997) 2656–2666, http://dx.doi.org/10.1161/01.CIR.96.8.2656.
[18] C. Valant, J. Robert Lane, P.M. Sexton, A. Christopoulos, The best of both worlds? Bitopic orthosteric/allosteric ligands of G protein-coupled receptors, Annu. Rev. Pharmacol. Toxicol. 52 (2012) 153–178, http://dx.doi.org/10.1146/annurev-pharmtox-010611-134514.
[19] J.R. Lane, P.M. Sexton, A. Christopoulos, Bridging the gap: bitopic ligands of G-protein-coupled receptors, Trends Pharmacol. Sci. 34 (2013) 59–66, http://dx.doi.org/10.1016/j.tips.2012.10.003.
[20] G. Vauquelin, G. Bricca, I. Van Liefde, Avidity and positive allosteric modulation/cooperativity act hand in hand to increase the residence time of bivalent receptor ligands, Fundam. Clin. Pharmacol. 28 (2013) 530–543, http://dx.doi.org/10.1111/fcp.12052.
[21] L. Aurelio, C. Valant, B.L. Flynn, P.M. Sexton, A. Christopoulos, P.J. Scammells, Allosteric modulators of the adenosine A1 receptor: synthesis and pharmacological evaluation of 4-substituted 2-amino-3-benzoylthiophenes, J. Med. Chem. 52 (2009) 4543–4547, http://dx.doi.org/10.1021/jm9002582.
[22] J.-A. Baltos, K.J. Gregory, P.J. White, P.M. Sexton, A. Christopoulos, L.T. May, Quantification of adenosine A1 receptor biased agonism: implications for drug discovery, Biochem. Pharmacol. 99 (2016) 101–112, http://dx.doi.org/10.1016/j.bcp.2015.11.013.
[23] C. Valant, L.T. May, L. Aurelio, C.H. Chuo, P.J. White, J.A. Baltos, et al., Separation of on-target efficacy from adverse effects through rational design of a bitopic adenosine receptor agonist, Proc. Natl. Acad. Sci. U.S.A. 111 (2014) 4614–4619, http://dx.doi.org/10.1073/pnas.1320962111.
[24] M.W. Alnouri, S. Jepards, A. Casari, A.C. Schiedel, S. Hinz, C.E. Müller, Selectivity is species-dependent: characterization of standard agonists and antagonists at

human, rat, and mouse adenosine receptors, Purinergic Signal. 11 (2015) 389–407, http://dx.doi.org/10.1007/s11302-015-9460-9.
[25] E.A. Vecchio, C.Y.R. Tan, K.J. Gregory, A. Christopoulos, P.J. White, L.T. May, Ligand-independent adenosine A2B receptor constitutive activity as a promoter of prostate cancer cell proliferation, J. Pharmacol. Exp. Ther. 357 (2016) 36–44, http://dx.doi.org/10.1124/jpet.115.230003.
[26] D.J. Autelitano, E.A. Woodcock, Selective activation of alpha1A-adrenergic receptors in neonatal cardiac myocytes is sufficient to cause hypertrophy and differential regulation of alpha1-adrenergic receptor subtype mRNAs, J. Mol. Cell. Cardiol. 30 (1998) 1515–1523, http://dx.doi.org/10.1006/jmcc.1998.0717.
[27] A. Phrommintikul, L. Tran, A. Kompa, B. Wang, A. Adrahtas, D. Cantwell, et al., Effects of a Rho kinase inhibitor on pressure overload induced cardiac hypertrophy and associated diastolic dysfunction, Am. J. Physiol. Heart Circ. Physiol. 294 (2008) H1804–H1814, http://dx.doi.org/10.1152/ajpheart.01078.2007.
[28] L.T. May, V.A. Avlani, C.J. Langmead, H.J. Herdon, M.D. Wood, P.M. Sexton, et al., Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors, Mol. Pharmacol. 72 (2007) 463–476, http://dx.doi.org/10.1124/mol.107.037630.
[29] L.T. May, T.J. Self, S.J. Briddon, S.J. Hill, The effect of allosteric modulators on the kinetics of agonist-G protein-coupled receptor interactions in single living cells, Mol. Pharmacol. 78 (2010) 511–523, http://dx.doi.org/10.1124/mol.110.064493.
[30] C. Yung-Chi, W.H. Prusoff, Relationship between the inhibition constant (K i) and the concentration of inhibitor which causes 50 per cent inhibition (I 50) of an enzymatic reaction, Biochem. Pharmacol. (1973), http://dx.doi.org/10.1016/0006-2952(73)90196-2.
[31] J. Sühnel, Parallel dose-response curves in combination experiments, Bull. Math. Biol. 60 (1998) 197–213, http://dx.doi.org/10.1006/bulm.1997.0009.
[32] S. Hinz, S.K. Lacher, B.F. Seibt, C.E. Muller, BAY60-6583 acts as a partial agonist at adenosine A2B receptors, J. Pharmacol. Exp. Ther. 349 (2014) 427–436, http://dx.doi.org/10.1124/jpet.113.210849.
[33] T. Steinfeld, M. Mammen, J.A.M. Smith, R.D. Wilson, J.R. Jasper, A novel multivalent ligand that bridges the allosteric and orthosteric binding sites of the M2 muscarinic receptor, Mol. Pharmacol. 72 (2007) 291–302, http://dx.doi.org/10.1124/mol.106.033746.
[34] S. Weyler, F. Fülle, M. Diekmann, B. Schumacher, S. Hinz, K.-N. Klotz, et al., Improving potency, selectivity, and water solubility of adenosine A1 receptor antagonists: xanthines modified at position 3 and related pyrimido[1,2,3-cd] purinediones, ChemMedChem 1 (2006) 891–902, http://dx.doi.org/10.1002/cmdc.200600066.
[35] J. Linden, T. Thai, H. Figler, X. Jin, A.S. Robeva, Characterization of human A2B adenosine receptors: radioligand binding, western blotting, and coupling to Gq in human embryonic kidney 293 cells and HMC-1 mast cells, Mol. Pharmacol. 56 (1999) 705–713.
[36] F.N. Ziyadeh, K. Sharma, M. Ericksen, G. Wolf, Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-beta, J. Clin. Invest. 93 (1994) 536–542, http://dx.doi.org/10.1172/JCI117004.
[37] R.K. Dubey, M. Rosselli, D.G. Gillespie, Z. Mi, E.K. Jackson, Extracellular 3′,5′-cAMP-adenosine pathway inhibits glomerular mesangial cell growth, J. Pharmacol. Exp. Ther. 333 (2010) 808–815, http://dx.doi.org/10.1124/jpet.110.166371.
[38] D.C. Crawford, A.V. Chobanian, P. Brecher, Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat, Circ. Res. 74 (1994) 727–739, http://dx.doi.org/10.1161/01.RES.74.4.727.
[39] R.G. Dean, L.C. Balding, R. Candido, W.C. Burns, Z. Cao, S.M. Twigg, et al., Connective tissue growth factor and cardiac fibrosis after myocardial infarction, J. Histochem. Cytochem. 53 (2005) 1245–1256, http://dx.doi.org/10.1369/jhc.4A6560.2005.
[40] B.B. Fredholm, E. Irenius, B. Kull, G. Schulte, Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells, Biochem. Pharmacol. 61 (2001) 443–448, http://dx.doi.org/10.1016/S0006-2952(00)00570-0.
[41] Z.-G. Gao, R. Balasubramanian, E. Kiselev, Q. Wei, K.A. Jacobson, Probing biased/partial agonism at the G protein-coupled A2B adenosine receptor, Biochem. Pharmacol. 90 (2014) 297–306, http://dx.doi.org/10.1016/j.bcp.2014.05.008.
[42] A. Christopoulos, Allosteric binding sites on cell-surface receptors: novel targets for drug discovery, Nat. Rev. Drug Discov. 1 (2002) 198–210, http://dx.doi.org/10.1038/nrd746.
[43] L.T. May, V.A. Avlani, P.M. Sexton, A. Christopoulos, Allosteric modulation of G protein-coupled receptors, Curr. Pharm. Des. 10 (2004) 2003–2013, http://dx.doi.org/10.2174/1381612043384303.
[44] T. Kenakin, A. Christopoulos, Signalling bias in new drug discovery: detection, quantification and therapeutic impact, Nat. Rev. Drug Discov. 12 (2013) 205–216, http://dx.doi.org/10.1038/nrd3954.
[45] J.W. Wisler, K. Xiao, A.R. Thomsen, R.J. Lefkowitz, Recent developments in biased agonism, Curr. Opin. Cell Biol. 27 (2014) 18–24, http://dx.doi.org/10.1016/j.ceb.2013.10.008.
[46] R.B. Mailman, GPCR functional selectivity has therapeutic impact, Trends Pharmacol. Sci. 28 (2007) 390–396, http://dx.doi.org/10.1016/j.tips.2007.06.002.
[47] M. Wakeno, T. Minamino, O. Seguchi, H. Okazaki, O. Tsukamoto, K.I. Okada, et al., Long-term stimulation of adenosine A2B receptors begun after myocardial infarction prevents cardiac remodeling in rats, Circulation 114 (2006) 1923–1932, http://dx.doi.org/10.1161/CIRCULATIONAHA.106.630087.
[48] S.A. Epperson, L.L. Brunton, I. Ramirez-Sanchez, F. Villarreal, Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts, Am. J. Physiol. Cell Physiol. 296 (2009) C1171–C1177, http://dx.doi.org/10.1152/ajpcell.00290.2008.
[49] J.P. Headrick, K.J. Ashton, R.B. Rose'Meyer, J.N. Peart, Cardiovascular adenosine receptors: expression, actions and interactions, Pharmacol. Ther. 140 (2013) 92–111, http://dx.doi.org/10.1016/j.pharmthera.2013.06.002.
[50] J.M. Schnee, W.A. Hsueh, Angiotensin II, adhesion, and cardiac fibrosis, Cardiovasc. Res. 46 (2000) 264–268, http://dx.doi.org/10.1016/S0008-6363(00)00044-4.
[51] M.J. Sopel, N.L. Rosin, T.D. Lee, J.-F. Legare, Myocardial fibrosis in response to Angiotensin II is preceded by the recruitment of mesenchymal progenitor cells, Lab. Invest. 91 (2010) 565–578, http://dx.doi.org/10.1038/labinvest.2010.190.
[52] U. Seeland, C. Haeuseler, R. Hinrichs, S. Rosenkranz, T. Pfitzner, K. Scharffetter-Kochanek, et al., Myocardial fibrosis in transforming growth factor-beta(1) (TGF-beta(1)) transgenic mice is associated with inhibition of interstitial collagenase, Eur. J. Clin. Invest. 32 (2002) 295–303, http://dx.doi.org/10.1046/j.1365-2362.2002.00985.
[53] A.V. Sigel, M. Centrella, M. Eghbali-Webb, Regulation of proliferative response of cardiac fibroblasts by transforming growth factor-beta 1, J. Mol. Cell. Cardiol. 28 (1996) 1921–1929, http://dx.doi.org/10.1006/jmcc.1996.0185.
[54] S. Lekawanvijit, A.R. Kompa, B.H. Wang, D.J. Kelly, H. Krum, Cardiorenal syndrome: the emerging role of protein-bound uremic toxins, Circ. Res. 111 (2012) 1470–1483, http://dx.doi.org/10.1161/CIRCRESAHA.112.278457.
[55] J. Chen, J.K. Chen, K. Nagai, D. Plieth, M. Tan, T.C. Lee, et al., EGFR signaling promotes TGF-beta-dependent renal fibrosis, J. Am. Soc. Nephrol. 23 (2012) 215–224, http://dx.doi.org/10.1681/ASN.2011070645.
[56] S. Lekawanvijit, A.R. Kompa, Y. Zhang, B.H. Wang, D.J. Kelly, H. Krum, Myocardial infarction impairs renal function, induces renal interstitial fibrosis, and increases renal KIM-1 expression: implications for cardiorenal syndrome, Am. J. Physiol. Heart Circ. Physiol. 302 (2012) H1884–H1893, http://dx.doi.org/10.1152/ajpheart.00967.2011.
[57] R.K. Dubey, D.G. Gillespie, Z. Mi, E.K. Jackson, Adenosine inhibits PDGF-induced growth of human glomerular mesangial cells via A2B receptors, Hypertension 46 (2005) 628–634, http://dx.doi.org/10.1161/01.HYP.0000178464.63393.88.
[58] Y. Liao, Activation of adenosine A1 receptor attenuates cardiac hypertrophy and prevents heart failure in murine left ventricular pressure-overload model, Circ. Res. 93 (2003) 759–766, http://dx.doi.org/10.1161/01.RES.0000094744.88220.62.

Chapter 6:

Novel Adenosine A_{2B} Receptor Signalling in Cardiac Fibroblasts – Uncovering Context-Specific Biased Agonism

6.1 Introduction

Cardiac fibroblasts constitute the largest population of interstitial cells in the adult mammalian heart and are essential for maintaining the structural and electro-mechanical integrity of the myocardium (Chen and Frangogiannis, 2013; Souders et al., 2009). They regulate the extracellular matrix (ECM) in response to various mechanical and hormonal stimuli, such as the cytokine transforming growth factor β1 (TGF-β1). TGF-β1 activates fibroblast cell-surface receptors to promote differentiation to myofibroblasts, the pro-fibrogenic phenotype that expresses the contractile protein α-smooth muscle actin (α-SMA) and produces collagen, the predominant ECM protein (Leask, 2007; Petrov et al., 2002; Porter and Turner, 2009). Myofibroblasts are the principle cell type responsible for maladaptive fibrosis, which is a hallmark feature of heart failure pathophysiology. As such, much research in recent years has focused on the regulation of myofibroblast activation and function in an effort to identify novel therapeutics for heart failure (See et al., 2005; Segura et al., 2012).

Signalling via the adenosineA_{2B} receptor (A_{2B}AR), represents one such novel pharmacological approach which has been demonstrated to have an important role in both the acute and chronic stages of cardiac fibrosis and tissue remodelling post myocardial infarction (MI). In the acute, *in vitro* setting, stimulation of A_{2B}ARs inhibits cardiac fibroblast proliferation (Dubey et al., 1997), collagen synthesis (Dubey et al., 1998) and decreases expression of pro-fibrotic gene markers including connective tissue growth factor (Vecchio et al., 2016a). However, prolonged A_{2B}AR activation in an *in vivo* setting appears to confer a paradoxical maladaptive increase in fibrosis and tissue remodelling. Chronic administration of a novel, highly selective A_{2B}AR antagonist, GS-6201, attenuates cardiac dysfunction and fibrosis in animal models of MI (Toldo et al., 2012; Zhang et al., 2014), effects which can be mimicked by A_{2B}AR gene knock out (Maas et al., 2008). The exact mechanism behind these paradoxical effects requires further

elucidation but may reflect changes in differential receptor coupling with changes in cellular background as the disease progresses. There is certainly precedence for context-dependent variations in intracellular signalling, as previous studies have demonstrated myofibroblasts have a reduced capacity to produce the anti-fibrotic second messenger cAMP when compared to unmodified fibroblasts (Lu et al., 2013; Yokoyama et al., 2008).

The aim of the current study therefore, was to investigate A_{2B}AR signalling in cardiac fibroblasts and assess the influence of conversion to a myofibroblast phenotype on signal transduction. Based on an established protocol known to promote α-SMA expression (a marker for myofibroblast differentiation) (Lijnen and Petrov, 2002; Swaney et al., 2005), isolated rat neonatal ventricular cardiac fibroblasts (NVCFs) were treated in the absence or presence of TGF-β1 in order to generate a cell population enriched with fibroblasts or myofibroblasts respectively. Given the A_{2B}AR is pleiotropically coupled to both G_s and G_q proteins, we examined two canonical GPCR signalling pathways using accumulation assays; G_s-mediated cAMP and G_q-mediated IP_1 accumulation (Fredholm et al., 2001a; Linden et al., 1999). Experiments detected changes in basal and A_{2B}AR agonist mediated signalling between the two conditions and it was demonstrated myofibroblasts had reduced G_s-cAMP accumulation but an enhanced capacity for G_q-IP_1 signalling. Interestingly, we identified a novel signalling pathway in which A_{2B}AR stimulation inhibited basal IP_1 accumulation, potentially downstream of protein kinase G (PKG) activation. In addition, in contrast to the non-selective adenosine receptor agonist NECA, we demonstrated the A_1AR/A_{2B}AR biased agonist, VCP746 (Baltos et al., 2016b; Vecchio et al., 2016a), retained activity even when A_{2B}AR signalling was diminished under myofibroblast (+TGF-β1) conditions. Thus, results for this chapter demonstrate a unique example of *'context-specific biased agonism,'* which we define as the ability of contextual alterations in cell biology to significantly influence ligand bias.

Specifically, this study has demonstrated that TGF-β1 treatment, and resultant conversion of fibroblasts to myofiboblasts, significantly influences the bias profile of VCP746 at the A_{2B}AR.

6.2 Materials & Methods

6.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin, antibiotic-antimycotic and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). The LANCE cAMP kit was purchased from PerkinElmer (Waltham, MA, USA) and the IP-One homogeneous time resolved fluorescence (HTRF) kit was obtained from Cisbio Bioassays (Codolet, France). Type II collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Recombinant human transforming growth factor-β1 (TGF-β1) was purchased from R&D Systems (Minneapolis, MN). Adenosine receptor ligands, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583) and 8-(4-(4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl))-1-propylxanthine (PSB-603) and inhibitor KT 5823 were from Tocris Bioscience (Bristol, UK). VCP746 was synthesized in-house as described previously (Valant et al., 2014). UBO-QIC was purchased from University of Bonn (Bonn, Germany). All other reagents were of analytical quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.2.2 Rat neonatal cardiac fibroblast isolation and cell culture

Animal experiments conformed to the requirements of the National Health and Medical Research Council of Australia's *Code of Practice for the Care and Use of Animals for Scientific Purposes* and were conducted in accordance with Monash Institute of Pharmacy and Pharmaceutical Sciences animal ethics committee-approved protocols. Isolation of primary

neonatal rat ventricular cardiac fibroblasts (NVCFs) from 1-2 day-old Sprague-Dawley rat pups was performed as previously described (Baltos et al., 2017; Laskowski et al., 2006). After digestion and separation of NVCFs from cardiomyocytes, culture dishes containing adherent NVCFs were twice washed with PBS and detached using trypsin/versene, centrifuged and resuspended in DMEM containing 1% FBS. NVCFs were subsequently seeded into 96-well plates pre-coated with 1% gelatin at a density of 2×10^4 cells/well and maintained at 37°C in a humidified incubator containing 5% CO_2. After 16 h culture media was removed and replaced with serum free DMEM in the presence or absence of TGF-β1 (10 ng/mL) for 48 h prior to assaying.

6.2.3 cAMP accumulation

Stimulation of cAMP accumulation in NVCFs was performed as previously described (Baltos et al., 2017). Results were analysed against a cAMP standard curve performed in parallel and expressed as cAMP concentration (pmol/well) or as a percentage of the response mediated by forskolin (10 μM) in the absence of TGF-β1 (10 ng/mL).

6.2.4 IP_1 accumulation

IP_1 accumulation was performed as previously described (Baltos et al., 2017; Vecchio et al., 2016b). When inhibitors or the A_{2B}AR antagonist PSB-603 were used, they were pre-incubated for 40 min prior to agonist stimulation. Data were analysed against an IP_1 standard curve performed in parallel and expressed as IP_1 concentration or as a percentage of the basal IP_1 concentration in the presence of TGF-β1 (10 ng/mL) as stated in the results.

6.2.5 Data Analysis

Curve fitting and statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA). Agonist concentration-response data were fitted to a three-parameter Hill equation:

$$Response = Basal + \frac{(E_{max} - Basal) \times [A]}{EC_{50} + [A]} \quad \text{Equation 1}$$

where basal is the baseline response in the absence of agonist, [A] is the concentration of agonist, and EC_{50} is the concentration of agonist required to generate a response halfway between the basal level and maximal effect (E_{max}).

Signalling bias was quantified by fitting the agonist concentration-response curves to the Black-Leff operational model of agonism to obtain transduction coefficients, $\log(\tau/K_A)$, using the equation as follows:

$$Response = \frac{E_m[\mathrm{A}]^n \tau^n}{[\mathrm{A}]^n \tau^n + ([\mathrm{A}] + K_\mathrm{A})^n} \quad \text{Equation 2}$$

where E_m describes the maximal response of the system, [A] is the agonist concentration, n is the 'transducer slope' that links agonist concentration to measured response, τ is an index of the coupling efficacy of the agonist and K_A is the functional equilibrium dissociation constant. Biased agonism was quantified as described previously (Baltos et al., 2016b; van der Westhuizen et al., 2014). All results were expressed as the mean ± standard error of the mean (SEM). Statistical significance was defined as $P<0.05$ as determined by t-test or two-way analysis of variance (ANOVA) with Sidak's or Dunnett's multiple comparisons post hoc analysis.

6.3 Results

6.3.1 Myofibroblasts have a reduced capacity for cAMP signalling

Conversion of NVCFs to profibrogenic myofibroblasts in the presence of TGF-β1 using an established protocol (10 ng/mL for 48 h; (Lijnen and Petrov, 2002; Swaney et al., 2005); details

of imaging and protocol optimisation are included in Appendix 2) revealed significant alterations in second messenger signalling between the two cell populations. Treatment of NVCFs with the direct adenylyl cyclase activator, forskolin (FSK), stimulated robust cAMP accumulation over the basal level, however this response to FSK was significantly diminished in myofibroblasts (Fig. 6.1A; $P < 0.001$; Two-way ANOVA, Sidak's multiple comparison's test). Incubation of NVCFs with the non-selective adenosine receptor agonist NECA, or the A_1/A_{2B}AR agonist VCP746 (Baltos et al., 2016b; Vecchio et al., 2016a), stimulated concentration-dependent cAMP accumulation with a maximal response that was significantly attenuated in myofibroblasts (Fig. 6.1B; $P < 0.05$; Paired t test). The A_{2B}AR is known to be the most abundantly expressed adenosine receptor in rat cardiac fibroblasts (Epperson et al., 2009). To establish the A_{2B}AR contribution to agonist-mediated cAMP accumulation, assays were repeated in the presence of the A_{2B}AR-selective antagonist, PSB-603. In both NVCFs and myofibroblasts, the response to NECA and VCP746 was virtually abolished when the cells were pre-incubated with the A_{2B}AR-selective antagonist, PSB-603 (Fig. 6.1C). Thus, we have established the A_{2B}AR is the main adenosine receptor subtype responsible for cAMP accumulation in fibroblasts and myofibroblasts and highlighted the myofibroblasts' reduced capacity for cAMP production in response to either forskolin or adenosine receptor agonists.

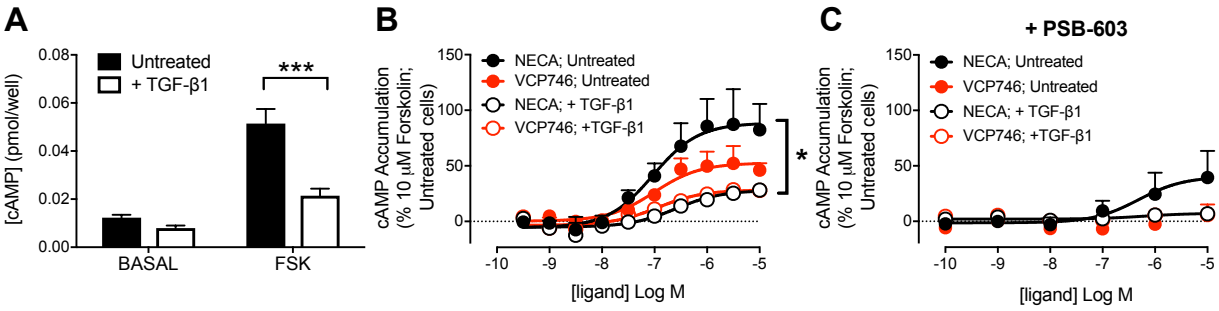

Fig. 6.1. NVCFs treated with TGF-β1 have a reduced capacity to produce cAMP upon incubation with forskolin or A_{2B}AR agonists. (A) NVCFs treated with forskolin (FSK; 10 μM) generated robust increases in cAMP accumulation. Upon conversion to a myofibroblast phenotype (treated with TGF-β1, 10 ng/mL for 48 h), cells had a significantly attenuated response to FSK as expressed as [cAMP] in pmol/well. ***P <0.001; Two-way ANOVA, Sidak's multiple comparison's test. (B) Adenosine receptor agonists, NECA and VCP746 increased

cAMP accumulation in a concentration-dependent manner in NVCFs (closed symbols) and myofibroblasts (open symbols), however had a significantly reduced maximal response in myofibroblasts. **P* <0.05; Paired t test. (C) NECA- and VCP746-mediated cAMP accumulation in NVCFs and myofibroblasts was virtually abolished in the presence of the A_{2B}AR-selective antagonist PSB-603 (1 μM). Data expressed as a percentage of the maximal response produced by 10 μM FSK in untreated NVCFs. Data represent the mean + SEM from 5-6 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

6.3.2 Myofibroblasts have an increased capacity for IP_x signalling and reveal A_{2B}AR-mediated inhibition of IP_x accumulation

As the A_{2B}AR is a pleiotropically-coupled receptor, capable of additionally interacting with $G_{q/11}$-proteins in endogenously expressing cells (Linden et al., 1999), we tested the ability of both adenosine receptor agonists and positive control, adenosine triphosphate (ATP) to stimulate inositol triphosphate (IP_3) production. Given the rapid degradation of IP_3 to inositol monophosphate (IP_1), IP_1 accumulation was used as a readout of pathway activation in NVCFs and converted myofibroblasts (+TGF-β1). There was a significant difference in baseline IP_1 production between the two cell types, with the myofibroblasts having a significantly higher basal IP_1 level (Fig. 6.2A; $P < 0.05$; Two-way ANOVA, Sidak's multiple comparison's test). In addition, incubation of NVCFs with ATP generated a robust increase in IP_1 accumulation that was significantly greater in the converted myofibroblasts (Fig. 6.2A; $P < 0.01$; Two-way ANOVA, Sidak's multiple comparison's test). Incubation of NVCFs and myofibroblasts with adenosine receptor agonists NECA and VCP746, generated paradoxical significant concentration-dependent inhibition of IP_1 accumulation below basal levels (Fig. 6.2B; $P < 0.05$; Paired t test on top and bottom plateau for each agonist). Agonist-stimulated inhibition of IP_1 accumulation was demonstrated to be A_{2B}AR-mediated as pre-incubation with PSB-603 abolished the effect (Fig. 6.2C).

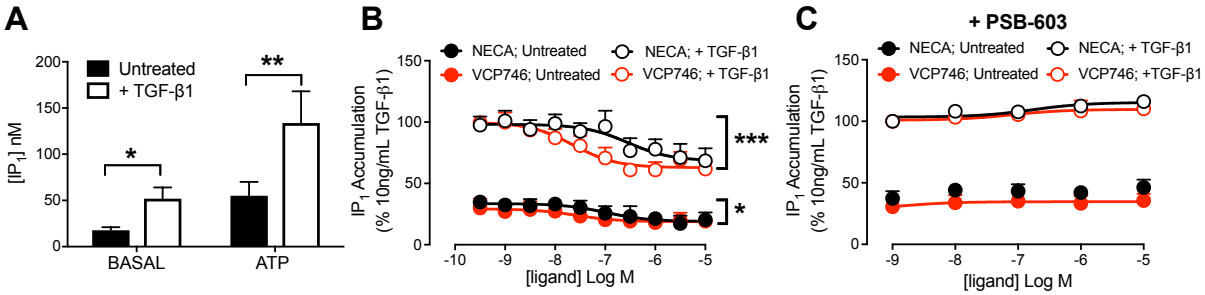

Fig. 6.2. TGF-β1-induced myofibroblasts have an increased capacity to produce IP_1. (A) Converted myofibroblasts (NVCFs treated with TGF-β1, 10 ng/mL for 48 h) produce significantly more IP_1 under basal and ATP-stimulated conditions than their untreated NVCF counterpart. (B) Adenosine receptor agonists, NECA and VCP746 significantly inhibit basal IP_1 accumulation in a concentration-dependent manner in NVCFs (closed symbols) and myofibroblasts (open symbols). **P* <0.05, ****P* <0.001; Paired t test on top and bottom plateau for each agonist. Results expressed as a percentage of the basal response in myofibroblasts (+TGF-β1) which is substantially elevated compared to NVCFs. (C) NECA- and VCP746-mediated inhibition of IP_1 accumulation in NVCFs and myofibroblasts is abolished in presence of the $A_{2B}AR$-selective antagonist PSB-603 (1 μM). **P* <0.05, ***P* <0.01; Two-way ANOVA, Sidak's multiple comparison's test. Data represent the mean + SEM from 4-5 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

6.3.3 VCP746 displays 'context-specific ligand bias'

It was evident the maximal $A_{2B}AR$-mediated responses in cAMP accumulation and IP_1 inhibition were altered in myofibroblasts when compared to untreated fibroblasts (Fig. 6.1B & 6.2B). In addition, the potency of NECA was significantly reduced in the presence of TGF-β1 in both assays (Fig. 6.3A; $P < 0.05$; t-test). In contrast, VCP746 retained potency under diseased conditions, which suggests it can maintain anti-fibrotic signal transduction in myofibroblasts (Fig. 6.3A). These differential changes in fibroblasts and myofibroblasts suggest VCP746, relative to NECA, displays context-specific biased agonism. Indeed, when VCP746 bias was quantified, (relative to the reference agonist, NECA and the reference pathway, cAMP accumulation), a significant increase in VCP746 bias towards IP_1 inhibition was observed in the presence of TGF-β1 treatment, with the bias factor increasing from 1.9 to 8.3 (Fig. 6.3B).

In the untreated fibroblasts, VCP746 had no significant bias for cAMP or IP_1 accumulation relative to the reference agonist NECA (Fig. 6.3B; Log (Bias Factor) 0.29 ± 0.34; $P > 0.05$; t test). In contrast, in the presence of TGF-β1, VCP746 was significantly biased toward inhibition of IP_1 over cAMP accumulation (Fig. 6.3B; Log (Bias Factor) 0.92 ± 0.22; $P < 0.05$; t test). These data suggest that the contextual alterations in cell biology have influenced ligand bias.

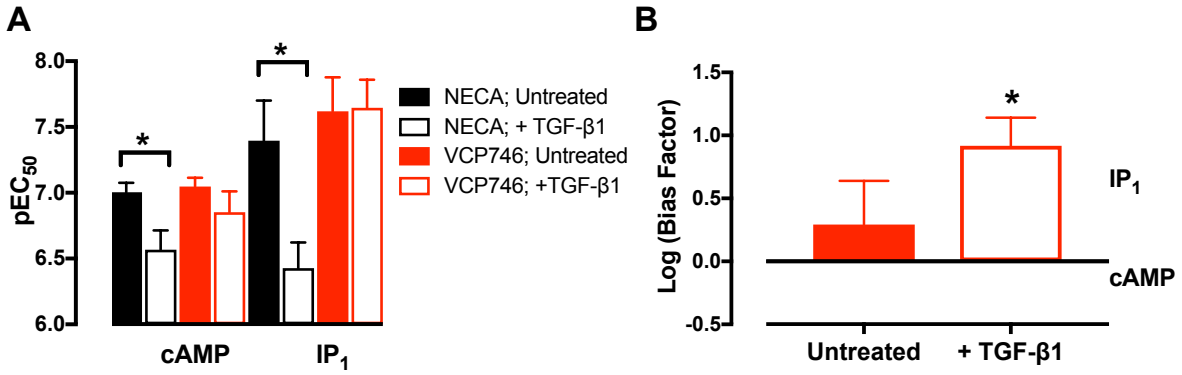

Fig. 6.3. VCP746 displays context-specific A_{2B}AR biased agonism. (A) In contrast to NECA, VCP746 potency for anti-fibrotic cAMP accumulation and IP_1 inhibition is unchanged upon fibroblast conversion to myofibroblasts (+TGF-β1, 10 ng/mL for 48 h). **P* <0.05, t-test. (B) VCP746 displays significant bias toward IP_1 inhibition relative to the reference ligand; NECA and the reference pathway; cAMP accumulation, only under TGF-β1 treated conditions. The non-Log (Bias Factor) for VCP746 increased significantly from 1.9 in untreated fibroblasts to 8.3 in cells treated with TGF-β1. **P* <0.05, t-test. Data represent the mean + SEM from 4-6 independent experiments performed in duplicate.

6.3.4 Agonist-mediated inhibition of IP_x accumulation is reversed with a PKG inhibitor

This is the first demonstration of GPCR-mediated inhibition of IP_x accumulation in cardiac fibroblasts and potentially represents a novel mechanism to decrease fibrosis. Given G_q-IP_3 signalling is known to be pro-fibrotic (Ju et al., 1998; Matsushita et al., 2014; Mende et al., 1998), we wanted to understand the mechanism behind the inhibition. In smooth muscle there is evidence that cAMP-dependent protein kinase A (PKA) and cGMP-dependent protein kinase G (PKG) can inhibit G_q-dependent phospholipase C (PLC) activity (Huang et al., 2006; Nalli et al., 2014). As such, we pre-incubated NVCFs and myofibroblasts with the PKA inhibitor;

PKI, the potent and selective PKG inhibitor; KT5823, or the G_q-protein inhibitor; UBO-QIC and determined basal and agonist mediated IP_1 accumulation. UBO-QIC inhibited basal and ATP-stimulated IP_1 accumulation, confirming the involvement of the G_q protein in pathway activation (Fig. 6.4 A&B). Interestingly, KT5823, but not PKI, significantly augmented the response to ATP, which would suggest an interaction between PKG (but not PKA) and G_q-signalling (Fig. 6.4B; $P < 0.001$; Two-way ANOVA, Dunnett's multiple comparison's test; compared to ATP level). In untreated fibroblasts, there was no clear reversal in the small, non-significant, A_{2B}AR agonist-mediated decrease in IP_1 accumulation with any inhibitor (Fig. 6.4 C&D; $P > 0.05$; Two-way ANOVA, Dunnett's multiple comparison's test; compared to NECA or VCP746 level respectively). In the myofibroblasts (+TGF-β1), KT5823 appeared to reverse the significant A_{2B}AR agonist-mediated inhibition of IP_1 accumulation, which again would support an interaction between PKG and G_q-signalling (Fig. 6.4 C&D; $P < 0.05$; Two-way ANOVA, Dunnett's multiple comparison's test; compared to NECA or VCP746 level respectively), however further studies are required to fully elucidate the mechanism.

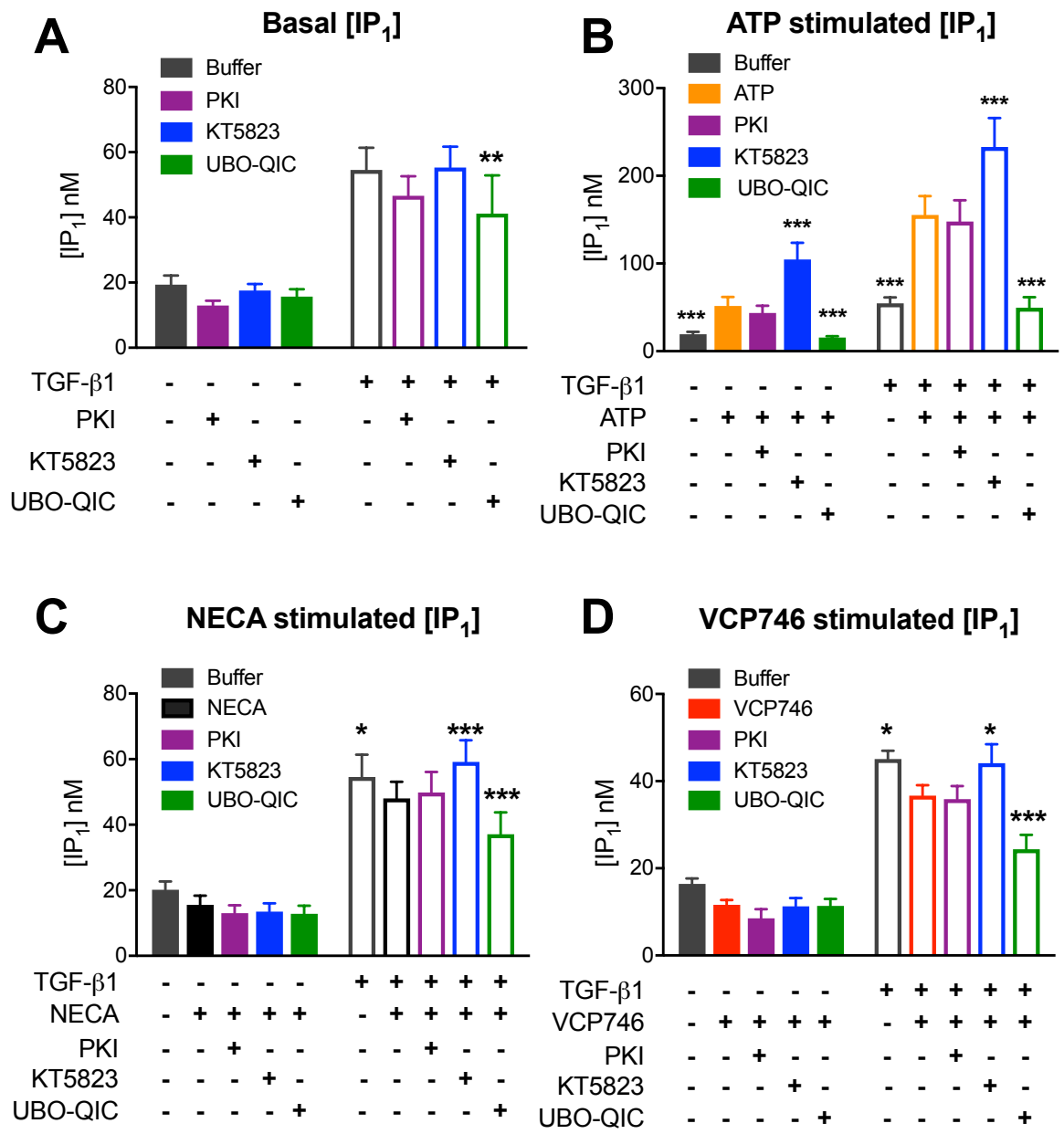

Fig. 6.4. Evidence of interaction between PKG and G_q-IP_1 signalling pathways in NVCFs and myofibroblasts. (A) Basal, (B) ATP (10 μM), (C) NECA (10 μM), or (D) VCP746 (1 μM) stimulated IP_1 accumulation in untreated NVCFs (closed bars) and converted myofibroblasts (+TGF-β1; open bars) in the presence or absence of pathway inhibitors; PKI (PKA inhibitor; 1 μM), KT5823 (PKG inhibitor; 1 μM) or UBO-QIC (G_q inhibitor; 1 μM). **P* <0.05, ***P* <0.01, ****P* <0.001; Two-way ANOVA, Dunnett's multiple comparison's test compared to buffer (A), ATP (B), NECA (C), or VCP746 (D) level. Data represent the mean + SEM from 5-7 independent experiments performed in duplicate.

6.3.5 Signalling profiles in NVCFs and myofibroblasts are conserved for other G_s-coupled GPCRs

To test whether the altered signalling profiles for NVCFs and myofibroblasts are confined to A_{2B}AR activation or more generalisable, we examined the activation of the more highly expressed, clinically relevant, G_s-coupled β_2-adrenergic receptor (β_2-AR) (Snead and Insel, 2012). The non-selective β-adrenergic receptor agonist isoprenaline (ISO), stimulated potent and robust cAMP accumulation that was in excess of the response generated by 10 μM FSK. Similar to the results observed with A_{2B}AR agonists, the maximal response to ISO was substantially reduced in myofibroblasts compared to NVCFs (Fig. 6.5A). Likewise, in the IP_1 accumulation assay, ISO stimulated concentration-dependent inhibition of basal IP_1 production that was more pronounced in myofibroblasts due to their substantially elevated baseline (Fig. 6.5B). The similar signalling profiles of both A_{2B}AR and β_2-AR agonists highlight global cellular changes between NVCFs and converted myofibroblasts. Furthermore, the inhibition of IP_x accumulation appears to be a general signalling pathway for G_s-coupled GPCRs in (myo)fibroblasts.

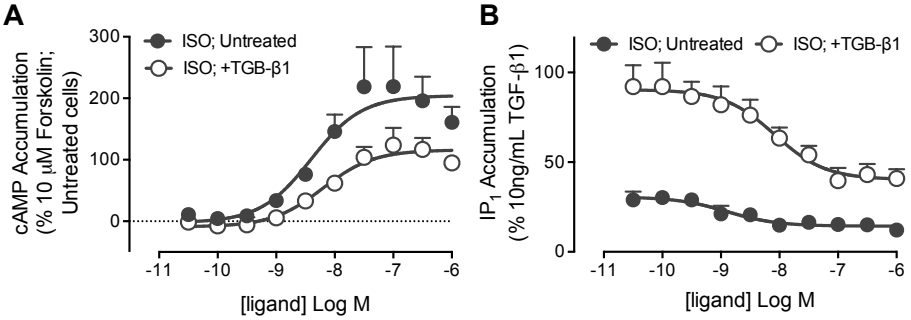

Fig. 6.5. β-adrenergic receptor agonist isoprenaline (ISO) stimulates similar signalling profiles to A_{2B}AR agonists in NVCFs and converted myofibroblasts. (A) ISO-stimulated cAMP accumulation in NVCFs (closed symbols) and myofibroblasts (open symbols) expressed as a percentage of the maximal response produced by 10 μM FSK in untreated NVCFs. (B) ISO inhibits basal IP_1 accumulation in NVCFs (closed symbols) and myofibroblasts (open symbols), with results expressed as a percentage of the basal response in myofibroblasts (+TGF-β1). Data represent the mean + SEM from 5 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

6.4 Discussion

This study has investigated the influence of disease context on A_{2B}AR signalling in cardiac fibroblasts in an effort to understand how both pro- and anti-fibrotic signal transduction could be attributed to this adenosine receptor subtype. We demonstrated that NVCFs treated with TGF-β1 had an altered signalling profile, which may be representative of the changes occurring when cardiac fibroblasts undergo a phenotypic change to myofibroblasts, the cell type associated with extracellular matrix remodelling in heart failure. We have shown that myofibroblasts have reduced G_s-cAMP production but enhanced G_q-IP_1 signalling in response to both basal and positive controls. The adenosine receptor agonists NECA and VCP746 stimulated cAMP production and an inhibition of IP_1 accumulation in both cell types via activation of the A_{2B}AR. These experiments additionally revealed 'context-specific bias' of VCP746 relative to NECA, with VCP746 better able to maintain its potency in the diseased myofibroblasts. Agonist inhibition of IP_1 accumulation appeared to involve crosstalk between G_q and PKG signalling, though further work is required to fully elucidate this novel mechanism. Interestingly, the signalling profiles in NVCFs and myofibroblasts appears to be maintained for other clinically relevant G_s-coupled GPCRs such as the β_2-adrenergic receptor (β_2-AR).

In order to observe any potential differences in signalling, experiments for this chapter required the development and optimization of a protocol for the conversion of isolated NVCFs to a myofibroblast phenotype. The cytokine TGF-β1 has been well established as a promoter of myofibroblasts differentiation, as measured by the presence α-SMA stress fibres, both *in vitro* and *in vivo* (Santiago et al., 2010; Vaughan et al., 2000). However, the culture conditions for fibroblasts are very important, as passage number, time in culture, plate coating and presence of FBS in the media can all influence the spontaneous conversion to myofibroblasts (as discussed in further detail in Appendix 2) (Arora et al., 1999; Santiago et al., 2010; Swaney et

al., 2005). Despite not observing clear differences in α-SMA staining content, possibly due to non-specific labelling (See Appendix 2; future studies could assess phenotypic changes such as changes in cell size and assembly of fibres), we are fairly confident our culturing protocol generated two different cell populations as evidenced by the significant differences in intracellular signalling. An important consideration is whether TGF-β1 was exerting its effect by simply changing cell number. Early reports in lung fibroblasts demonstrated that TGF-β1 increased collagen content without increasing fibroblast proliferation over a 48 h period (Fine and Goldstein, 1987). In addition, if it was just an effect of increasing cell number, then we would expect to see an increase in both IP_1 and cAMP accumulation in the myofibroblasts, which was not the case.

In agreement with previous studies demonstrating myofibroblasts have a reduced ability to produce the anti-fibrotic second messenger cAMP (Lu et al., 2013; Swaney et al., 2005; Yokoyama et al., 2008), we have demonstrated that the response to forskolin, A_{2B}AR agonists NECA and VCP746 and the β_2-AR agonist isoprenaline were diminished in NVCFs treated with TGF-β1. Previous work has demonstrated this is most probably due to global changes in expression of adenylyl cyclase and phosphodiesterase isoforms when cells are converted to the myofibroblast phenotype (Lu et al., 2013). We also examined the accumulation of IP_1 as the A_{2B}AR is known to promiscuously couple to $G_{q/11}$ proteins (Linden et al., 1999), but also because $G_{q/11}$ signalling downstream of GPCRs such as the angiotensin II and endothelin receptors is more broadly recognised as pro-fibrotic (Ju et al., 1998; Matsushita et al., 2014; Mende et al., 1998; Schumacher et al., 2016). Myofibroblasts had a significantly elevated basal and ATP-stimulated level of IP_1 accumulation which would suggest $G_{q/11}$ signalling is enhanced in this cell type, though the mechanism behind this is still to be investigated. Surprisingly, we identified a concentration-dependent inhibition of IP_1 accumulation in the presence of both

A_{2B}AR and β_2-AR agonists which may represent a novel mechanism to dampen $G_{q/11}$ signalling in cardiac fibroblasts. Previous reports in smooth muscle cells have identified a possible mechanism of interaction whereby PKA and PKG can inhibit PLC activity and thus inhibit G_q-IP_1 signalling (Huang et al., 2006; Nalli et al., 2014). Using selective inhibitors, we identified PKG but not PKA, as having a role in the cross talk with G_q signalling in NVCFs and myofibroblasts (Fig. 6.6). Blockade of PKG appeared to prevent A_{2B}AR agonist-mediated inhibition of IP_1 accumulation, though the exact mechanism remains to be fully elucidated. One possible explanation may involve A_{2B}AR-mediated activation of nitric oxide synthase (NOS) in endothelial cells that may also be present in the fibroblast preparations. Production of nitric oxide (NO) is known to stimulate cGMP-PKG in fibroblasts, which may then go on to inhibit PLC activity and subsequent IP_1 production (Calderone et al., 1998; Du et al., 2015).

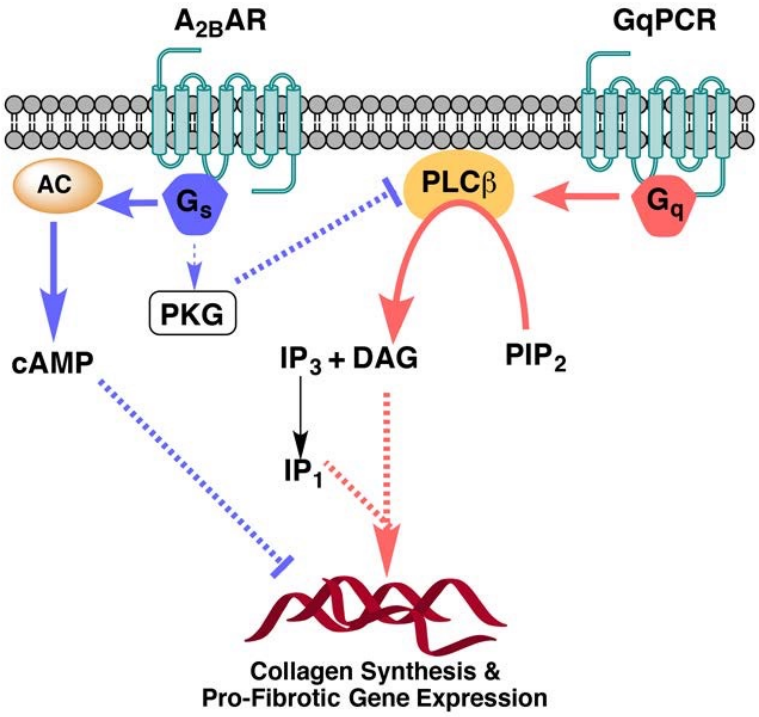

Fig. 6.6. Working hypothesis for the possible mechanism of A_{2B}AR-mediated Gs crosstalk with pro-fibrotic Gq signalling in fibroblasts.

Data from signalling assays also revealed, for the first time, context-specific bias. NECA had a lower potency for cAMP accumulation and inhibition of IP_1 accumulation in myofibroblasts whereas VCP746 maintained its potency under disease conditions. This suggests biased agonists such as VCP746 may be less influenced by disease context and may retain A_{2B}AR anti-fibrotic signalling even when prototypical A_{2B}AR agonists are less efficacious. Importantly, this illustrates the need to consider disease context in the rational-design of agonists and is especially pertinent to cardiovascular disease because risk factors such as advancing age and diabetes and post-MI pathobiology can significantly alter cardiac cell background, membrane environment and adenosine receptor expression (Headrick et al., 2013).

These studies have provided the groundwork to understand A_{2B}AR signalling in fibroblasts and myofibroblasts. In addition to stimulating anti-fibrotic cAMP production we have also demonstrated a novel mechanism by which A_{2B}AR activation can inhibit pro-fibrotic G_q-IP_x signal transduction. These *in vitro* experiments have not provided mechanistic insight into how both anti- and pro-fibrotic signal transduction could be downstream of A_{2B}AR activation. An obvious explanation is that *in vitro* conditions may not reflect the true course of disease progression *in vivo* due to the exclusion of the inflammatory response and loss of whole organ complexity. It is evident however, that prototypical A_{2B}AR agonists lose efficacy in myofibroblasts compared to fibroblasts and this may contribute to the loss of agonist effect in the chronic disease setting. Excitingly, we have provided the first evidence to suggest that a biased agonist such as VCP746 may be able to, at least partially, overcome the influence of disease context by maintaining potency in desired pathways and thus may represent a valid therapeutic approach to target fibrosis in heart failure.

Chapter 7:

General Discussion

The 'low-affinity' adenosine A_{2B} receptor (A_{2B}AR) was presumed to be quiescent under normal physiological conditions and had, up until recently, been a neglected member of the adenosine receptor family (Feoktistov and Biaggioni, 1997). We now know the A_{2B}AR represents an exciting emerging target in many pathological disease states. In particular, those characterised by conditions of elevated adenosine concentrations associated with reduced oxygen supply or increased metabolic demand such as in tissue ischaemia, fibrosis, inflammation and cancer (Cekic et al., 2011; Dubey et al., 1998; Eltzschig et al., 2013; Ham and Rees, 2008; Ma et al., 2010; Zimmerman et al., 2013). Pharmacological characterisation of the A_{2B}AR has lagged behind the other adenosine receptor subtypes. However, it has become apparent that a clearer understanding of A_{2B}AR pharmacology and signalling is required in order for the full therapeutic potential of the A_{2B}AR to be revealed. The main aim of my thesis therefore was to do just that, to investigate new paradigms of A_{2B}AR signalling and explore how these could be targeted or possibly exploited in the context of pathophysiology.

The first interesting discovery of the thesis came about through the observation that heterologous expression of the A_{2B}AR had grossly elevated basal levels of cAMP production when compared to the other adenosine receptor subtypes in the same FlpInCHO cell background. Incubation with A_{2B}AR 'antagonists' demonstrated concentration-dependent decreases in baseline cAMP even in the presence of ADA, which led to the reclassification of these agents as 'inverse agonists'. It is important to note that at low endogenous concentrations, adenosine is cleared predominantly via adenosine kinase rather than ADA and the constant production and transport of adenosine into the extracellular space means that a trace amount of adenosine will remain (Lloyd and Fredholm, 1995). However, our data demonstrates that the A_{2B}AR is not activated by these low nanomolar concentrations of adenosine, with the addition of ADA (1 U/mL) abolishing the cAMP accumulation stimulated in the presence of exogenous

adenosine (1 nM – 10 μM; Fig. 2B). Thus it is more likely the inverse agonists decrease basal cAMP through inhibiting constitutive activity as opposed to endogenous adenosine. Thus studies for Chapter 2 led to the confirmation that the human wild type A_{2B}AR does indeed have a detectable level of constitutive activity. On its own, this finding may simply reflect the greater likelihood of detecting GPCR constitutive activity when they are heterologously overexpressed (Milligan, 2003). But the observation that inverse agonism could also be measured in prostate cancer cells expressing physiologically relevant levels of the A_{2B}AR, suggested there may indeed be a pathological role for A_{2B}AR constitutive activity. A caveat of this was that the prostate cancer cell line first had to be exposed to a period of hypoxia to facilitate the upregulation of the A_{2B}AR, which is known to be under the control of HIF-1α (Kong et al., 2006). While hypoxia was obviously achieved under artificial experimental conditions, this is still pathologically relevant because a defining feature of solid tumours is that the cells exist in a state of reduced oxygen perfusion and constant hypoxia (Ryzhov et al., 2008; Subarsky and Hill, 2003). These studies also demonstrated the A_{2B}AR antagonist PSB-603 could inhibit basal cell growth in an environment devoid of endogenous adenosine, which suggested A_{2B}AR ligand-independent activity contributes to driving prostate cancer cell proliferation. It is important to note that a subsequent publication has also identified that PSB-603 alters cellular metabolism in colorectal cancer cells. However these effects occurred via an adenosine receptor-independent mechanism, which may have implications for our results in the cell viability MTT assay (Mølck et al., 2016). In light of other studies using A_{2B}AR knock out or knockdown to show attenuated tumour growth (Cekic et al., 2011; Kasama et al., 2015; Ryzhov et al., 2008), it is evident that the A_{2B}AR, whether through constitutive activity or elevated adenosine concentrations in the tumour microenvironment, plays an important role in modulating cancer cell growth of a variety of solid tumours. Collectively, these findings highlight that A_{2B}AR inverse agonists may provide a novel therapeutic approach as a

pharmacological adjuvant in cancer treatment. The wider implications for the high level of A_{2B}AR constitutive activity is that the A_{2B}AR may in fact have an unappreciated role in physiology, even in the settings where endogenous adenosine concentrations were thought to be insufficient for receptor activation.

The next major area of research involved the investigation of A_{2B}AR interactions with other adenosine receptors in the setting of cardioprotection. Recent studies have identified that for full A_1AR-mediated protection against ischaemia-reperfusion injury there is a requirement for the coincident activation of the A_2AR subtypes (Urmaliya et al., 2009; 2010b; Zhan et al., 2011). Using an *ex vivo* model we demonstrated in Langendorff-perfused isolated rat hearts that adenosine receptor-mediated reductions in infarct size and recovery of cardiac function could be blocked by either an A_1AR or an A_{2B}AR antagonist. It was hypothesised that cooperative adenosine receptor signalling could be as consequence of signal convergence at downstream effector proteins or from interactions occurring more proximally at the receptor level across a dimeric interface. Precedence for the presence of adenosine receptor dimers and higher order oligomers comes from forerunner studies in the brain demonstrating assembly of adenosine receptors into heteromers, both with members of the adenosine receptor family and other GPCRs such as the dopamine D_2 receptor (Casadó et al., 2010; Ciruela et al., 2006a; Ferré et al., 2004). Within neonatal cardiomyocytes we demonstrated alterations in radiolabelled antagonist dissociation kinetics, occurring via allosteric interactions between the two binding sites of the A_1AR and the A_{2B}AR, which supports the hypothesis of a A_1AR-A_{2B}AR heteromer. The physical association of adenosine receptor subtypes displayed a functional consequence in NVCMs as blockade of the A_{2B}AR could attenuate A_1AR-$G_{i/o}$-mediated pERK1/2 signalling. Interestingly, attempts to further investigate the physical association of the adenosine receptor subtypes using heterologous expression of tagged receptors to enable the use of high powered

resonance energy transfer and imaging techniques, displayed no evidence of functional interactions. This may be due to a number of reasons, including the different stoichiometry of A_1AR to A_{2B}ARs in FlpInCHO cells compared to cardiomyocytes. It may also be due to a lack of all required binding partners for oligomeric complex formation or the absence of compartmentalisation and the complex membrane environment present in primary cardiomyocytes. As such, future studies into adenosine receptor interactions should continue to focus on providing evidence of physical association in native tissues. Given the potential for crossover of even the most subtype-selective adenosine receptor agonists and antagonists, a powerful next step for confirmation of dimerization would be to demonstrate specific pharmacological properties unique to the dimer that are altered in the absence of one of the subunits. Preferably this would be validated with the use of RNA interference technology or in cardiomyocytes obtained from receptor knock-out animals (Pin et al., 2007). The take away message from this chapter is that it is important to consider the A_{2B}AR (and indeed the A_1AR) not as a single receptor entity, rather as a dynamic protein capable of interacting with other receptors and binding partners. This also means the environment in which the A_{2B}AR exists can have an important influence on pharmacology.

One of the contributing reasons as to why the A_{2B}AR remained so poorly characterised until recently, was due to a paucity of pharmacological tools, in particular high affinity agonists (Fredholm et al., 2001a). Work for this thesis has reclassified two A_1AR ligands, capadenoson and VCP746 as A_1AR/A_{2B}AR agonists, which importantly expands the pharmacological toolbox to explore the role of the A_{2B}AR in pathophysiology. Capadenoson was identified based on its structural similarity to the prototypical A_{2B}AR agonist BAY60-6583 and was rigorously characterised in heterologous and endogenous expression systems. We demonstrated that capadenoson displayed a unique signalling profile that preferentially stimulated cAMP

accumulation over other intracellular pathways in both A_{2B}AR-FlpInCHO cells and HEK293T cells. In addition, capadenoson strongly stimulated A_{2B}AR cAMP accumulation in primary neonatal cardiac fibroblasts and cardiomyocytes. This suggests some of the beneficial cardioprotective effects previously demonstrated by capadenoson *in vivo* (Sabbah et al., 2013), may in fact be downstream of A_{2B}AR rather than A_1AR activation. We also reclassified the rationally designed A_1AR agonist VCP746 as having additional activity at the A_{2B}AR. Remarkably at the A_{2B}AR, VCP746 had higher affinity and higher potency than the prototypical A_{2B}AR agonists NECA and BAY60-6583. VCP746 was identified as having a bivalent mode of receptor engagement, most probably due to its extended structure which combines two separate moieties. As well as being a potent agonist across a number of signalling pathways in a heterologous expression system, VCP746 attenuated ANGII and TGF-β1 stimulated collagen synthesis and pro-fibrotic gene expression in neonatal cardiac fibroblasts via an A_{2B}AR mechanism. As such, work for these chapters has reclassified capadenoson and VCP746 as dual A_1AR/A_{2B}AR agonists and identified two new pharmacological tools to assess the role of A_{2B}AR signalling in pathophysiology. Excitingly, both ligands have shown promise as cardioprotective therapies (Sabbah et al., 2013; Valant et al., 2014), which may result from their favourable dual agonist profile, or their ability to promote distinct signalling outcomes from the adenosine-like agonist NECA at the A_{2B}AR. It is of interest that VCP746 and capadenoson were both A_1AR agonists that we have now shown to have good A_{2B}AR activity. This is somewhat surprising because within the adenosine receptor family, the A_1AR and A_{2B}AR do not share the highest degree of sequence conservation. Based on receptor sequence, an A_1AR agonist would be anticipated to have cross-reactivity with the A_3AR rather than the A_{2B}AR (Fredholm et al., 2000; Jacobson and Gao, 2006). Therefore, it would be interesting to determine the conserved binding residues or pockets between the two receptor subtypes using capadenoson and VCP746 as pharmacological probes. Given the structural basis for subtype

selectivity between the A_1AR and A_{2A}AR has just recently been discovered by our lab (Glukhova et al., 2017), elucidating the structural similarity between the A_1AR and A_{2B}AR would further aid in the pursuit of rationally-designed adenosine receptor ligands.

One such disease context where rationally-designed A_{2B}AR agonists would be desirable is in the treatment of cardiac fibrosis. Both pro- and anti-fibrotic signal transduction have been attributed to A_{2B}AR activation (Dubey et al., 1998; Toldo et al., 2012), highlighting the need to better understand A_{2B}AR signalling. This required the development and optimisation of an *in vitro* model to examine A_{2B}AR signalling in normal and diseased cardiac fibroblasts. We demonstrated in diseased myofibroblasts (fibroblasts treated with TGF-β1), the A_{2B}AR ligands NECA and VCP746 had a reduced capacity for cAMP production, which supports previous findings showing attenuated accumulation of anti-fibrotic cAMP in myofibroblasts as a result of altered adenylyl cyclase expression (Lu et al., 2013). Interestingly, we also identified myofibroblasts had an elevated basal production of IP_1 that surprisingly could be inhibited by A_{2B}AR stimulation, via a pathway that appeared to involve activation of protein kinase G. The exact mechanism requires further elucidation but may represent an unappreciated pathway by which G_s-coupled receptors dampen detrimental fibrotic G_q-mediated signalling. These studies provide a starting point to understand A_{2B}AR-mediated intracellular signalling in fibroblasts and has identified a good working model to further investigate how this signalling may alter under disease conditions, with future work ideally focusing on fibrotic endpoints such as collagen synthesis and pro-fibrotic gene expression.

One promising approach to selectively target desired A_{2B}AR signal transduction is biased agonism. Ligand bias is a rapidly emerging phenomenon in the GPCR field and is the concept that describes the ability of different ligands to stabilise distinct conformations of a given

receptor, such that only a subset of possible signalling pathways are engaged to the relative exclusion of others (Kenakin et al., 2012; Shonberg et al., 2014). Biased agonism promises a mechanism through which we can design drugs that promote therapeutically beneficial outcomes while avoiding 'on target' side effects. However, this can only really be achieved when the GPCR signalling pathways that contribute to a particular disease state are well understood. Unfortunately, this is usually not the case and in reality remains a fundamental barrier to the development of novel therapeutically-efficacious biased agonists. The other difficulty lies in translating promising *in vitro* characterisation and quantification of biased agonism into therapeutic efficacy *in vivo.* This has been highlighted by the recent disappointing results from the Phase IIb clinical trial that examined the promising preclinical biased angiotensin II type 1 receptor ligand, TRV027 for the treatment of acute heart failure (Felker et al., 2015).

One of the emerging themes in the field, which may perhaps underpin some of the issues with translation of biased agonists, is that the system in which we quantify bias may not be reflective of the system in which we intend to use the drug. For example, the ligand bias in model cell systems or more physiologically-relevant primary cells may be different to that observed for the receptor within the disease context. This idea of context-specific bias describes how the receptor environment, including changes to the membrane composition, signalling partners and effector proteins may alter the subset of receptor conformations stabilised by different ligands. In other words, the disease context has the potential to engender a different bias profile. This was observed for VCP746 when we compared its signalling profile relative to the reference agonist NECA in isolated cardiac fibroblasts and treated myofibroblasts (+TGF-β1). VCP746 maintained its activity and potency, even when NECA-mediated A_{2B}AR signalling was diminished under diseased conditions (+TGF-β1). Disease context is especially pertinent with

regards to myocardial infarction, as risk factors such as advancing age, diabetes and post-MI pathobiology can significantly alter cardiac cell background, membrane environment and adenosine receptor expression (Headrick et al., 2013). An important caveat when defining and quantifying context-dependent biased agonism is that all ligands cannot be impacted equally. For example, an equivalent decrease in agonist efficacy/potency due to the global down-regulation of cAMP production in myofibroblasts compared to fibroblasts (Lu et al., 2013), simply reflects a change in the system bias rather than context-dependent bias. However, given that we have seen ligand-dependent changes in two pathways under two different conditions; we have clear evidence that relative to NECA, VCP746 displays context-specific biased agonism.

In summary, this thesis has provided unprecedented insights into A_{2B}AR pharmacology through investigation of novel signalling paradigms including constitutive activity, dimerization and biased agonism. It is hoped that the enhanced understanding of A_{2B}AR signalling in the setting of pathophysiology and the identification of novel ligands for this adenosine receptor subtype, will provide new therapeutic opportunities for targeting the A_{2B}AR in the future.

References

Abbracchio, M. P., Brambilla, R., Ceruti, S., Kim, H. O., Lubitz, von, D. K., Jacobson, K. A., et al. (1995). G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. *Mol. Pharmacol.* 48, 1038–1045.

Aherne, C. M., Kewley, E. M., and Eltzschig, H. K. (2011). The resurgence of A2B adenosine receptor signaling. *Biochim. Biophys. Acta, Biomembr.* 1808, 1329–1339. doi:10.1016/j.bbamem.2010.05.016.

Akbar, M., Okajima, F., Tomura, H., Shimegi, S., and Kondo, Y. (1994). A single species of A1 adenosine receptor expressed in Chinese hamster ovary cells not only inhibits cAMP accumulation but also stimulates phospholipase C and arachidonate release. *Mol. Pharmacol.* 45, 1036–1042.

Angers, S., Salahpour, A., and Bouvier, M. (2002). Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu. Rev. Pharmacol. Toxicol.* 42, 409–435. doi:10.1146/annurev.pharmtox.42.091701.082314.

Anvari, F., Sharma, A. K., Fernandez, L. G., Hranjec, T., Ravid, K., Kron, I. L., et al. (2010). Tissue-derived proinflammatory effect of adenosine A2B receptor in lung ischemia–reperfusion injury. *J. Thorac. Cardiovasc. Surg.* 140, 871–877. doi:10.1016/j.jtcvs.2010.06.051.

Arora, P. D., Narani, N., and McCulloch, C. A. (1999). The compliance of collagen gels regulates transforming growth factor-beta induction of alpha-smooth muscle actin in fibroblasts. *Am. J. Pathol.* 154, 871–882.

Asakura, M., Asanuma, H., Kim, J., Liao, Y., Nakamaru, K., Fujita, M., et al. (2007). Impact of adenosine receptor signaling and metabolism on pathophysiology in patients with chronic heart failure. *Hypertens. Res.* 30, 781–787. doi:10.1291/hypres.30.781.

Ashton, K. J., Holmgren, K., Peart, J., Lankford, A. R., Matherne, G. P., Grimmond, A. S., et al. (2003). Effects of A1 adenosine receptor overexpression on normoxic and post-ischemic gene expression. *Cardiovasc. Res.* 57, 715–726. doi:10.1016/S0008-6363(02)00738-1.

Auchampach, J. A., Jin, X., Wan, T. C., Caughey, G. H., and Linden, J. (1997). Canine mast cell adenosine receptors: cloning and expression of the A3 receptor and evidence that degranulation is mediated by the A2B receptor. *Mol. Pharmacol.* 52, 846–860.

Aurelio, L., Valant, C., Flynn, B. L., Sexton, P. M., Christopoulos, A., and Scammells, P. J. (2009). Allosteric modulators of the adenosine A1 receptor: synthesis and pharmacological evaluation of 4-substituted 2-amino-3-benzoylthiophenes. *J. Med. Chem.* 52, 4543–4547. doi:10.1021/jm9002582.

Azdad, K., Gall, D., Woods, A. S., Ledent, C., Ferré, S., and Schiffmann, S. N. (2008). Dopamine D2 and adenosine A2A receptors regulate NMDA-mediated excitation in accumbens neurons through A2A–D2 receptor heteromerization. *Neuropsychopharmacology* 34, 972–986. doi:10.1038/npp.2008.144.

Ballarín, M., Fredholm, B. B., Ambrosio, S., and Mahy, N. (1991). Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta Physiol. Scand.* 142, 97–103. doi:10.1111/j.1748-1716.1991.tb09133.x.

Baltos, J. A., Paoletta, S., Nguyen, A. T. N., Gregory, K. J., Tosh, D. K., Christopoulos, A., et al. (2016a). Structure-activity analysis of biased agonism at the human adenosine A3 receptor. *Mol. Pharmacol.* 90, 12–22. doi:10.1124/mol.116.103283.

Baltos, J.-A., Gregory, K. J., White, P. J., Sexton, P. M., Christopoulos, A., and May, L. T. (2016b). Quantification of adenosine A1 receptor biased agonism: Implications for drug discovery. *Biochem. Pharmacol.* 99, 101–112. doi:10.1016/j.bcp.2015.11.013.

Baltos, J.-A., Vecchio, E. A., Harris, M. A., Qin, C. X., Ritchie, R. H., Christopoulos, A., et al. (2017). Capadenoson, a clinically trialed partial adenosine A1 receptor agonist, can stimulate adenosine A2B receptor biased agonism. *Biochem. Pharmacol.* doi:10.1016/j.bcp.2017.03.014.

Baraldi, P. G., Preti, D., Tabrizi, M. A., Fruttarolo, F., Saponaro, G., Baraldi, S., et al. (2007). N6-[(Hetero)aryl/(cyclo)alkyl-carbamoyl-methoxy-phenyl]-(2-chloro)-5′-N-ethylcarboxamido-adenosines: The first example of adenosine-related structures with potent agonist activity at the human A2B adenosine receptor. *Bioorg. Med. Chem.* 15, 2514–2527. doi:10.1016/j.bmc.2007.01.055.

Baraldi, P. G., Romagnoli, R., Preti, D., Fruttarolo, F., Carrion, M. D., and Tabrizi, M. A. (2006). Ligands for A2B adenosine receptor subtype. *Curr. Med. Chem.* 13, 3467–3482. doi:10.2174/092986706779010306.

Baraldi, P. G., Tabrizi, M. A., Fruttarolo, F., and Romagnoli, R. (2009). Recent improvements in the development of A2B adenosine receptor agonists. *Purinergic Signal.* 5, 3–19.

Bayer Study to investigate the effect of the A1 agonist capadenoson on ventricular HR in patients with persistent or permanent atrial fibrillation by administration of capadenoson in a dose of 4 mg once daily for five days. In: ClinicalTrial.gov [Internet]. Bethesda (MD): National Library of Medicine (US). *clinicaltrials.gov*. Available at: https://clinicaltrials.gov/show/NCT00568945 [Accessed October 25, 2016].

Bertarelli, D. C. G., Diekmann, M., Hayallah, A. M., Rüsing, D., Iqbal, J., Preiss, B., et al. (2006). Characterization of human and rodent native and recombinant adenosine A2B receptors by radioligand binding studies. *Purinergic Signal.* 2, 559–571. doi:10.1007/s11302-006-9012-4.

Beukers, M. W., Chang, L. C. W., Frijtag Drabbe Künzel, von, J. K., Mulder-Krieger, T., Spanjersberg, R. F., Brussee, J., et al. (2004a). New, non-adenosine, high-potency agonists for the human adenosine A2B receptor with an improved selectivity profile compared to the reference agonist N-Ethylcarboxamidoadenosine. *J. Med. Chem.* 47, 3707–3709. doi:10.1021/jm049947s.

Beukers, M. W., Dulk, den, H., van Tilburg, E. W., Brouwer, J., and IJzerman, A. P. (2000). Why are A2B receptors low-affinity adenosine receptors? Mutation of Asn273 to Tyr increases affinity of human A2B receptor for 2-(1-Hexynyl)adenosine. *Mol. Pharmacol.* 58, 1349–1356. doi:10.1124/mol.58.6.1349.

Beukers, M. W., Meurs, I., and IJzerman, A. P. (2006). Structure-affinity relationships of adenosine A2B receptor ligands. *Med. Res. Rev.* 26, 667–698. doi:10.1002/med.20069.

Beukers, M. W., van Oppenraaij, J., van der Hoorn, P. P. W., Blad, C. C., Dulk, den, H.,

Brouwer, J., et al. (2004b). Random mutagenesis of the human adenosine A2B receptor followed by growth selection in yeast. Identification of constitutively active and gain of function mutations. *Mol. Pharmacol.* 65, 702–710. doi:10.1124/mol.65.3.702.

Black, J. W., and Leff, P. (1983). Operational models of pharmacological agonism. *Proc. R. Soc. B* 220, 141–162.

Boateng, S., and Sanborn, T. (2013). Acute myocardial infarction. *Disease-a-Month* 59, 83–96. doi:10.1016/j.disamonth.2012.12.004.

Bond, R. A., and Ijzerman, A. P. (2006). Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol. Sci.* 27, 92–96. doi:10.1016/j.tips.2005.12.007.

Borrmann, T., Hinz, S., Bertarelli, D. C. G., Li, W., Florin, N. C., Scheiff, A. B., et al. (2009). 1-Alkyl-8-(piperazine-1-sulfonyl)phenylxanthines: development and characterization of adenosine A2B receptor antagonists and a new radioligand with subnanomolar affinity and subtype specificity. *J. Med. Chem.* 52, 3994–4006. doi:10.1021/jm900413e.

Bradley, S. J., and Tobin, A. B. (2016). Design of next-generation G protein–coupled receptor drugs: linking novel pharmacology and in vivo animal models. *Annu. Rev. Pharmacol. Toxicol.* 56, 535–559. doi:10.1146/annurev-pharmtox-011613-140012.

Briddon, S. J., Gandía, J., Amaral, O. B., Ferré, S., Lluís, C., Franco, R., et al. (2008). Plasma membrane diffusion of G protein-coupled receptor oligomers. *Biochim. Biophys. Acta, Mol. Cell Res.* 1783, 2262–2268. doi:10.1016/j.bbamcr.2008.07.006.

Bruns, R. F., Lu, G. H., and Pugsley, T. A. (1986). Characterization of the A2 adenosine receptor labeled by [3H]NECA in rat striatal membranes. *Mol. Pharmacol.* 29, 331–346.

Cabello, N., Gandía, J., Bertarelli, D. C. G., Watanabe, M., Lluís, C., Franco, R., et al. (2009). Metabotropic glutamate type 5, dopamine D2 and adenosine A2A receptors form higher-order oligomers in living cells. *J. Neurochem.* 109, 1497–1507. doi:10.1111/j.1471-4159.2009.06078.x.

Calderone, A., Thaik, C. M., Takahashi, N., Chang, D. L., and Colucci, W. S. (1998). Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. *J. Clin. Invest.* 101, 812–818. doi:10.1172/JCI119883.

Camelliti, P., Green, C. R., LeGrice, I., and Kohl, P. (2004). Fibroblast network in rabbit sinoatrial node: structural and functional identification of homogeneous and heterogeneous cell coupling. *Circ. Res.* 94, 828–835. doi:10.1161/01.RES.0000122382.19400.14.

Canals, M., Burgueño, J., Marcellino, D., Cabello, N., Canela, E. I., Mallol, J., et al. (2004). Homodimerization of adenosine A2A receptors: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Neurochem.* 88, 726–734.

Canals, M., Marcellino, D., Fanelli, F., Ciruela, F., de Benedetti, P., Goldberg, S. R., et al. (2003). Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Biol. Chem.* 278, 46741–46749. doi:10.1074/jbc.M306451200.

Carriba, P., Navarro, G., Ciruela, F., Ferré, S., Casadó, V., Agnati, L., et al. (2008). Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat. Methods* 5, 727–733. doi:10.1038/nmeth.1229.

Casadó, V., Barrondo, S., Spasic, M., Callado, L. F., Mallol, J., Canela, E., et al. (2010). Gi protein coupling to adenosine A1-A2A receptor heteromers in human brain caudate nucleus. *J. Neurochem.*, 972–980. doi:10.1111/j.1471-4159.2010.06810.x.

Cekic, C., Sag, D., Li, Y., Theodorescu, D., Strieter, R. M., and Linden, J. (2011). Adenosine A2B receptor blockade slows growth of bladder and breast tumors. *J. Immunol.* 188, 198–205. doi:10.4049/jimmunol.1101845.

Cerione, R. A., Codina, J., Benovic, J. L., Lefkowitz, R. J., Birnbaumer, L., and Caron, M. G. (1984). The mammalian Beta 2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. *Biochemistry*.

Chalmers, D. T., and Behan, D. P. (2002). The use of constitutively active GPCRs in drug discovery and functional genomics. *Nat. Rev. Drug Discov.* 1, 599–608. doi:10.1038/nrd872.

Chan, E. S. L., and Cronstein, B. N. (2009). Adenosine in fibrosis. *Mod. Rheumatol.* 20, 114–122. doi:10.1007/s10165-009-0251-4.

Chandrasekera, P. C., Wan, T. C., Gizewski, E. T., Auchampach, J. A., and Lasley, R. D. (2013). Adenosine A1 receptors heterodimerize with β1- and β2-adrenergic receptors creating novel receptor complexes with altered G protein coupling and signaling. *Cell. Signalling* 25, 736–742. doi:10.1016/j.cellsig.2012.12.022.

Chen, J.-F., Eltzschig, H. K., and Fredholm, B. B. (2013). Adenosine receptors as drug targets — what are the challenges? *Nat. Rev. Drug Discov.* 12, 265–286. doi:10.1038/nrd3955.

Chen, W., and Frangogiannis, N. G. (2013). Fibroblasts in post-infarction inflammation and cardiac repair. *Biochim. Biophys. Acta* 1833, 945–953. doi:10.1016/j.bbamcr.2012.08.023.

Chen, Y., Epperson, S., Makhsudova, L., Ito, B., Suarez, J., Dillmann, W., et al. (2004). Functional effects of enhancing or silencing adenosine A2B receptors in cardiac fibroblasts. *Am. J. Physiol. Heart Circ. Physiol.* 287, H2478–86. doi:10.1152/ajpheart.00217.2004.

Ciruela, F., Casado, V., Mallol, J., Canela, E. I., Lluis, C., and Franco, R. (1995). Immunological identification of A1 adenosine receptors in brain cortex. *J. Neurosci. Res.* 42, 818–828. doi:10.1002/jnr.490420610.

Ciruela, F., Casadó, V., Rodrigues, R. J., Luján, R., Burgueño, J., Canals, M., et al. (2006a). Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. *J. Neurosci.* 26, 2080–2087. doi:10.1523/JNEUROSCI.3574-05.2006.

Ciruela, F., Ferré, S., Casado, V., Cortés, A., Cunha, R. A., Lluis, C., et al. (2006b). Heterodimeric adenosine receptors: a device to regulate neurotransmitter release. *Cell. Mol. Life Sci.* 63, 2427–2431. doi:10.1007/s00018-006-6216-2.

Cohen, M. V., Yang, X., and Downey, J. M. (2010). A2B adenosine receptors can change their spots. *Br. J. Pharmacol.* 159, 1595–1597. doi:10.1111/j.1476-5381.2010.00668.x.

Cooper, J., Hill, S. J., and Alexander, S. P. (1997). An endogenous A2B adenosine receptor coupled to cyclic AMP generation in human embryonic kidney (HEK 293) cells. *Br. J. Pharmacol.* 122, 546–550. doi:10.1038/sj.bjp.0701401.

Corriden, R., Kilpatrick, L. E., Kellam, B., Briddon, S. J., and Hill, S. J. (2014). Kinetic analysis of antagonist-occupied adenosine-A3 receptors within membrane microdomains of individual cells provides evidence of receptor dimerization and allosterism. *FASEB J.* 28, 4211–4222. doi:10.1096/fj.13-247270.

Corvol, J. C., Studler, J. M., Schonn, J. S., Girault, J. A., and Hervé, D. (2001). Galpha(olf) is necessary for coupling D1 and A2A receptors to adenylyl cyclase in the striatum. *J. Neurochem.* 76, 1585–1588.

Costa, T., and Cotecchia, S. (2005). Historical review: Negative efficacy and the constitutive activity of G-protein-coupled receptors. *Trends Pharmacol. Sci.* 26, 618–624. doi:10.1016/j.tips.2005.10.009.

Cronstein, B. N. (2011). Adenosine receptors and fibrosis: a translational review. *F1000Prime Rep.* 3:21 doi:10.3410/B3-21.

Csóka, B., Németh, Z. H., Rosenberger, P., Eltzschig, H. K., Spolarics, Z., Pacher, P., et al. (2010). A2B adenosine receptors protect against sepsis-induced mortality by dampening excessive inflammation. *J. Immunol.* 185, 542–550. doi:10.4049/jimmunol.0901295.

Csóka, B., Selmeczy, Z., Koscso, B., Németh, Z. H., Pacher, P., Murray, P. J., et al. (2012). Adenosine promotes alternative macrophage activation via A2A and A2B receptors. *FASEB J.* 26, 376–386. doi:10.1096/fj.11-190934.

Dalrymple, M. B., Pfleger, K. D. G., and Eidne, K. A. (2008). G protein-coupled receptor dimers: Functional consequences, disease states and drug targets. *Pharmacol. Ther.* 118, 359–371. doi:10.1016/j.pharmthera.2008.03.004.

Daly, J. W., Butts-Lamb, P., and Padgett, W. (1983). Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell. Mol. Neurobiol.* 3, 69-80

De Lean, A., Stadel, J. M., and Lefkowitz, R. J. (1980). A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J. Biol. Chem.* 255, 7108-7117

de Ligt, R. A. F., Rivkees, S. A., Lorenzen, A., Leurs, R., and Ijzerman, A. P. (2005). A "locked-on," constitutively active mutant of the adenosine A1 receptor. *Eur. J. Pharmacol.* 510, 1–8. doi:10.1016/j.ejphar.2005.01.007.

de Zwart, M., Link, R., Frijtag Drabbe Kunzela, von, J. K., Cristalli, G., Jacobson, K. A., Townsend-Nicholson, A., et al. (1998). A functional screening of adenosine analogues at the adenosine A2B receptor: a search for potent agonists. *Nucleosides Nucleotides* 17, 969–985. doi:10.1080/07328319808004215.

Desmet, C. J., Gallenne, T., Prieur, A., Reyal, F., Visser, N. L., Wittner, B. S., et al. (2013). Identification of a pharmacologically tractable Fra-1/ADORA2B axis promoting breast cancer metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5139–5144. doi:10.1073/pnas.1222085110.

Deussen, A., Borst, M., and Schrader, J. (1988). Formation of S-adenosylhomocysteine in the heart. I: An index of free intracellular adenosine. *Circ. Res.* 63, 240–249.

Dixon, A. K., Gubitz, A. K., Sirinathsinghji, D. J., Richardson, P. J., and Freeman, T. C. (1996). Tissue distribution of adenosine receptor mRNAs in the rat. *Br. J. Pharmacol.* 118, 1461–1468.

Dong, Q., Ginsberg, H. N., and Erlanger, B. F. (2001). Overexpression of the A1 adenosine receptor in adipose tissue protects mice from obesity-related insulin resistance. *Diabetes, Obes. Metab.* 3, 360–366.

Dorsam, R. T., and Gutkind, J. S. (2007). G-protein-coupled receptors and cancer. *Nat. Rev. Cancer* 7, 79–94. doi:10.1038/nrc2069.

Drury, A. N., and Szent-Györgyi, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J. Physiol. (Lond.)*.

Du, X., Ou, X., Song, T., Zhang, W., Cong, F., Zhang, S., et al. (2015). Adenosine A2B receptor stimulates angiogenesis by inducing VEGF and eNOS in human microvascular endothelial cells. *Exp. Biol. Med.* 240, 1472–1479. doi:10.1371/journal.pone.0040365.

Dubey, R. K., Gillespie, D. G., and Jackson, E. K. (1998). Adenosine inhibits collagen and protein synthesis in cardiac fibroblasts: role of A2B receptors. *Hypertension* 31, 943–948. doi:10.1161/01.HYP.31.4.943.

Dubey, R. K., Gillespie, D. G., Mi, Z., and Jackson, E. K. (1997). Exogenous and endogenous adenosine inhibits fetal calf serum–induced growth of rat cardiac fibroblasts: role of A2B receptors. *Circulation* 96, 2656–2666. doi:10.1161/01.CIR.96.8.2656.

Dubey, R. K., Gillespie, D. G., Mi, Z., and Jackson, E. K. (2001a). Endogenous cyclic AMP-adenosine pathway regulates cardiac fibroblast growth. *Hypertension* 37, 1095–1100. doi:10.1161/01.HYP.37.4.1095.

Dubey, R. K., Gillespie, D. G., Zacharia, L. C., Mi, Z., and Jackson, E. K. (2001b). A2B receptors mediate the antimitogenic effects of adenosine in cardiac fibroblasts. *Hypertension* 37, 716–721. doi:10.1161/01.HYP.37.2.716.

Dunwiddie, T. V., and Masino, S. A. (2001). The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* 24, 31–55. doi:10.1146/annurev.neuro.24.1.31.

Dux, E., Fastbom, J., Ungerstedt, U., Rudolphi, K., and Fredholm, B. B. (1990). Protective effect of adenosine and a novel xanthine derivative propentofylline on the cell damage after bilateral carotid occlusion in the gerbil hippocampus. *Brain Res.* 516, 248–256.

Eckle, T., Faigle, M., Grenz, A., Laucher, S., and Thompson, L. F. (2008). A2B adenosine receptor dampens hypoxia-induced vascular leak. *Blood*. doi:10.1182/blood-2007-10.

Eckle, T., Hartmann, K., Bonney, S., Reithel, S., Mittelbronn, M., Walker, L. A., et al. (2012). Adora2b-elicited *Per2* stabilization promotes a HIF- dependent metabolic switch crucial for myocardial adaptation to ischemia. *Nat. Med.* 18, 774–782. doi:10.1038/nm.2728.

Eckle, T., Krahn, T., Grenz, A., Kohler, D., Mittelbronn, M., Ledent, C., et al. (2007). Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. *Circulation* 115, 1581–1590. doi:10.1161/CIRCULATIONAHA.106.669697.

Ehlert, F. J., Suga, H., and Griffin, M. T. (2011). Analysis of agonism and inverse agonism in functional assays with constitutive activity: estimation of orthosteric ligand affinity constants for active and inactive receptor states. *J. Pharmacol. Exp. Ther.* 338, 671–686. doi:10.1124/jpet.111.179309.

Eisenstein, A., Patterson, S., and Ravid, K. (2015). The many faces of the A2B adenosine receptor in cardiovascular and metabolic diseases. *J. Cell. Physiol.*, n/a–n/a. doi:10.1002/jcp.25043.

Eltzschig, H. K. (2009). Adenosine: an old drug newly discovered. *Anesthesiology* 111, 904–915. doi:10.1097/ALN.0b013e3181b060f2.

Eltzschig, H. K., Bonney, S. K., and Eckle, T. (2013). Attenuating myocardial ischemia by targeting A2B adenosine receptors. *Trends Mol. Med.* 19, 345–354. doi:10.1016/j.molmed.2013.02.005.

Eltzschig, H. K., Rivera-Nieves, J., and Colgan, S. P. (2009). Targeting the A2B adenosine receptor during gastrointestinal ischemia and inflammation. *Expert Opin. Ther. Targets* 13, 1267–1277. doi:10.1517/14728220903241666.

Elzein, E., Kalla, R. V., Li, X., Perry, T., Gimbel, A., Zeng, D., et al. (2008). Discovery of a novel A2B adenosine receptor antagonist as a clinical candidate for chronic inflammatory airway diseases. *J. Med. Chem.* 51, 2267–2278. doi:10.1021/jm7014815.

Englert, M., Quitterer, U., and Klotz, K.-N. (2002). Effector coupling of stably transfected human A3 adenosine receptors in CHO cells. *Biochem. Pharmacol.* 64, 61–65.

Epperson, S. A., Brunton, L. L., Ramirez-Sanchez, I., and Villarreal, F. (2009). Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts. *Am. J. Physiol., Cell Physiol.* 296, C1171–C1177. doi:10.1152/ajpcell.00290.2008.

Fang, Y., and Olah, M. E. (2007). Cyclic AMP-dependent, protein kinase A-independent activation of extracellular signal-regulated kinase 1/2 following adenosine receptor stimulation in human umbilical vein endothelial cells: role of exchange protein activated by cAMP 1 (Epac1). *J. Pharmacol. Exp. Ther.* 322, 1189–1200. doi:10.1124/jpet.107.119933.

Felker, G. M., Butler, J., Collins, S. P., Cotter, G., Davison, B. A., Ezekowitz, J. A., et al. (2015). Heart failure therapeutics on the basis of a biased ligand of the Angiotensin-2 type 1 receptor. Rationale and design of the BLAST-AHF Study (Biased Ligand of the Angiotensin Receptor Study in Acute Heart Failure). *JACC. Heart Fail.* 3, 193–201. doi:10.1016/j.jchf.2014.09.008.

Feng, W., Song, Y., Chen, C., Lu, Z. Z., and Zhang, Y. (2009). Stimulation of adenosine A2B

receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-δ-P38 signalling pathway. *Br. J. Pharmacol.* 159, 1598–1607. doi:10.1111/j.1476-5381.2009.00558.x.

Feoktistov, I. (2002). Differential expression of adenosine receptors in human endothelial cells: role of A2B receptors in angiogenic factor regulation. *Circ. Res.* 90, 531–538. doi:10.1161/01.RES.0000012203.21416.14.

Feoktistov, I., and Biaggioni, I. (1995). Adenosine A2B receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J. Clin. Invest.* 96, 1979–1986. doi:10.1172/JCI118245.

Feoktistov, I., and Biaggioni, I. (1997). Adenosine A2B receptors. *Pharmacol. Rev.* 49, 381–402.

Feoktistov, I., and Biaggioni, I. (2011). Role of adenosine A2B receptors in inflammation. *Adv. Pharmacol.*

Feoktistov, I., Goldstein, A. E., and Biaggioni, I. (1999). Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol. Pharmacol.* 55, 726–734.

Feoktistov, I., Murray, J. J., and Biaggioni, I. (1994). Positive modulation of intracellular Ca2+ levels by adenosine A2B receptors, prostacyclin, and prostaglandin E1 via a cholera toxin-sensitive mechanism in human erythroleukemia cells. *Mol. Pharmacol.* 45, 1160-1167

Fernández-Dueñas, V., Gómez-Soler, M., López-Cano, M., Taura, J. J., Ledent, C., Watanabe, M., et al. (2014). Uncovering caffeine's adenosine A2A receptor inverse agonism in experimental parkinsonism. *ACS Chem. Biol.* 9, 2496–2501. doi:10.1021/cb5005383.

Ferrari, D., Gambari, R., Idzko, M., Mu ller, T., Albanesi, C., Pastore, S., et al. (2016). Purinergic signaling in scarring. *FASEB J.* 30, 3–12. doi:10.1096/fj.15-274563.

Ferré, S., Casado, V., Devi, L. A., Filizola, M., Jockers, R., Lohse, M. J., et al. (2014). G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. *Pharmacol. Rev.* 66, 413–434. doi:10.1124/pr.113.008052.

Ferré, S., Ciruela, F., Canals, M., Marcellino, D., Burgueño, J., Casadó, V., et al. (2004). Adenosine A2A-dopamine D2 receptor–receptor heteromers. Targets for neuro-psychiatric disorders. *Parkinsonism Relat. Disord.* 10, 265–271. doi:10.1016/j.parkreldis.2004.02.014.

Figler, R. A., Wang, G., Srinivasan, S., Jung, D. Y., Zhang, Z., Pankow, J. S., et al. (2011). Links between insulin resistance, adenosine A2B receptors, and inflammatory markers in mice and humans. *Diabetes* 60, 669–679. doi:10.2337/db10-1070.

Fine, A., and Goldstein, R. H. (1987). The effect of transforming growth factor-beta on cell proliferation and collagen formation by lung fibroblasts. *J. Biol. Chem.*

Flacke, J. P., Flacke, H., Appukuttan, A., Palisaar, R. J., Noldus, J., Robinson, B. D., et al. (2013). Type 10 soluble adenylyl cyclase is overexpressed in prostate carcinoma and controls proliferation of prostate cancer cells. *J. Biol. Chem.* 288, 3126–3135. doi:10.1074/jbc.M112.403279.

Franco, R., Casadó, V., Cortés, A., Pérez-Capote, K., Mallol, J., Canela, E., et al. (2008). Novel pharmacological targets based on receptor heteromers. *Brain Res. Rev.* 58, 475–482. doi:10.1016/j.brainresrev.2008.06.002.

Franco, R., Ferré, S., Agnati, L., Torvinen, M., Ginés, S., Hillion, J., et al. (2000). Evidence for adenosine/dopamine receptor interactions: indications for heteromerization. *Neuropsychopharmacology* 23, S50–S59.

Frank, A., Bonney, M., Bonney, S., Weitzel, L., Koeppen, M., and Eckle, T. (2012). Myocardial ischemia reperfusion injury: from basic science to clinical bedside. *Semin. Cardiothorac. Vasc. Anesth.* 16, 123–132. doi:10.1177/1089253211436350.

Fredholm, B. B. (2007). Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ.* 14, 1315–1323. doi:10.1038/sj.cdd.4402132.

Fredholm, B. B. (2014). Adenosine—a physiological or pathophysiological agent? *J. Mol. Med.* 92, 201–206. doi:10.1007/s00109-013-1101-6.

Fredholm, B. B., Arslan, G., Halldner, L., Kull, B., Schulte, G., and Wasserman, W. (2000). Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedebergs Arch. Pharmacol.* 362, 364–374. doi:10.1007/s002100000313.

Fredholm, B. B., Chen, J.-F., Masino, S. A., and Vaugeois, J.-M. (2005). Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. *Annu. Rev. Pharmacol. Toxicol.* 45, 385–412. doi:10.1146/annurev.pharmtox.45.120403.095731.

Fredholm, B. B., IJzerman, A. P., Jacobson, K. A., Klotz, K. N., and Linden, J. (2001a). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 53, 527–552.

Fredholm, B. B., IJzerman, A. P., Jacobson, K. A., Linden, J., and Müller, C. E. (2011). International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. *Pharmacol. Rev.* 63, 1–34. doi:10.1124/pr.110.003285.

Fredholm, B. B., Irenius, E., Kull, B., and Schulte, G. (2001b). Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem. Pharmacol.* 61, 443–448.

Fredriksson, R., Lagerström, M. C., Lundin, L.-G., and Schiöth, H. B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.* 63, 1256–1272. doi:10.1124/mol.63.6.1256.

Freissmuth, M., Selzer, E., and Schütz, W. (1991). Interactions of purified bovine brain A1-adenosine receptors with G-proteins. Reciprocal modulation of agonist and antagonist binding. *Biochem. J.* 275 (Pt 3), 651–656.

Freund, S., Ungerer, M., and Lohse, M. J. (1994). A1 adenosine receptors expressed in CHO-cells couple to adenylyl cyclase and to phospholipase C. *Naunyn-Schmiedebergs Arch. Pharmacol.* 350, 49–56.

Friedman, S. L., Sheppard, D., Duffield, J. S., and Violette, S. (2013). Therapy for fibrotic diseases: nearing the starting line. *Sci Transl Med* 5, 167sr1. doi:10.1126/scitranslmed.3004700.

Frohlich, G. M., Meier, P., White, S. K., Yellon, D. M., and Hausenloy, D. J. (2013). Myocardial reperfusion injury: looking beyond primary PCI. *Eur. Heart J.* 34, 1714–1722. doi:10.1093/eurheartj/eht090.

Futschik, M., Jeffs, A., Pattison, S., Kasabov, N., Sullivan, M., Merrie, A., et al. (2002). Gene expression profiling of metastatic and nonmetastatic colorectal cancer cell lines. *Genome Lett.* 1, 26–34. doi:10.1166/gl.2002.005.

Gandía, J., Galino, J., Amaral, O. B., Soriano, A., Lluís, C., Franco, R., et al. (2008). Detection of higher-order G protein-coupled receptor oligomers by a combined BRET–BiFC technique. *FEBS Letters* 582, 2979–2984. doi:10.1016/j.febslet.2008.07.045.

Gao, Z. G., and Jacobson, K. A. (2008). Translocation of arrestin induced by human A 3 adenosine receptor ligands in an engineered cell line: comparison with G protein-dependent pathways. *Pharmacol. Res.*

Gao, Z., Chen, T., Weber, M. J., and Linden, J. (1999). A2B adenosine and P2Y2 receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 Cells: cross-talk between cyclic AMP and protein kinase C pathways. *J. Biol. Chem.* 274, 5972–5980. doi:10.1074/jbc.274.9.5972.

Gao, Z.-G., Balasubramanian, R., Kiselev, E., Wei, Q., and Jacobson, K. A. (2014). Probing biased/partial agonism at the G protein-coupled A2B adenosine receptor. *Biochem. Pharmacol.* 90, 297–306. doi:10.1016/j.bcp.2014.05.008.

Gerwins, P., and Fredholm, B. B. (1995). Activation of adenosine A1 and bradykinin receptors increases protein kinase C and phospholipase D activity in smooth muscle cells. *Naunyn-Schmiedebergs Arch. Pharmacol.* 351, 186–193.

Gessi, S. (2005). expression, pharmacological profile, and functional coupling of A2B receptors in a recombinant system and in peripheral blood cells using a novel selective antagonist radioligand, [^{3}H]MRE 2029-F20. *Mol. Pharmacol.* 67, 2137–2147. doi:10.1124/mol.104.009225.

Ginés, S., Hillion, J., Torvinen, M., Le Crom, S., Casado, V., Canela, E. I., et al. (2000). Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8606–8611. doi:10.1073/pnas.150241097.

Glukhova, A., Thal, D. M., Nguyen, A. T., Vecchio, E. A., Jörg, M., Scammells, P. J., et al. (2017). Structure of the adenosine A1 receptor reveals the basis for subtype selectivity. *Cell* 168, 867–877.e13. doi:10.1016/j.cell.2017.01.042.

Gödecke, A. (2009). cAMP: fuel for extracellular adenosine formation? *Br. J. Pharmacol.* 153, 1087–1089. doi:10.1038/bjp.2008.7.

Gracia, E., Moreno, E., Cortés, A., Lluís, C., Mallol, J., McCormick, P. J., et al. (2013). Homodimerization of adenosine A1 receptors in brain cortex explains the biphasic effects of caffeine. *Neuropharmacology* 71, 56–69. doi:10.1016/j.neuropharm.2013.03.005.

Grant, M. B., Davis, M. I., Caballero, S., Feoktistov, I., Biaggioni, I., and Belardinelli, L. (2001). Proliferation, migration, and ERK activation in human retinal endothelial cells through A2B adenosine receptor stimulation. *Invest. Ophthalmol. Visual Sci.*

Grenz, A., Kim, J. H., Bauerle, J. D., Tak, E., Eltzschig, H. K., and Clambey, E. T. (2012). Adora2B adenosine receptor signaling protects during acute kidney injury via inhibition of neutrophil-dependent TNF-α release. *J. Immunol.* 189, 4566–4573.

Grube, K., Rüdebusch, J., Xu, Z., Böckenholt, T., Methner, C., Müller, T., et al. (2011). Evidence for an intracellular localization of the adenosine A2B receptor in rat cardiomyocytes. *Basic Res. Cardiol.* 106, 385–396. doi:10.1007/s00395-011-0151-6.

Hagberg, H., Andersson, P., Lacarewicz, J., Jacobson, I., Butcher, S., and Sandberg, M. (1987). Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. *J. Neurochem.* 49, 227–231.

Ham, J., and Rees, D. A. (2008). The adenosine A2B receptor: its role in inflammation. *Endocr., Metab. Immune Disord.: Drug Targets* 8, 244–254. doi:10.2174/187153008786848303.

Hart, M. L., Grenz, A., Gorzolla, I. C., Schittenhelm, J., Dalton, J. H., and Eltzschig, H. K. (2011). Hypoxia-inducible factor-1 -dependent protection from intestinal ischemia/reperfusion injury involves ecto-5'-nucleotidase (CD73) and the A2B adenosine receptor. *J. Immunol.* 186, 4367–4374. doi:10.4049/jimmunol.0903617.

Hart, M. L., Jacobi, B., Schittenhelm, J., Henn, M., and Eltzschig, H. K. (2009). Cutting edge: A2B adenosine receptor signaling provides potent protection during intestinal ischemia/reperfusion injury. *J. Immunol.* 182, 3965–3968. doi:10.4049/jimmunol.0802193.

Haskó, G., Csóka, B., Németh, Z. H., Vizi, E. S., and Pacher, P. (2009). A2B adenosine receptors in immunity and inflammation. *Trends Immunol.* 30, 263–270. doi:10.1016/j.it.2009.04.001.

Haskó, G., Linden, J., Cronstein, B., and Pacher, P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat. Rev. Drug Discov.* 7, 759–770. doi:10.1038/nrd2638.

Hausenloy, D. J. (2013). Cardioprotection techniques: preconditioning, postconditioning and remote con-ditioning (basic science). *Curr. Pharm. Des.* 19, 4544–4563.

Hausenloy, D. J., and Yellon, D. M. (2013). Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *J. Clin. Invest.* 123, 92–100. doi:10.1172/JCI62874.

Hausenloy, D., and Yellon, D. M. (2004). New directions for protecting the heart against ischaemia–reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc. Res.* 61, 448–460. doi:10.1016/j.cardiores.2003.09.024.

Hayallah, A. M., Sandoval-Ramírez, J., Reith, U., Schobert, U., Preiss, B., Schumacher, B., et al. (2002). 1,8-disubstituted xanthine derivatives: synthesis of potent A2B-selective adenosine receptor antagonists. *J. Med. Chem.* 45, 1500–1510. doi:10.1021/jm011049y.

He, W., Wilder, T., and Cronstein, B. N. (2013). Rolofylline, an adenosine A1 receptor antagonist, inhibits osteoclast differentiation as an inverse agonist. *Br. J. Pharmacol.* 170, 1167–1176. doi:10.1111/bph.12342.

Headrick, J. P., and Lasley, R. D. (2009). Adenosine receptors and reperfusion injury of the heart. *Handb. Exp. Pharmacol.*, 189–214. doi:10.1007/978-3-540-89615-9_7.

Headrick, J. P., and Willis, R. J. (1988). Mediation by adenosine of bradycardia in rat heart during graded global ischaemia. *Pfluegers Arch.*

Headrick, J. P., Ashton, K. J., Rose'Meyer, R. B., and Peart, J. N. (2013). Cardiovascular adenosine receptors: Expression, actions and interactions. *Pharmacol. Ther.* 140, 92–111. doi:10.1016/j.pharmthera.2013.06.002.

Headrick, J. P., Hack, B., and Ashton, K. J. (2003). Acute adenosinergic cardioprotection in ischemic-reperfused hearts. *Am. J. Physiol. Heart Circ. Physiol.* 285, H1797–818. doi:10.1152/ajpheart.00407.2003.

Herenbrink, C. K., Sykes, D. A., Donthamsetti, P., Canals, M., Coudrat, T., Shonberg, J., et al. (2016). The role of kinetic context in apparent biased agonism at GPCRs. *Nat. Commun.* 7, 1–14. doi:10.1038/ncomms10842.

Herrera, C., Casado, V., Ciruela, F., Schofield, P., Mallol, J., Lluis, C., et al. (2001). Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Mol. Pharmacol.* 59, 127–134.

Hinz, S., Lacher, S. K., Seibt, B. F., and Muller, C. E. (2014). BAY60-6583 acts as a partial agonist at adenosine A2B receptors. *J. Pharmacol. Exp. Ther.* 349, 427–436. doi:10.1124/jpet.113.210849.

Hodavance, S. Y., Gareri, C., Torok, R. D., and Rockman, H. A. (2016). G protein-coupled receptor biased agonism. *J. Cardiovasc. Pharmacol.*, 1. doi:10.1097/FJC.0000000000000356.

Huang, J., Zhou, H., Mahavadi, S., Sriwai, W., and Murthy, K. S. (2006). Inhibition of G q-dependent PLC-beta1 activity by PKG and PKA is mediated by phosphorylation of RGS4 and GRK2. *Am. J. Physiol. Cell. Physiol.* 292, C200–C208. doi:10.1152/ajpcell.00103.2006.

Huerter, M. E., Sharma, A. K., Zhao, Y., Charles, E. J., Kron, I. L., and Laubach, V. E. (2016). Attenuation of pulmonary ischemia-reperfusion injury by adenosine A2B receptor antagonism. *Ann. Thorac. Surg.* 102, 385–393. doi:10.1016/j.athoracsur.2016.02.060.

Iannone, R., Miele, L., Maiolino, P., Pinto, A., and Morello, S. (2013). Blockade of A2B adenosine receptor reduces tumor growth and immune suppression mediated by myeloid-derived suppressor cells in a mouse model of melanoma. *Neoplasia* 15, 1400–IN14. doi:10.1593/neo.131748.

Ibrisimovic, E., Drobny, H., Yang, Q., Höfer, T., Boehm, S., Nanoff, C., et al. (2012). Constitutive activity of the A2A adenosine receptor and compartmentalised cyclic AMP signalling fine-tune noradrenaline release. *Purinergic Signal.* 8, 677–692. doi:10.1007/s11302-012-9298-3.

Jackson, E. K., and Raghvendra, D. K. (2004). The extracellular cyclic AMP-adenosine pathway in renal physiology. *Annu. Rev. Physiol.* 66, 571–599. doi:10.1146/annurev.physiol.66.032102.111604.

Jackson, E. K., Mi, Z., and Dubey, R. K. (2007). The extracellular cAMP-adenosine pathway significantly contributes to the in vivo production of adenosine. *J. Pharmacol. Exp. Ther.* 320, 117–123. doi:10.1124/jpet.106.112748.

Jacobson, K. A., and Gao, Z.-G. (2006). Adenosine receptors as therapeutic targets. *Nat. Rev. Drug Discov.* 5, 247–264. doi:10.1038/nrd1983.

Jajoo, S., Mukherjea, D., Kumar, S., Sheth, S., Kaur, T., Rybak, L. P., et al. (2009). Role of beta-arrestin1/ERK MAP kinase pathway in regulating adenosine A1 receptor desensitization and recovery. *Am. J. Physiol. Cell. Physiol.* 298, C56–C65. doi:10.1152/ajpcell.00190.2009.

Ji, X. D., and Jacobson, K. A. (1999). Use of the triazolotriazine [3H]ZM 241385 as a radioligand at recombinant human A2B adenosine receptors. *Drug Des. Discovery* 16, 217–226.

Ji, X.-D., Kim, Y.-C., Ahern, D. G., Linden, J., and Jacobson, K. A. (2001). [3 H] MRS 1754, a selective antagonist radioligand for A2B adenosine receptors. *Biochem. Pharmacol.* 61, 657–663.

Jordan, B. A., and Devi, L. A. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 399, 697–700. doi:10.1038/21441.

Jordan, J. E., Zhao, Z. Q., Sato, H., Taft, S., and Vinten-Johansen, J. (1997). Adenosine A2 receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence. *J. Pharmacol. Exp. Ther.* 280, 301–309.

Jörg, M., Glukhova, A., Abdul-Ridha, A., Vecchio, E. A., Nguyen, A. T. N., Sexton, P. M., et al. (2016). Novel irreversible agonists acting at the A1 adenosine receptor. *J. Med. Chem.*, acs.jmedchem.6b01561. doi:10.1021/acs.jmedchem.6b01561.

Ju, H., Zhao, S., Tappia, P. S., Panagia, V., and Dixon, I. M. (1998). Expression of Gq alpha and PLC-beta in scar and border tissue in heart failure due to myocardial infarction. *Circulation* 97, 892–899.

Kalla, R. V., and Zablocki, J. (2009). Progress in the discovery of selective, high affinity A2B adenosine receptor antagonists as clinical candidates. *Purinergic Signal.* 5, 21–29. doi:10.1007/s11302-008-9119-x.

Kanno, T., Gotoh, A., Fujita, Y., Nakano, T., and Nishizaki, T. (2012). A_3 adenosine receptor mediates apoptosis in 5637 human bladder cancer cells by G_q protein/PKC-dependent AIF upregulation. *Cell. Physiol. Biochem.* 30, 1159–1168.

Karmouty-Quintana, H., Philip, K., Acero, L. F., Chen, N. Y., Weng, T., Molina, J. G., et al. (2014). Deletion of ADORA2B from myeloid cells dampens lung fibrosis and pulmonary hypertension. *FASEB J.* doi:10.1096/fj.14-260182.

Karmouty-Quintana, H., Xia, Y., and Blackburn, M. R. (2013). Adenosine signaling during acute and chronic disease states. *J. Mol. Med.* 91, 173–181. doi:10.1007/s00109-013-0997-1.

Kasama, H., Sakamoto, Y., Kasamatsu, A., Okamoto, A., Koyama, T., Minakawa, Y., et al. (2015). Adenosine A2B receptor promotes progression of human oral cancer. *BMC Cancer*, 1–12. doi:10.1186/s12885-015-1577-2.

Kenakin, T. (2004). Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol. Pharmacol.* 65, 2–11. doi:10.1124/mol.65.1.2.

Kenakin, T. (2011). Functional selectivity and biased receptor signaling. *J. Pharmacol. Exp. Ther.* 336, 296–302. doi:10.1124/jpet.110.173948.

Kenakin, T., and Christopoulos, A. (2013). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discov.* 12, 205–216. doi:10.1038/nrd3954.

Kenakin, T., Watson, C., Muniz-Medina, V., Christopoulos, A., and Novick, S. (2012). A simple method for quantifying functional selectivity and agonist bias. *ACS Chem. Neurosci.* 3, 193–203. doi:10.1021/cn200111m.

Khajehali, E., Malone, D. T., Glass, M., Sexton, P. M., Christopoulos, A., and Leach, K. (2015). Biased agonism and biased allosteric modulation at the CB1 cannabinoid receptor. *Mol. Pharmacol.* 88, 368–379. doi:10.1124/mol.115.099192.

Khoa, N. D., Montesinos, M. C., Williams, A. J., Kelly, M., and Cronstein, B. N. (2003). Th1 cytokines regulate adenosine receptors and their downstream signaling elements in human microvascular endothelial cells. *J. Immunol.* 171, 3991–3998. doi:10.4049/jimmunol.171.8.3991.

Kim, S.-A., Marshall, M. A., Melman, N., Kim, H. S., Müller, C. E., Linden, J., et al. (2002). structure−activity relationships at human and rat A2B adenosine receptors of xanthine derivatives substituted at the 1-, 3-, 7-, and 8-positions. *J. Med. Chem.* 45, 2131–2138. doi:10.1021/jm0104318.

Kim, Y.-C., Ji, X.-D., Melman, N., Linden, J., and Jacobson, K. A. (2000). Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A2B adenosine receptors. *J. Med. Chem.* 43, 1165–1172. doi:10.1021/jm990421v.

Klaasse, E. C., IJzerman, A. P., Grip, W. J., and Beukers, M. W. (2007). Internalization and desensitization of adenosine receptors. *Purinergic Signal.* 4, 21–37. doi:10.1007/s11302-007-9086-7.

Klinger, M., Kuhn, M., Just, H., Stefan, E., Palmer, T., Freissmuth, M., et al. (2002). Removal of the carboxy terminus of the A2A-adenosine receptor blunts constitutive activity: differential effect on cAMP accumulation and MAP kinase stimulation. *Naunyn-Schmiedebergs Arch. Pharmacol.* 366, 287–298. doi:10.1007/s00210-002-0617-z.

Kloner, R. A., Forman, M. B., Gibbons, R. J., Ross, A. M., Alexander, R. W., and Stone, G. W. (2006). Impact of time to therapy and reperfusion modality on the efficacy of adenosine in acute myocardial infarction: the AMISTAD-2 trial. *Eur. Heart J.* 27, 2400–2405.

doi:10.1093/eurheartj/ehl094.

Kniazeff, J., Prézeau, L., Rondard, P., Pin, J.-P., and Goudet, C. (2011). Dimers and beyond: The functional puzzles of class C GPCRs. *Pharmacol. Ther.* 130, 9–25. doi:10.1016/j.pharmthera.2011.01.006.

Koeppen, M., Harter, P. N., Bonney, S., Bonney, M., Reithel, S., Zachskorn, C., et al. (2012). Adora2b signaling on bone marrow derived cells dampens myocardial ischemia-reperfusion injury. *Anesthesiology* 116, 1245–1257 doi:10.1097/ALN.0b013e318255793c.

Kolachala, V. L., Ruble, B. K., Vijay-Kumar, M., Wang, L., Mwangi, S., Figler, H. E., et al. (2008). Blockade of adenosine A2B receptors ameliorates murine colitis. *Br. J. Pharmacol.* 155, 127–137. doi:10.1038/bjp.2008.227.

Kolachala, V., Asamoah, V., Wang, L., Obertone, T. S., Ziegler, T. R., Merlin, D., et al. (2005). TNF-α upregulates adenosine 2B (A2B) receptor expression and signaling in intestinal epithelial cells: a basis for A2BR overexpression in colitis. *Cell. Mol. Life Sci.* 62, 2647–2657. doi:10.1007/s00018-005-5328-4.

Kollias-Baker, C. A., Ruble, J., Jacobson, M., Harrison, J. K., Ozeck, M., Shryock, J. C., et al. (1997). Agonist-independent effect of an allosteric enhancer of the A1 adenosine receptor in CHO cells stably expressing the recombinant human A1 receptor. *J. Pharmacol. Exp. Ther.* 281, 761–768.

Kong, P., Christia, P., and Frangogiannis, N. G. (2013). The pathogenesis of cardiac fibrosis. *Cell. Mol. Life Sci.* 71, 549–574. doi:10.1007/s00018-013-1349-6.

Kong, T., Westerman, K. A., Faigle, M., Eltzschig, H. K., and Colgan, S. P. (2006). HIF-dependent induction of adenosine A2B receptor in hypoxia. *FASEB J.* 20, 2242–2250. doi:10.1096/fj.06-6419com.

Kopecky, S. L., Aviles, R. J., Bell, M. R., Lobl, J. K., Tipping, D., Frommell, G., et al. (2003). A randomized, double-blinded, placebo-controlled, dose-ranging study measuring the effect of an adenosine agonist on infarct size reduction in patients undergoing primary percutaneous transluminal coronary angioplasty: the ADMIRE (AmP579 delivery for myocardial infarction REduction) study. *Am. Heart J.* 146, 146–152. doi:10.1016/S0002-8703(03)00172-8.

Koski, G., Streaty, R. A., and Klee, W. A. (1982). Modulation of sodium-sensitive GTPase by partial opiate agonists. An explanation for the dual requirement for Na+ and GTP in inhibitory regulation of adenylate cyclase. *J. Biol. Chem.* 257, 14035–14040.

Kull, B., Svenningsson, P., and Fredholm, B. B. (2000). Adenosine A2A receptors are colocalized with and activate Golf in rat striatum. *Mol. Pharmacol.*

Kuno, A., Critz, S. D., Cui, L., Solodushko, V., Yang, X.-M., Krahn, T., et al. (2007). Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine A2b-dependent signaling during early reperfusion. *J. Mol. Cell. Cardiol.* 43, 262–271. doi:10.1016/j.yjmcc.2007.05.016.

Lagerström, M. C., and Schiöth, H. B. (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat. Rev. Drug Discov.*

Langemeijer, E. V., Verzijl, D., Dekker, S. J., and Ijzerman, A. P. (2013). Functional selectivity of adenosine A1 receptor ligands? *Purinergic Signal.* 9, 91–100. doi:10.1007/s11302-012-9334-3.

Laskowski, A., Woodman, O., Cao, A., Drummond, G., Marshall, T., Kaye, D., et al. (2006). Antioxidant actions contribute to the antihypertrophic effects of atrial natriuretic peptide in neonatal rat cardiomyocytes. *Cardiovasc. Res.* 72, 112–123. doi:10.1016/j.cardiores.2006.07.006.

Lasley, R. D., Kristo, G., Keith, B. J., and Mentzer, R. M. (2006). The A2A/A2B receptor antagonist ZM-241385 blocks the cardioprotective effect of adenosine agonist pretreatment in in vivo rat myocardium. *Am. J. Physiol. Heart Circ. Physiol.* 292, H426–H431. doi:10.1152/ajpheart.00675.2006.

Lasley, R. D., Rhee, J. W., Van Wylen, D. G., and Mentzer, R. M., Jr (1990). Adenosine A1 receptor mediated protection of the globally ischemic isolated rat heart. *J. Mol. Cell. Cardiol.* 22, 39–47.

Layland, J., Carrick, D., Lee, M., Oldroyd, K., and Berry, C. (2014). Adenosine: Physiology, pharmacology, and clinical applications. *JACC Cardiovasc. Interv.* 7, 581–591. doi:10.1016/j.jcin.2014.02.009.

Leask, A. (2007). TGFβ, cardiac fibroblasts, and the fibrotic response. *Cardiovasc. Res.* 74, 207–212. doi:10.1016/j.cardiores.2006.07.012.

Leff, P. (1995). The two-state model of receptor activation. *Trends Pharmacol. Sci.* 16, 89–97.

Lefkowitz, R. J., Cotecchia, S., Samama, P., and Costa, T. (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.* 14, 303–307. doi:10.1016/0165-6147(93)90048-O.

Li, Q., Ye, K., Blad, C. C., Dulk, den, H., Brouwer, J., IJzerman, A. P., et al. (2006). ZM241385, DPCPX, MRS1706 are inverse agonists with different relative intrinsic efficacies on constitutively active mutants of the human adenosine A2B receptor. *J. Pharmacol. Exp. Ther.* 320, 637–645. doi:10.1124/jpet.106.111203.

Li, S., Huang, S., and Peng, S.-B. (2005). Overexpression of G protein-coupled receptors in cancer cells: involvement in tumor progression. *Int. J. Oncol.* 27, 1329–1339.

Lijnen, P., and Petrov, V. (2002). Transforming growth factor-beta 1-induced collagen production in cultures of cardiac fibroblasts is the result of the appearance of myofibroblasts. *Methods Find Exp Clin Pharmacol* 24, 333–344.

Linden, J., Thai, T., Figler, H., Jin, X., and Robeva, A. S. (1999). Characterization of human A2B adenosine receptors: radioligand binding, western blotting, and coupling to Gq in human embryonic kidney 293 cells and HMC-1 mast cells. *Mol. Pharmacol.* 56, 705–713.

Lloyd, H. G., and Fredholm, B. B. (1995). Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem. Int.* 26, 387–395.

Londos, C., Cooper, D. M., and Wolff, J. (1980). Subclasses of external adenosine receptors.

Proc. Natl. Acad. Sci. U.S.A. 77, 2551–2554.

Lu, D., and Insel, P. A. (2014). Cellular mechanisms of tissue fibrosis. 6. Purinergic signaling and response in fibroblasts and tissue fibrosis. *Am. J. Physiol., Cell Physiol.* 306, C779–C788. doi:10.1152/ajpcell.00381.2013.

Lu, D., Aroonsakool, N., Yokoyama, U., Patel, H. H., and Insel, P. A. (2013). Increase in cellular cyclic AMP concentrations reverses the profibrogenic phenotype of cardiac myofibroblasts: a novel therapeutic approach for cardiac fibrosis. *Mol. Pharmacol.* 84, 787–793. doi:10.1124/mol.113.087742.

Luttrell, L. M., Maudsley, S., and Bohn, L. M. (2015). Fulfilling the promise of "biased" G protein-coupled receptor agonism. *Mol. Pharmacol.* 88, 579–588. doi:10.1124/mol.115.099630.

Ma, D.-F., Kondo, T., Nakazawa, T., Nio, D.-F., Mochizuki, K., Kawasaki, T., et al. (2010). Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells. *Hum. Pathol.* 41, 1550–1557. doi:10.1016/j.humpath.2010.04.008.

Maas, J. E., Koupenova, M., Ravid, K., and Auchampach, J. A. (2008). Abstract 4831: The A2B adenosine receptor contributes to post-infarction heart failure. *Circulation* 118, S_946.

Matharu, A.-L., Mundell, S. J., Benovic, J. L., and Kelly, E. (2001). Rapid agonist-induced desensitization and internalization of the A2B adenosine receptor is mediated by a serine residue close to the COOH terminus. *J. Biol. Chem.* 276, 30199–30207. doi:10.1074/jbc.M010650200.

Matsushita, N., Kashihara, T., Shimojo, H., Suzuki, S., Nakada, T., Takeishi, Y., et al. (2014). Cardiac overexpression of constitutively active Galphaq causes angiotensin II type1 receptor activation, leading to progressive heart failure and ventricular arrhythmias in transgenic mice. *PLoS ONE* 9, e106354. doi:10.1371/journal.pone.0106354.s001.

May, L. T., Avlani, V. A., Langmead, C. J., Herdon, H. J., Wood, M. D., Sexton, P. M., et al. (2007a). Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors. *Mol. Pharmacol.* 72, 463–476. doi:10.1124/mol.107.037630.

May, L. T., Bridge, L. J., Stoddart, L. A., Briddon, S. J., and Hill, S. J. (2011). Allosteric interactions across native adenosine-A3 receptor homodimers: quantification using single-cell ligand-binding kinetics. *FASEB J.* 25, 3465–3476. doi:10.1096/fj.11-186296.

May, L. T., Leach, K., Sexton, P. M., and Christopoulos, A. (2007b). Allosteric Modulation of G Protein–Coupled Receptors. *Annu. Rev. Pharmacol. Toxicol.* 47, 1–51. doi:10.1146/annurev.pharmtox.47.120505.105159.

McIntosh, V. J., and Lasley, R. D. (2012). Adenosine receptor-mediated cardioprotection: are all 4 subtypes required or redundant? *J. Cardiovasc. Pharmacol. Ther.* 17, 21–33. doi:10.1177/1074248410396877.

Mende, U., Kagen, A., Cohen, A., Aramburu, J., Schoen, F. J., and Neer, E. J. (1998). Transient cardiac expression of constitutively active Galphaq leads to hypertrophy and dilated cardiomyopathy by calcineurin-dependent and independent pathways. *Proc. Natl. Acad.*

Sci. U.S.A 95, 13893–13898.

Merighi, S., Benini, A., Mirandola, P., Gessi, S., Varani, K., Simioni, C., et al. (2007). Caffeine inhibits adenosine-induced accumulation of hypoxia-inducible factor-1 , vascular endothelial growth factor, and interleukin-8 expression in hypoxic human colon cancer cells. *Mol. Pharmacol.* 72, 395–406. doi:10.1124/mol.106.032920.

Merighi, S., Simioni, C., Gessi, S., Varani, K., Mirandola, P., Tabrizi, M. A., et al. (2009). Adenosine receptors modulate vascular endothelial growth factor and interleukin-8 expression in human melanoma cells treated with etoposideand doxorubicin. *Neoplasia* 11, 1064–1073. doi:10.1593/neo.09768.

Merkle, D., and Hoffmann, R. (2011). Roles of cAMP and cAMP-dependent protein kinase in the progression of prostate cancer: Cross-talk with the androgen receptor. *Cell. Signalling* 23, 507–515. doi:10.1016/j.cellsig.2010.08.017.

Methner, C., Schmidt, K., Cohen, M. V., Downey, J. M., and Krieg, T. (2010). Both A2a and A2b adenosine receptors at reperfusion are necessary to reduce infarct size in mouse hearts. *Am. J. Physiol. Heart Circ. Physiol.* 299, H1262–H1264. doi:10.1152/ajpheart.00181.2010.

Milligan, G. (2003). Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol. Pharmacol.* 64, 1271–1276.

Milligan, G. (2006). G-protein-coupled receptor heterodimers: pharmacology, function and relevance to drug discovery. *Drug Discovery Today* 11, 541–549. doi:10.1016/j.drudis.2006.04.007.

Milligan, G. (2007). G protein-coupled receptor dimerisation: Molecular basis and relevance to function. *Biochim. Biophys. Acta, Biomembr.* 1768, 825–835. doi:10.1016/j.bbamem.2006.09.021.

Milligan, G., and IJzerman, A. P. (2000). Stochastic multidimensional hypercubes and inverse agonism. *Trends Pharmacol. Sci.* 21, 362–363.

Mirabet, M., Herrera, C., Cordero, O. J., Mallol, J., Lluis, C., and Franco, R. (1999). Expression of A2B adenosine receptors in human lymphocytes: their role in T cell activation. *J. Cell Sci.* 112 (Pt 4), 491–502.

Mittal, D., Sinha, D., Barkauskas, D., Young, A., Kalimutho, M., Stannard, K., et al. (2016). Adenosine 2B receptor expression on cancer cells promotes metastasis. *Cancer Res.* 76, 4372–4382. doi:10.1158/0008-5472.CAN-16-0544.

Mogul, D. J., Adams, M. E., and Fox, A. P. (1993). Differential activation of adenosine receptors decreases N-type but potentiates P-type Ca2+ current in hippocampal CA3 neurons. *Neuron* 10, 327–334.

Moriyama, K., and Sitkovsky, M. V. (2010). Adenosine A2A receptor is involved in cell surface expression of A2B receptor. *J. Biol. Chem.* 285, 39271–39288. doi:10.1074/jbc.M109.098293.

Mousavi, S., Panjehpour, M., Izadpanahi, M. H., and Aghaei, M. (2015). Expression of

adenosine receptor subclasses in malignant and adjacent normal human prostate tissues. *Prostate*, n/a–n/a. doi:10.1002/pros.22955.

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., et al. (2016). Heart disease and stroke statistics-2016 update: A report from the American Heart Association. *Circulation* 133, e38–360. doi:10.1161/CIR.0000000000000350.

Mundell, S. J., Matharu, A.-L., Kelly, E., and Benovic, J. L. (2000). Arrestin isoforms dictate differential kinetics of A2B adenosine receptor trafficking. *Biochemistry* 39, 12828–12836. doi:10.1021/bi0010928.

Mølck, C., Ryall, J., Failla, L. M., Coates, J. L., Pascussi, J.-M., Heath, J. K., et al. (2016). The A2B adenosine receptor antagonist PSB-603 promotes oxidative phosphorylation and ROS production in colorectal cancer cells via adenosine receptor-independent mechanism. *Cancer Lett.* 383, 135–143. doi:10.1016/j.canlet.2016.09.018.

Nalli, A. D., Kumar, D. P., Al-Shboul, O., Mahavadi, S., Kuemmerle, J. F., Grider, J. R., et al. (2014). Regulation of Gβγi-dependent PLC-β3 activity in smooth muscle: inhibitory phosphorylation of PLC-β3 by PKA and PKG and stimulatory phosphorylation of Gαi-GTPase-activating protein RGS2 by PKG. *Cell Biochem. Biophys.* 70, 867–880. doi:10.1007/s12013-014-9992-6.

Neer, E. J. (1995). Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* 80, 249–257.

Newby, A. C. (1984). Adenosine and the concept of "retaliatory metabolites." *Trends Biochem. Sci.* 9, 42–44.

Németh, Z. H., Bleich, D., Csóka, B., Pacher, P., Mabley, J. G., Himer, L., et al. (2007). Adenosine receptor activation ameliorates type 1 diabetes. *FASEB J.* 21, 2379–2388. doi:10.1096/fj.07-8213com.

Németh, Z. H., Lutz, C. S., Csoka, B., Deitch, E. A., Leibovich, S. J., Gause, W. C., et al. (2005). Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism. *The Journal of ...*.

Nguyen, A. T. N., Vecchio, E. A., Thomas, T., Nguyen, T. D., Aurelio, L., Scammells, P. J., et al. (2016). Role of the second extracellular loop of the adenosine A1 receptor on allosteric modulator binding, signaling, and cooperativity. *Mol. Pharmacol.* 90, 715–725. doi:10.1124/mol.116.105015.

Noji, T., Karasawa, A., and Kusaka, H. (2004). Adenosine uptake inhibitors. *Eur. J. Pharmacol.* 495, 1–16. doi:10.1016/j.ejphar.2004.05.003.

Novitskaya, T., Chepurko, E., Covarrubias, R., Novitskiy, S., Ryzhov, S. V., Feoktistov, I., et al. (2016). Extracellular nucleotide regulation and signaling in cardiac fibrosis. *J. Mol. Cell. Cardiol.* 93, 47–56. doi:10.1016/j.yjmcc.2016.02.010.

Novitskiy, S. V., Ryzhov, S., Zaynagetdinov, R., Goldstein, A. E., Huang, Y., Tikhomirov, O. Y., et al. (2008). Adenosine receptors in regulation of dendritic cell differentiation and function. *Blood* 112, 1822–1831. doi:10.1182/blood-2008-02-136325.

Ntantie, E., Gonyo, P., Lorimer, E. L., Hauser, A. D., Schuld, N., McAllister, D., et al. (2013). An adenosine-mediated signaling pathway suppresses prenylation of the GTPase rap1b and promotes cell scattering. *Sci. Signaling* 6, ra39–ra39. doi:10.1126/scisignal.2003374.

Ortore, G., and Martinelli, A. (2010). A2B receptor ligands: past, present and future trends. *Curr. Top. Med. Chem.* 10, 923–940.

Pacheco, R., Martinez-Navio, J. M., Lejeune, M., Climent, N., Oliva, H., Gatell, J. M., et al. (2005). CD26, adenosine deaminase, and adenosine receptors mediate costimulatory signals in the immunological synapse. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9583–9588. doi:10.1073/pnas.0501050102.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., et al. (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289, 739–745.

Palmer, T. M., Gettys, T. W., and Stiles, G. L. (1995a). Differential interaction with and regulation of multiple G-proteins by the rat A3 adenosine receptor. *J. Biol. Chem.* 270, 16895–16902.

Palmer, T. M., Poucher, S. M., Jacobson, K. A., and Stiles, G. L. (1995b). 125I-4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl)phenol, a high affinity antagonist radioligand selective for the A2a adenosine receptor. *Mol. Pharmacol.* 48, 970–974.

Pantazi, E., Bejaoui, M., Folch-Puy, E., Adam, R., and Roselló-Catafau, J. (2016). Advances in treatment strategies for ischemia reperfusion injury. *Expert Opin. Pharmacother.* 17, 169–179. doi:10.1517/14656566.2016.1115015.

Park, P. S.-H., and Palczewski, K. (2005). Diversifying the repertoire of G protein-coupled receptors through oligomerization. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8793–8794. doi:10.1073/pnas.0504016102.

Park, P. S.-H., Lodowski, D. T., and Palczewski, K. (2008). Activation of G protein–coupled receptors: beyond two-state models and tertiary conformational changes. *Annu. Rev. Pharmacol. Toxicol.* 48, 107–141. doi:10.1146/annurev.pharmtox.48.113006.094630.

Parra, S., and Bond, R. A. (2007). Inverse agonism: from curiosity to accepted dogma, but is it clinically relevant? *Curr. Opin. Pharmacol.* 7, 146–150. doi:10.1016/j.coph.2006.10.005.

Peart, J. N., and Headrick, J. P. (2007). Adenosinergic cardioprotection: Multiple receptors, multiple pathways. *Pharmacol. Ther.* 114, 208–221. doi:10.1016/j.pharmthera.2007.02.004.

Pedroza, M., Schneider, D. J., Karmouty-Quintana, H., Coote, J., Shaw, S., Corrigan, R., et al. (2011). Interleukin-6 contributes to inflammation and remodeling in a model of adenosine mediated lung injury. *PLoS ONE* 6, e22667. doi:10.1371/journal.pone.0022667.s006.

Peeters, M. C., Li, Q., Elands, R., van Westen, G. J. P., Lenselink, E. B., Müller, C. E., et al. (2014). Domains for activation and inactivation in G protein-coupled receptors – A mutational analysis of constitutive activity of the adenosine A2B receptor. *Biochem. Pharmacol.* 92, 348–357. doi:10.1016/j.bcp.2014.08.022.

Petrov, V. V., Fagard, R. H., and Lijnen, P. J. (2002). Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension* 39, 258–263. doi:10.1161/hy0202.103268.

Philipp, S., Yang, X.-M., Cui, L., Davis, A. M., Downey, J. M., and Cohen, M. V. (2006). Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2B receptor cascade. *Cardiovasc. Res.* 70, 308–314. doi:10.1016/j.cardiores.2006.02.014.

Pierce, K. D., Furlong, T. J., Selbie, L. A., and Shine, J. (1992). Molecular cloning and expression of an adenosine A2B receptor from human brain. *Biochem. Biophys. Res. Commun.* 187, 86–93.

Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002). Seven-transmembrane receptors. *Nat. Rev. Mol. Cell Biol.* 3, 639–650. doi:10.1038/nrm908.

Pin, J. P., Neubig, R., Bouvier, M., Devi, L., Filizola, M., Javitch, J. A., et al. (2007). International Union of Basic and Clinical Pharmacology. LXVII. Recommendations for the recognition and nomenclature of G protein-coupled receptor heteromultimers. *Pharmacol. Rev.* 59, 5–13. doi:10.1124/pr.59.1.5.

Porter, K. E., and Turner, N. A. (2009). Cardiac fibroblasts: At the heart of myocardial remodeling. *Pharmacol. Ther.* 123, 255–278. doi:10.1016/j.pharmthera.2009.05.002.

Prinster, S. C. (2005). Heterodimerization of G protein-coupled receptors: specificity and functional significance. *Pharmacol. Rev.* 57, 289–298. doi:10.1124/pr.57.3.1.

Quintana, M., Kahan, T., and Hjemdahl, P. (2004). Pharmacological prevention of reperfusion injury in acute myocardial infarction. *Am. J. Cardiovasc. Drugs* 4, 159–167.

Rajagopal, S., Ahn, S., Rominger, D. H., Gowen-MacDonald, W., Lam, C. M., DeWire, S. M., et al. (2011). Quantifying ligand bias at seven-transmembrane receptors. *Mol. Pharmacol.* 80, 367–377. doi:10.1124/mol.111.072801.

Regan, S. E., Broad, M., Byford, A. M., Lankford, A. R., Cerniway, R. J., Mayo, M. W., et al. (2003). A1 adenosine receptor overexpression attenuates ischemia-reperfusion-induced apoptosis and caspase 3 activity. *Am. J. Physiol. Heart Circ. Physiol.* 284, H859–66. doi:10.1152/ajpheart.00251.2002.

Reichelt, M. E., Shanu, A., Willems, L., Witting, P. K., Ellis, N. A., Blackburn, M. R., et al. (2009). Endogenous adenosine selectively modulates oxidant stress via the A1 receptor in ischemic hearts. *Antioxid. Redox Signal.* 11, 2641–2650. doi:10.1089/ARS.2009.2644.

Reiter, E., Ahn, S., Shukla, A. K., and Lefkowitz, R. J. (2012). Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors. *Annu. Rev. Pharmacol. Toxicol.* 52, 179–197. doi:10.1146/annurev.pharmtox.010909.105800.

Rivkees, S. A., and Reppert, S. M. (1992). RFL9 encodes an A2B-adenosine receptor. *Mol. Endocrinol.* 6, 1598–1604. doi:10.1210/mend.6.10.1333049.

Roberts, V. S., Cowan, P. J., Alexander, S. I., Robson, S. C., and Dwyer, K. M. (2014a). The role of adenosine receptors A2A and A2B signaling in renal fibrosis. *Kidney Int.* 86, 685–692. doi:10.1038/ki.2014.244.

Roberts, V., Lu, B., Dwyer, K. M., and Cowan, P. J. (2014b). Adenosine receptor expression in the development of renal fibrosis following ischemic injury. *Transplant Proc.* 46, 3257–3261. doi:10.1016/j.transproceed.2014.09.151.

Rosenbaum, D. M., Rasmussen, S. G. F., and Kobilka, B. K. (2009). The structure and function of G-protein-coupled receptors. *Nature* 459, 356–363. doi:10.1038/nature08144.

Rosentreter, U., Henning, R., Bauser, M., Krämer, T., Vaupel, A., Hübsch, W., et al. (2001). Substituted 2-thio-3,5-dicyano-4-aryl-6-aminopyridines and the use thereof as adenosine reeceptor ligands.

Ross, A. M., Gibbons, R. J., Stone, G. W., Kloner, R. A., and Alexander, R. W. (2005). A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *J. Am. Coll. Cardiol.* 45, 1775–1780. doi:10.1016/j.jacc.2005.02.061.

Ryzhov, S., Biktasova, A., Goldstein, A. E., Zhang, Q., Biaggioni, I., Dikov, M. M., et al. (2013). Role of JunB in adenosine A2B receptor-mediated vascular endothelial growth factor production. *Mol. Pharmacol.* 85, 62–73. doi:10.1124/mol.113.088567.

Ryzhov, S., Goldstein, A. E., Biaggioni, I., and Feoktistov, I. (2006). Cross-talk between Gs- and Gq-coupled pathways in regulation of interleukin-4 by A2B adenosine receptors in human mast cells. *Mol. Pharmacol.* 70, 727–735. doi:10.1124/mol.106.022780.

Ryzhov, S., Novitskiy, S. V., Zaynagetdinov, R., Goldstein, A. E., Carbone, D. P., Biaggioni, I., et al. (2008). Host A2B adenosine receptors promote carcinoma growth. *Neoplasia* 10, 987–995.

Sabbah, H. N., Gupta, R. C., Kohli, S., Wang, M., Rastogi, S., Zhang, K., et al. (2013). Chronic therapy with a partial adenosine A1-receptor agonist improves left ventricular function and remodeling in dogs with advanced heart failure. *Circ. Heart Fail.* 6, 563–571. doi:10.1161/CIRCHEARTFAILURE.112.000208.

Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993). A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.* 268, 4625–4636.

Santiago, J.-J., Dangerfield, A. L., Rattan, S. G., Bathe, K. L., Cunnington, R. H., Raizman, J. E., et al. (2010). Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: Expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. *Dev. Dyn.* 239, 1573–1584. doi:10.1002/dvdy.22280.

Sassi, Y., Ahles, A., Truong, D.-J. J., Baqi, Y., Lee, S.-Y., Husse, B., et al. (2014). Cardiac myocyte–secreted cAMP exerts paracrine action via adenosine receptor activation. *J. Clin. Invest.* 124, 5385–5397. doi:10.1172/JCI74349DS1.

Sattin, A., and Rall, T. W. (1970). The effect of adenosine and adenine nucleotides on the cyclic adenosine 3“, 5-”phosphate content of guinea pig cerebral cortex slices. *Mol. Pharmacol.* 6, 13–23.

Savinainen, J. R., Saario, S. M., Niemi, R., Järvinen, T., and Laitinen, J. T. (2003). An optimized approach to study endocannabinoid signaling: evidence against constitutive

activity of rat brain adenosine A1 and cannabinoid CB1 receptors. *Br. J. Pharmacol.* 140, 1451–1459. doi:10.1038/sj.bjp.0705577.

Sawynok, J. (2016). Adenosine receptor targets for pain. *Neuroscience* 338, 1–18. doi:10.1016/j.neuroscience.2015.10.031.

Schnee, J. M., and Hsueh, W. A. (2000). Angiotensin II, adhesion, and cardiac fibrosis. *Cardiovasc. Res.* 46, 264–268. doi:10.1016/S0008-6363(00)00044-4.

Schubert, P., Komp, W., and Kreutzberg, G. W. (1979). Correlation of 5'-nucleotidase activity and selective transneuronal transfer of adenosine in the hippocampus. *Brain Res.* 168, 419–424.

Schulte, G., and Fredholm, B. B. (2000). Human adenosine A1, A2A, A2B, and A3 receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol. Pharmacol.* 58, 477–482.

Schulte, G., and Fredholm, B. B. (2003a). Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell. Signalling* 15, 813–827. doi:10.1016/S0898-6568(03)00058-5.

Schulte, G., and Fredholm, B. B. (2003b). The Gs-coupled adenosine A2B receptor recruits divergent pathways to regulate ERK1/2 and p38. *Exp. Cell Res.* 290, 168–176. doi:10.1016/S0014-4827(03)00324-0.

Schumacher, S. M., Gao, E., Cohen, M., Lieu, M., Chuprun, J. K., and Koch, W. J. (2016). A peptide of the RGS domain of GRK2 binds and inhibits Gα(q) to suppress pathological cardiac hypertrophy and dysfunction. *Sci. Signaling* 9, ra30. doi:10.1126/scisignal.aae0549.

Searl, T. J., and Silinsky, E. M. (2012). Evidence for constitutively-active adenosine receptors at mammalian motor nerve endings. *Eur. J. Pharmacol.* 685, 38–41. doi:10.1016/j.ejphar.2012.04.008.

See, F., Kompa, A., Martin, J., Lewis, D. A., and Krum, H. (2005). Fibrosis as a therapeutic target post-myocardial infarction. *Curr. Pharm. Des.* 11, 477–487. doi:10.2174/1381612053382098.

Segura, A. M., Frazier, O. H., and Buja, L. M. (2012). Fibrosis and heart failure. *Heart Failure Rev.* 19, 173–185. doi:10.1007/s10741-012-9365-4.

Seifert, R., and Wenzel-Seifert, K. (2002). Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.* 366, 381–416. doi:10.1007/s00210-002-0588-0.

Seo, S. W., Koeppen, M., Bonney, S., Gobel, M., Thayer, M., Harter, P. N., et al. (2015). Differential tissue-specific function of adora2b in cardioprotection. *J. Immunol.* doi:10.4049/jimmunol.1402288.

Sepúlveda, C., Palomo, I., and Fuentes, E. (2016). Role of adenosine A2B receptor overexpression in tumor progression. *Life Sci.* 166, 92–99. doi:10.1016/j.lfs.2016.10.008.

Shonberg, J., Lopez, L., Scammells, P. J., Christopoulos, A., Capuano, B., and Lane, J. R. (2014). Biased agonism at G protein-coupled receptors: the promise and the challenges-a medicinal chemistry perspective. *Med. Res. Rev.* 34, 1286–1330. doi:10.1002/med.21318.

Shryock, J. C., Ozeck, M. J., and Belardinelli, L. (1998). Inverse agonists and neutral antagonists of recombinant human A1 adenosine receptors stably expressed in Chinese hamster ovary cells. *Mol. Pharmacol.* 53, 886–893.

Shyrock, J. C., and Belardinelli, L. (1997). Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am. J. Cardiol.* 79, 2–10. doi:10.1016/S0002-9149(97)00256-7.

Sitaraman, S. V. (2002). The adenosine A2B receptor is recruited to the plasma membrane and associates with E3KARP and ezrin upon agonist stimulation. *J. Biol. Chem.* 277, 33188–33195. doi:10.1074/jbc.M202522200.

Sitaraman, S. V., Merlin, D., Wang, L., Wong, M., Gewirtz, A. T., Si-Tahar, M., et al. (2001). Neutrophil-epithelial crosstalk at the intestinal lumenal surface mediated by reciprocal secretion of adenosine and IL-6. *J. Clin. Invest.* 107, 861–869. doi:10.1172/JCI11783.

Snead, A. N., and Insel, P. A. (2012). Defining the cellular repertoire of GPCRs identifies a profibrotic role for the most highly expressed receptor, protease-activated receptor 1, in cardiac fibroblasts. *FASEB J.* 26, 4540–4547. doi:10.1096/fj.12-213496.

Sommerschild, H. T., and Kirkebøen, K. A. (2000). Adenosine and cardioprotection during ischaemia and reperfusion–an overview. *Acta Anaesthesiol. Scand.* 44, 1038–1055.

Souders, C. A., Bowers, S. L. K., and Baudino, T. A. (2009). Cardiac fibroblast: The renaissance cell. *Circ. Res.* 105, 1164–1176. doi:10.1161/CIRCRESAHA.109.209809.

St Hilaire, C., Koupenova, M., Carroll, S. H., Smith, B. D., and Ravid, K. (2008). TNF-α upregulates the A2B adenosine receptor gene: The role of NAD(P)H oxidase 4. *Biochem. Biophys. Res. Commun.* 375, 292–296. doi:10.1016/j.bbrc.2008.07.059.

Stagg, J., Divisekera, U., McLaughlin, N., Sharkey, J., Pommey, S., Denoyer, D., et al. (2010). Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 1547–1552. doi:10.1073/pnas.0908801107.

Stallaert, W., Christopoulos, A., and Bouvier, M. (2011). Ligand functional selectivity and quantitative pharmacology at G protein-coupled receptors. *Expert Opin. Drug Discov.* 6, 811–825. doi:10.1016/S0165-6147(98)01240-1.

Stehle, J. H., Rivkees, S. A., Lee, J. J., Weaver, D. R., Deeds, J. D., and Reppert, S. M. (1992). Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. *Mol. Endocrinol.* 6, 384–393.

Sternini, C., Spann, M., Anton, B., Keith, D. E., Bunnett, N. W., Zastrow, von, M., et al. (1996). Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 93, 9241–9246.

Stewart, M., Steinig, A. G., Ma, C., Song, J.-P., McKibben, B., Castelhano, A. L., et al. (2004). [3H]OSIP339391, a selective, novel, and high affinity antagonist radioligand for adenosine

A2B receptors. *Biochem. Pharmacol.* 68, 305–312. doi:10.1016/j.bcp.2004.03.026.

Strange, P. G. (2002). Mechanisms of inverse agonism at G-protein-coupled receptors. *Trends Pharmacol. Sci.* 23, 89–95.

Stuart, S. D. F., De Jesus, N. M., Lindsey, M. L., and Ripplinger, C. M. (2016). The crossroads of inflammation, fibrosis, and arrhythmia following myocardial infarction. *J. Mol. Cell. Cardiol.* 91, 114–122. doi:10.1016/j.yjmcc.2015.12.024.

Subarsky, P., and Hill, R. P. (2003). The hypoxic tumour microenvironment and metastatic progression. *Clin. Exp. Metastasis* 20, 237–250.

Sun, C. X. (2006). Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *J. Clin. Invest.* 116, 2173–2182. doi:10.1172/JCI27303.

Sun, Y., and Huang, P. (2016). Adenosine A2B receptor: From cell biology to human diseases. *Front. Chem.* 4, 1329. doi:10.4049/jimmunol.0900515.

Sun, Y., Hu, W., Yu, X., Liu, Z., Tarran, R., Ravid, K., et al. (2016). Actinin-1 binds to the C-terminus of A2B adenosine receptor (A2BAR) and enhances A2BAR cell-surface expression. *Biochem. J.* doi:10.1042/BCJ20160272.

Surendra, H., Diaz, R. J., Harvey, K., Tropak, M., Callahan, J., Hinek, A., et al. (2013). Interaction of δ and κ opioid receptors with adenosine A1 receptors mediates cardioprotection by remote ischemic preconditioning. *J. Mol. Cell. Cardiol.* 60, 142–150. doi:10.1016/j.yjmcc.2013.04.010.

Swaney, J. S., Roth, D. M., Olson, E. R., Naugle, J. E., Meszaros, J. G., and Insel, P. A. (2005). Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* 102, 437–442. doi:10.1073/pnas.0408704102.

Tendera, M., Gaszewska-Żurek, E., Parma, Z., Ponikowski, P., Jankowska, E., Kawecka-Jaszcz, K., et al. (2012). The new oral adenosine A1 receptor agonist capadenoson in male patients with stable angina. *Clin. Res. Cardiol.* 101, 585–591. doi:10.1007/s00392-012-0430-8.

Thornton, J. D., Liu, G. S., Olsson, R. A., and Downey, J. M. (1992). Intravenous pretreatment with A1-selective adenosine analogues protects the heart against infarction. *Circulation* 85, 659–665. doi:10.1161/01.CIR.85.2.659.

Tian, Y., Piras, B. A., Kron, I. L., French, B. A., and Yang, Z. (2015). Adenosine 2B receptor activation reduces myocardial reperfusion injury by promoting anti-inflammatory macrophages differentiation via PI3K/Akt pathway. *Oxid. Med. Cell. Longevity*, 1–8. doi:10.1155/2015/585297.

Toldo, S., Zhong, H., Mezzaroma, E., Van Tassell, B. W., Kannan, H., Zeng, D., et al. (2012). GS-6201, a selective blocker of the A2B adenosine receptor, attenuates cardiac remodeling after acute myocardial infarction in the mouse. *J. Pharmacol. Exp. Ther.* 343, 587–595. doi:10.1124/jpet.111.191288.

Tracey, W. R., Magee, W., Masamune, H., Oleyneck, J. J., and Hill, R. J. (1998). Selective activation of adenosine A3 receptors with N6-(3-chlorobenzyl)-5′-N-methylcarboxamidoadenosine (CB-MECA) provides cardioprotection via KATP channel activation *Cardiovasc. Res.* 40, 138-145

Trussell, L. O., and Jackson, M. B. (1985). Adenosine-activated potassium conductance in cultured striatal neurons. *Proc. Natl. Acad. Sci. U.S.A.* 82, 4857-4861.

Tyndall, J. D. A., and Sandilya, R. (2005). GPCR agonists and antagonists in the clinic. *Med. Chem.* 1, 405–421.

Urmaliya, V. B., Church, J. E., Coupar, I. M., Rose'Meyer, R. B., Pouton, C. W., and White, P. J. (2009). Cardioprotection induced by adenosine A1 receptor agonists in a cardiac cell ischemia model involves cooperative activation of adenosine A2A and A2B receptors by endogenous adenosine. *J. Cardiovasc. Pharmacol.* 53, 424–433.

Urmaliya, V. B., Pouton, C. W., Devine, S. M., Haynes, J. M., Warfe, L., Scammells, P. J., et al. (2010a). A novel highly selective adenosine A1 receptor agonist VCP28 reduces ischemia injury in a cardiac cell line and ischemia-reperfusion injury in isolated rat hearts at concentrations that do not affect heart rate. *J. Cardiovasc. Pharmacol.* 56, 282–292. doi:10.1097/FJC.0b013e3181eb8563.

Urmaliya, V. B., Pouton, C. W., Ledent, C., Short, J. L., and White, P. J. (2010b). Cooperative cardioprotection through adenosine A1 and A2A receptor agonism in ischemia-reperfused isolated mouse heart. *J. Cardiovasc. Pharmacol.* 56, 379–388.

Valant, C., Aurelio, L., Devine, S. M., Ashton, T. D., White, J. M., Sexton, P. M., et al. (2012). Synthesis and characterization of novel 2-amino-3-benzoylthiophene derivatives as biased allosteric agonists and modulators of the adenosine A 1receptor. *J. Med. Chem.* 55, 2367–2375. doi:10.1021/jm201600e.

Valant, C., May, L. T., Aurelio, L., Chuo, C. H., White, P. J., Baltos, J. A., et al. (2014). Separation of on-target efficacy from adverse effects through rational design of a bitopic adenosine receptor agonist. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4614–4619. doi:10.1073/pnas.1320962111.

van Calker, D., Müller, M., and Hamprecht, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* 33, 999–1005.

van der Hoeven, D., Wan, T. C., Gizewski, E. T., Kreckler, L. M., Maas, J. E., Van Orman, J., et al. (2011). A role for the low-affinity A2B adenosine receptor in regulating superoxide generation by murine neutrophils. *J. Pharmacol. Exp. Ther.* 338, 1004–1012. doi:10.1124/jpet.111.181792.

van der Westhuizen, E. T., Breton, B., Christopoulos, A., and Bouvier, M. (2014). Quantification of ligand bias for clinically relevant 2-adrenergic receptor ligands: implications for drug taxonomy. *Mol. Pharmacol.* 85, 492–509. doi:10.1124/mol.113.088880.

Vaughan, M., Howard, E. W., and Tomasek, J. J. (2000). Transforming growth factor-β1 promotes the morphological and functional differentiation of the myofibroblast. *Exp. Cell*

Res. 257, 180–189. doi:10.1006/excr.2000.4869.

Vecchio, E. A., Chuo, C. H., Baltos, J.-A., Ford, L., Scammells, P. J., Wang, B. H., et al. (2016a). The hybrid molecule, VCP746, is a potent adenosine A2B receptor agonist that stimulates anti-fibrotic signalling. *Biochem. Pharmacol.*, 1–11. doi:10.1016/j.bcp.2016.08.007.

Vecchio, E. A., Tan, C. Y. R., Gregory, K. J., Christopoulos, A., White, P. J., and May, L. T. (2016b). Ligand-independent adenosine A2B receptor constitutive activity as a promoter of prostate cancer cell proliferation. *J. Pharmacol. Exp. Ther.* 357, 36–44. doi:10.1124/jpet.115.230003.

Vecchio, E. A., White, P. J., and May, L. T. (2017). Targeting adenosine receptors for the treatment of cardiac fibrosis. *Front Pharmacol* 8, 1–8. doi:10.3389/fphar.2017.00243.

Vidi, P. A., Chemel, B. R., Hu, C. D., and Watts, V. J. (2008a). Ligand-dependent oligomerization of dopamine D2 and adenosine A2A receptors in living neuronal cells. *Mol. Pharmacol.* 74, 544–551. doi:10.1124/mol.108.047472.

Vidi, P.-A., Chen, J., Irudayaraj, J. M. K., and Watts, V. J. (2008b). Adenosine A2A receptors assemble into higher-order oligomers at the plasma membrane. *FEBS Letters* 582, 3985–3990. doi:10.1016/j.febslet.2008.09.062.

Vilardaga, J.-P., Steinmeyer, R., Harms, G. S., and Lohse, M. J. (2005). Molecular basis of inverse agonism in a G protein–coupled receptor. *Nat Chem Biol* 1, 25–28. doi:10.1038/nchembio705.

Villarreal, F., Epperson, S. A., Ramirez-Sanchez, I., Yamazaki, K. G., and Brunton, L. L. (2009). Regulation of cardiac fibroblast collagen synthesis by adenosine: roles for Epac and PI3K. *Am. J. Physiol., Cell Physiol.* 296, C1178–C1184. doi:10.1152/ajpcell.00291.2008.

Violin, J. D., Crombie, A. L., Soergel, D. G., and Lark, M. W. (2014). Biased ligands at G-protein-coupledreceptors: promise and progress. *Trends Pharmacol. Sci.* 35, 308–316. doi:10.1016/j.tips.2014.04.007.

Wakeno, M., Minamino, T., Seguchi, O., Okazaki, H., Tsukamoto, O., Okada, K. I., et al. (2006). Long-term stimulation of adenosine A2B receptors begun after myocardial infarction prevents cardiac remodeling in rats. *Circulation* 114, 1923–1932. doi:10.1161/CIRCULATIONAHA.106.630087.

Wang, L. (2004). Agonist-induced polarized trafficking and surface expression of the adenosine 2b receptor in intestinal epithelial cells: role of SNARE proteins. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287, G1100–G1107. doi:10.1152/ajpgi.00164.2004.

Watson, M. J., Lee, S. L., Marklew, A. J., Gilmore, R. C., Gentzsch, M., Sassano, M. F., et al. (2016). The cystic fibrosis transmembrane conductance regulator (CFTR) uses its C-terminus to regulate the A2B adenosine receptor. *Sci. Rep.*, 1–13. doi:10.1038/srep27390.

Watson, M. J., Worthington, E. N., Clunes, L. A., Rasmussen, J. E., Jones, L., and Tarran, R. (2011). Defective adenosine-stimulated cAMP production in cystic fibrosis airway epithelia: a novel role for CFTR in cell signaling. *FASEB J.* 25, 2996–3003.

doi:10.1096/fj.11-186080.

Wei, Q., Costanzi, S., Balasubramanian, R., Gao, Z.-G., and Jacobson, K. A. (2013). A2B adenosine receptor blockade inhibits growth of prostate cancer cells. *Purinergic Signal.* 9, 271–280. doi:10.1007/s11302-012-9350-3.

Weinberg, J. M., and Venkatachalam, M. A. (2012). Preserving postischemic reperfusion in the kidney: a role for extracellular adenosine. *J. Clin. Invest.* 122, 493–496. doi:10.1172/JCI60957.

Welihinda, A. A., Kaur, M., Greene, K., Zhai, Y., and Amento, E. P. (2016). Cellular Signalling. *Cell. Signalling* 28, 552–560. doi:10.1016/j.cellsig.2016.02.010.

Weyler, S., Fülle, F., Diekmann, M., Schumacher, B., Hinz, S., Klotz, K.-N., et al. (2006). Improving potency, selectivity, and water solubility of adenosine A1 receptor antagonists: xanthines modified at position 3 and related pyrimido[1,2,3-cd]purinediones. *ChemMedChem* 1, 891–902. doi:10.1002/cmdc.200600066.

Whistler, J. L., and Zastrow, von, M. (1998). Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9914–9919.

Wilson, C. N., Nadeem, A., Spina, D., Brown, R., Page, C. P., and Mustafa, S. J. (2009). Adenosine receptors and asthma. *Handb. Exp. Pharmacol.*, 329–362. doi:10.1007/978-3-540-89615-9_11.

Wisler, J. W., DeWire, S. M., Whalen, E. J., Violin, J. D., Drake, M. T., Ahn, S., et al. (2007). A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16657–16662. doi:10.1073/pnas.0707936104.

Xaus, J., Mirabet, M., Lloberas, J., Soler, C., Lluis, C., Franco, R., et al. (1999). IFN-gamma up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J. Immunol.* 162, 3607–3614.

Xi, J., McIntosh, R., Shen, X., Lee, S., Chanoit, G., Criswell, H., et al. (2009). Adenosine A2A and A2B receptors work in concert to induce a strong protection against reperfusion injury in rat hearts. *J. Mol. Cell. Cardiol.* 47, 684–690. doi:10.1016/j.yjmcc.2009.08.009.

Xiang, Y., Rybin, V. O., Steinberg, S. F., and Kobilka, B. (2002). Caveolar localization dictates physiologic signaling of beta 2-adrenoceptors in neonatal cardiac myocytes. *J. Biol. Chem.* 277, 34280–34286. doi:10.1074/jbc.M201644200.

Yan, L., Burbiel, J. C., Maaß, A., and Müller, C. E. (2003). Adenosine receptor agonists: from basic medicinal chemistry to clinical development. *Expert Opin. Emerging Drugs* 8, 537–576.

Yang, D., Zhang, Y., Nguyen, H. G., Koupenova, M., Chauhan, A. K., Makitalo, M., et al. (2006). The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. *J. Clin. Invest.* 116, 1913–1923. doi:10.1172/JCI27933DS1.

Yang, X., Xin, W., Yang, X.-M., Kuno, A., Rich, T. C., Cohen, M. V., et al. (2011). A2B adenosine receptors inhibit superoxide production from mitochondrial complex I in rabbit cardiomyocytes via a mechanism sensitive to Pertussistoxin. *Br. J. Pharmacol.* 163, 995–

1006. doi:10.1111/j.1476-5381.2011.01288.x.

Yang, Y., Qiu, Y., Wang, W., Xiao, W., Liang, H., Zhang, C., et al. (2014). Adenosine A2B receptor modulates intestinal barrier function under hypoxic and ischemia/reperfusion conditions. *Int. J. Clin. Exp. Pathol.* 7, 2006–2018.

Yang, Z. (2005). Infarct-sparing effect of A2A-adenosine receptor activation is due primarily to its action on lymphocytes. *Circulation* 111, 2190–2197. doi:10.1161/01.CIR.0000163586.62253.A5.

Yang, Z., Cerniway, R. J., Byford, A. M., Berr, S. S., French, B. A., and Matherne, G. P. (2002). Cardiac overexpression of A1-adenosine receptor protects intact mice against myocardial infarction. *Am. J. Physiol. Heart Circ. Physiol.* 282, H949–H955. doi:10.1152/ajpheart.00741.2001.

Yokoyama, U., Patel, H. H., Lai, N. C., Aroonsakool, N., Roth, D. M., and Insel, P. A. (2008). The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6386–6391. doi:10.1073/pnas.0801490105.

Young, J. D., Yao, S. Y. M., Sun, L., Cass, C. E., and Baldwin, S. A. (2008). Human equilibrative nucleoside transporter (ENT) family of nucleoside and nucleobase transporter proteins. *Xenobiotica* 38, 995–1021. doi:10.1080/00498250801927427.

Zhan, E., McIntosh, V. J., and Lasley, R. D. (2011). Adenosine A2A and A2B receptors are both required for adenosine A1 receptor-mediated cardioprotection. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1183–H1189. doi:10.1152/ajpheart.00264.2011.

Zhang, H., Zhong, H., Everett, T. H., Wilson, E., Chang, R., Zeng, D., et al. (2014). Blockade of A2B adenosine receptor reduces left ventricular dysfunction and ventricular arrhythmias 1 week after myocardial infarction in the rat model. *Heart Rhythm* 11, 101–109. doi:10.1016/j.hrthm.2013.10.023.

Zhong, H., Belardinelli, L., Maa, T., and Zeng, D. (2005). Synergy between A2B adenosine receptors and hypoxia in activating human lung fibroblasts. *Am. J. Respir. Cell Mol. Biol.* 32, 2–8. doi:10.1165/rcmb.2004-0103OC.

Zhong, H., Belardinelli, L., Maa, T., Feoktistov, I., Biaggioni, I., and Zeng, D. (2004). A(2B) adenosine receptors increase cytokine release by bronchial smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* 30, 118–125. doi:10.1165/rcmb.2003-0118OC.

Zhou, Q. Y., LI, C., Olah, M. E., Johnson, R. A., Stiles, G. L., and Civelli, O. (1992). Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7432–7436.

Zhou, Y., Schneider, D. J., and Blackburn, M. R. (2009). Adenosine signaling and the regulation of chronic lung disease. *Pharmacol. Ther.* 123, 105–116. doi:10.1016/j.pharmthera.2009.04.003.

Zhou, Y., Schneider, D. J., Morschl, E., Song, L., Pedroza, M., Karmouty-Quintana, H., et al. (2011). Distinct roles for the A2B adenosine receptor in acute and chronic stages of bleomycin-induced lung injury. *J. Immunol.* 186, 1097–1106. doi:10.4049/jimmunol.1002907.

Zimmerman, M. A., Grenz, A., Tak, E., Kaplan, M., Ridyard, D., Brodsky, K. S., et al. (2013). Signaling through hepatocellular A2B adenosine receptors dampens ischemia and reperfusion injury of the liver. *Proc. Natl. Acad. Sci. U.S.A* 110, 12012–12017. doi:10.1073/pnas.1221733110.

Zylka, M. J. (2011). Pain-relieving prospects for adenosine receptors and ectonucleotidases. *Trends in Molecular Medicine* 17, 188–196. doi:10.1016/j.molmed.2010.12.006.

Appendix 1:

Targeting Adenosine Receptors for the Treatment of Cardiac Fibrosis: Mini Review

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Frontiers in Pharmacology **2017**, In Press

Chapter 1: Section *1.4.2 Fibrosis,* taken and ammended from the following mini review

frontiers in Pharmacology

MINI REVIEW
published: xx April 2017
doi: 10.3389/fphar.2017.00243

Targeting Adenosine Receptors for the Treatment of Cardiac Fibrosis

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Adenosine is a ubiquitous molecule with key regulatory and cytoprotective mechanisms at times of metabolic imbalance in the body. Among a plethora of physiological actions, adenosine has an important role in attenuating ischaemia-reperfusion injury and modulating the ensuing fibrosis and tissue remodeling following myocardial damage. Adenosine exerts these actions through interaction with four adenosine G protein-coupled receptors expressed in the heart. The adenosine A_{2B} receptor (A_{2B}AR) is the most abundant adenosine receptor (AR) in cardiac fibroblasts and is largely responsible for the influence of adenosine on cardiac fibrosis. *In vitro* and *in vivo* studies demonstrate that acute A_{2B}AR stimulation can decrease fibrosis through the inhibition of fibroblast proliferation and reduction in collagen synthesis. However, in contrast, there is also evidence that chronic A_{2B}AR antagonism reduces tissue fibrosis. This review explores the opposing pro- and anti-fibrotic activity attributed to the activation of cardiac ARs and investigates the therapeutic potential of targeting ARs for the treatment of cardiac fibrosis.

Keywords: adenosine, adenosine A_{2B} receptor, cardiac fibrosis, fibroblast, collagen synthesis, cAMP, myocardial infarction, heart failure

OPEN ACCESS

Edited by:
Tim David Hewitson, Royal Melbourne Hospital, Australia

Reviewed by:
Jason N. Peart, Griffith University, Australia
Nazareno Paolocci, Johns Hopkins University, USA

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Specialty section:
This article was submitted to Cardiovascular and Smooth Muscle Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *28 February 2017*
Accepted: *18 April 2017*
Published: *xx April 2017*

Citation:
Vecchio EA, White PJ and May LT (2017) Targeting Adenosine Receptors for the Treatment of Cardiac Fibrosis. Front. Pharmacol. 8:243. doi: 10.3389/fphar.2017.00243

INTRODUCTION

Cardiac fibroblasts form the largest population of interstitial cells in the adult mammalian heart (Chen and Frangogiannis, 2013). They have an essential role in the regulation of the extracellular matrix (ECM), which is crucial for maintaining the structural integrity of the myocardium and for electro-mechanical signal transduction (Camelliti et al., 2004; Souders et al., 2009). Cardiac fibroblasts are regulated by various mechanical and hormonal stimuli, in particular growth factors such as angiotensin II (ANGII) and the cytokine transforming growth factor β (TGFβ). ANGII and TGFβ can activate fibroblast cell-surface receptors to promote differentiation to myofibroblasts, the pro-fibrogenic phenotype that express the contractile protein α-smooth muscle actin (α-SMA) and exhibit enhanced secretory, migratory and proliferative properties (Schnee and Hsueh, 2000; Petrov et al., 2002; Leask, 2007; Porter and Turner, 2009; Lu and Insel, 2014). Following a myocardial infarction (MI), fibroblasts promote essential matrix deposition for proper tissue repair and scar formation to ensure structural integrity of the infarct zone. However, aberrant ECM deposition and excessive myofibroblast accumulation extending beyond the area of the original insult is responsible for maladaptive fibrosis leading to cardiac dysfunction, a hallmark feature of heart failure pathophysiology (See et al., 2005; Segura et al., 2012; Ferrari et al., 2016). Heart failure remains a major cause of mortality and morbidity in the western world with an estimated 50% 5 years survival rate after diagnosis (Mozaffarian et al., 2016). This highlights both the limitations

of current therapeutic management and the crucial need for new and innovative therapies for the treatment and prevention of heart failure. Extracellular nucleotides and nucleosides have recently been implicated as important mediators of fibroblast homeostasis and as such purinergic signaling has been investigated for its role in cardiac fibrosis. AMP catabolites, including inosine and oxypurines have also been shown to contribute to cardiac fibrosis and diastolic stiffening in some animal models of heart failure (Paolocci et al., 2006). The role of nucleotide (ATP, ADP, UTP) signaling in tissue fibrosis has been comprehensively reviewed previously (Lu and Insel, 2014; Ferrari et al., 2016; Novitskaya et al., 2016), therefore the current review will focus the modulation of cardiac fibrosis mediated by the nucleoside adenosine and adenosine receptors (ARs).

ADENOSINE SIGNALING IN THE HEART

Adenosine is a ubiquitous purine nucleoside that is an important regulator of cardiac function. Adenosine is described as a 'retaliatory metabolite' owing to its enhanced local release and ability to restore energy balance during times of cellular and metabolic stress (Newby, 1984; Shyrock and Belardinelli, 1997). The well-characterized cytoprotective actions have resulted in large clinical trials for adenosine and adenosine derivatives for the treatment of ischaemia-reperfusion injury post-MI (Kopecky et al., 2003; Ross et al., 2005; Forman et al., 2006). In addition to a clear role in cardioprotection, adenosine exerts a multitude of actions on the physiological regulation of the heart, including coronary vasodilation, heart rate control and AV nodal conduction, angiogenesis, myocardial hypertrophy and remodeling and fibrosis (Auchampach and Bolli, 1999; Peart and Headrick, 2007; Headrick et al., 2013). The myriad of cardiovascular effects stimulated by adenosine occur via activation of specific cell surface ARs. The AR family is comprised of four Class A G protein-coupled receptors (GPCRs), the A_1, A_{2A}, A_{2B} and A_3ARs. They exert distinct pharmacological actions through differential coupling to intracellular G proteins; the A_1AR and A_3AR preferentially activate $G_{i/o}$ proteins to inhibit adenylyl cyclase activity and subsequent cAMP production, while the A_{2A}AR and A_{2B}AR preferentially stimulate G_s proteins to activate adenylyl cyclase activity and increase cAMP accumulation (**Figure 1**) (Fredholm et al., 2001). The A_{2B}AR has also been shown to stimulate robust $G_{q/11}$ protein activation in some cell types (Feoktistov and Biaggioni, 1997; Linden et al., 1999). ARs, and the A_{2B}AR in particular, have also been shown to couple to additional transmembrane and intracellular proteins, which may influence downstream signal transduction (Mundell and Benovic, 2000; Fredholm et al., 2001; Sun and Huang, 2016). All four ARs are expressed in the heart and synchronous activation of multiple subtypes results in both complementary and opposing signal transduction for the fine-tuned regulation of cardiac function. Interestingly, both pro- and anti-fibrotic actions have been attributed to AR activation, which highlights both the complexity and ensuing challenges faced when targeting ARs for the treatment of cardiac fibrosis (Chan and Cronstein, 2009; Cronstein, 2011; Karmouty-Quintana et al., 2013). To date, the preponderance of evidence has implicated the A_{2B}AR in cardiac fibrosis (Epperson et al., 2009; Headrick et al., 2013; Novitskaya et al., 2016). Therefore, this review will explore the current understanding of the role of AR signaling in augmenting or attenuating cardiac fibrosis, with a focus on the predominant subtype implicated, the A_{2B}AR.

A_{2B}AR-MEDIATED ANTI-FIBROTIC SIGNAL TRANSDUCTION

Studies in isolated rat cardiac fibroblasts first proposed the A_{2B}AR as the subtype responsible for mediating adenosine's inhibitory actions on fetal calf serum-stimulated fibroblast proliferation (Dubey et al., 1997) and collagen and protein synthesis (Dubey et al., 1998). The role of the A_{2B}AR in adenosine-mediated anti-fibrotic signal transduction was later confirmed via antisense oligonucleotide A_{2B}AR silencing, which resulted in increased cell proliferation and basal collagen synthesis in cardiac fibroblasts (Dubey et al., 2001b). Similarly, A_{2B}AR overexpression had the opposite effect, significantly decreasing collagen and protein synthesis (Chen et al., 2004). The second messenger cAMP, has been shown to have a central role in inhibiting fibroblast and myofibroblast activity (Swaney et al., 2005; Lu et al., 2013). Accordingly, A_{2B}AR-mediated cAMP accumulation stimulated in fibroblasts by the non-selective AR agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) (Epperson et al., 2009) can reduce ANGII-stimulated collagen synthesis via an exchange factor directly activated by cAMP (Epac) and phosphoinositol-3 kinase (PI3K) dependent pathway (**Figure 1**) (Villarreal et al., 2009). In addition to effects on collagen synthesis, A_{2B}AR stimulation has been shown to decrease mRNA expression of pro-fibrotic gene markers including collagen I and connective tissue growth factor (CTGF) (Vecchio et al., 2016). Of specific importance to ARs, a positive feedback loop has been identified whereby β-adrenoceptor-stimulated cAMP can be secreted by fibroblasts or cardiac myocytes and metabolized in the extracellular space to adenosine to activate A_2ARs, thus exerting further inhibitory effects on fibroblast growth and function (Dubey et al., 2001a; Sassi et al., 2014).

Commensurate with the *in vitro* findings, an *in vivo* study in rats demonstrated chronic administration of the stable adenosine analog, 2-chloroadenosine (CADO) or the adenosine uptake inhibitor, dipyridamole, initiated 1 week after permanent ligation of the left anterior descending (LAD) coronary artery, protected against cardiac remodeling and reduced markers of fibrosis such as collagen volume fraction and matrix metalloproteinase gene expression (Wakeno et al., 2006). The effects of CADO on fibrotic and haemodynamic parameters were abolished in the presence of the selective A_{2B}AR antagonist MRS1754, but not selective antagonists for the other AR subtypes (Wakeno et al., 2006). Together, these studies suggest a salutary effect of A_{2B}AR activation on cardiac fibrosis, an effect which may be lost upon

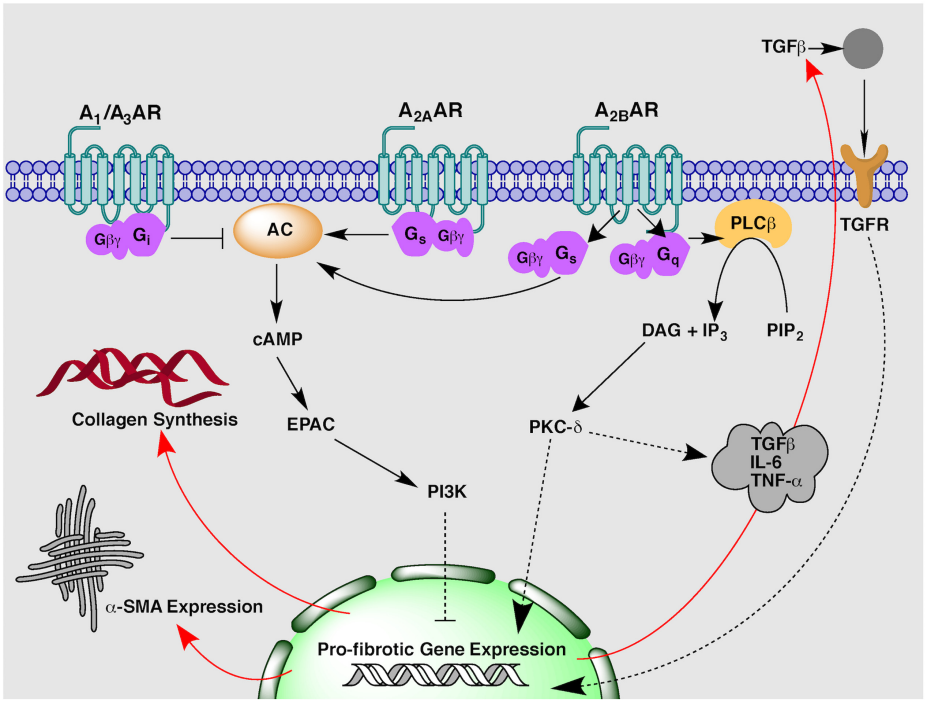

FIGURE 1 | An overview of proposed adenosine receptor-mediated intracellular signaling pathways implicated in the regulation of cardiac fibrosis.

A_{2B}AR downregulation as observed in hearts taken from human patients with chronic heart failure (Asakura et al., 2007).

A_{2B}AR-MEDIATED PRO-FIBROTIC SIGNAL TRANSDUCTION

While the majority of *in vitro* studies have identified an anti-fibrotic role for the A_{2B}AR, recent studies have demonstrated A_{2B}AR blockade appears to be beneficial within *in vivo* models of cardiac remodeling and fibrosis. In an *in vivo* mouse model of MI involving permanent coronary artery ligation, chronic administration of a novel, highly selective A_{2B}AR antagonist, GS-6201, significantly reduced cardiac enlargement and dysfunction compared to vehicle-treated mice (Toldo et al., 2012). Similarly in an *in vivo* rat myocardial ischaemia-reperfusion model, GS-6201 improved ejection fraction and decreased fibrosis in the non-infarct and border zones with the greatest effect observed when GS-6201 was given 1 week rather 1 day after MI (Zhang et al., 2014). A pro-fibrotic role for the A_{2B}AR has been supported by a study in A_{2B}AR knock-out ($A_{2B}AR^{-/-}$) mice that demonstrate the A_{2B}AR contributes to post-infarction heart failure (Maas et al., 2008). $A_{2B}AR^{-/-}$ mice had improved end diastolic pressure and reduced interstitial fibrosis when compared to wild-type mice 8 weeks after permanent left coronary ligation. Systolic blood pressure and infarct size remained the same between knock-out and wild-type animals suggesting the A_{2B}AR contributes to heart failure pathology via post-infarction remodeling and reactive fibrosis rather than acute cardioprotection (Maas et al., 2008). The mechanism underlying the pro-fibrotic activity of the A_{2B}AR may involve the pro-inflammatory effects mediated by this AR subtype. Blockade of the A_{2B}AR inhibits caspase-1 activity and leukocyte infiltrate (Toldo et al., 2012), and attenuates secretion of pro-fibrotic and pro-inflammatory mediators such as TGFβ, tissue necrosis factor α (TNF-α) and interleukin-6 (IL-6) post-MI via a PKC-δ pathway (**Figure 1**) (Feng et al., 2009; Toldo et al., 2012; Zhang et al., 2014). A pro-inflammatory role of the A_{2B}AR is reported by studies in other organ systems, in particular the lung where elevated adenosine concentrations and A_{2B}AR activity promotes chronic fibrosis and inflammation in asthma and chronic obstructive pulmonary disease (Sun, 2006; Chan and Cronstein, 2009; Zhou et al., 2009; Karmouty-Quintana et al., 2013). Given the inflammatory response is intricately linked

to the regulation of tissue fibrosis, it is perhaps unsurprising therefore, that the A_{2B}AR has been implicated as a promoter of cardiac fibrosis *in vivo* (Ham and Rees, 2008; Kong et al., 2013; Stuart et al., 2016).

A_1AR MODULATION OF CARDIAC FIBROSIS

The protective role of A_1AR activation in cardiac remodeling appears to be largely attributed to the beneficial effects on cardiomyocyte hypertrophy rather than effects on fibrosis (Liao et al., 2003; Sassi et al., 2014; Chuo et al., 2016). A study using a non-selective adenosine analog (CADO) in mice subject to 4 weeks of chronic pressure overload via transverse aortic constriction (TAC), demonstrated reduced myocardial and perivascular fibrosis and hypertrophy compared to saline-treated mice (Liao et al., 2003). Attenuation of myocardial hypertrophy was A_1AR-mediated, as the anti-hypertrophic effects were reversed in the presence of an A_1AR-selective antagonist. As similar antagonist studies were not reported for measures of cardiac fibrosis (Liao et al., 2003), it cannot be ruled out that the anti-fibrotic effects were mediated by another AR subtype, in particular the A_{2B}AR. However, recent studies using more A_1AR-selective agonists do suggest an involvement of the A_1AR in cardiac fibrosis. A study of heart failure in dogs demonstrated capadenoson, an A_1AR partial agonist, decreased interstitial fibrosis (Sabbah et al., 2013). Similarly, activation of the A_1AR with a selective agonist N^6-cyclopentyladenosine (CPA), attenuated left ventricular collagen content and markers of fibrosis in response to α_1-adrenergic stimulation *in vivo* (Puhl et al., 2016).

Activation of the A_1AR has been recognized as central to the acute cardioprotective actions of adenosine (McIntosh and Lasley, 2012; Headrick et al., 2013). In agreement, overexpression of the A_1AR protects mice against acute ischaemic events, with cardiac infarct size markedly reduced in transgenic compared to wild-type animals (Yang et al., 2002). Paradoxically, however, chronic A_1AR cardiac overexpression in older mice (20 weeks) has been associated with enhanced baseline cardiac fibrosis and dilated cardiomyopathy (Funakoshi et al., 2006). Additionally, a study investigating myocardial fibrosis secondary to chronic renal failure demonstrated that an A_1AR-selective antagonist, SLV320, normalized cardiac collagen I and III content in the hearts of rats that had undergone a nephrectomy (Kalk et al., 2007). These studies may suggest chronic A_1AR stimulation reduces the cardiac resistance to non-ischaemic stress and may promote fibrosis, however, the conflicting evidence highlights the need for further studies to fully elucidate the role of this AR subtype in cardiac fibrosis.

A_{2A}AR MODULATION OF CARDIAC FIBROSIS

Separating the contribution of A_{2B}AR-mediated fibrotic signaling from that of A_{2A}AR activation has been difficult owing to the paucity of early subtype selective agonists and antagonists. Genetic alteration of the A_{2A}AR demonstrated that cardiac-specific overexpression of the A_{2A}AR in mice was protective against pressure-induced heart failure, attenuating fibrosis and improving cardiac function (Hamad et al., 2012). A more recent study demonstrated high A_{2A}AR expression in mouse cardiac fibroblasts stimulated the accumulation of the anti-fibrotic second messenger cAMP (Sassi et al., 2014), though perhaps to a lesser extent than the A_{2B}AR (Epperson et al., 2009). Combined with the known anti-inflammatory actions of the A_{2A}AR in the heart (Linden, 2001; Haskó et al., 2008), there is certainly valid grounds to suggest that A_{2A}AR signaling would attenuate cardiac fibrosis. However, further work is needed to clarify the exact role of A_{2A}AR, as stimulation of this receptor subtype has also been demonstrated to have pro-fibrotic effects in other organs such as the liver and skin (Chan et al., 2006a,b; Perez-Aso et al., 2014).

A_3AR MODULATION OF CARDIAC FIBROSIS

Comparatively few studies have investigated the role of the A_3AR in cardiac fibrosis, which is unsurprising given early studies examining the A_3AR (and A_1AR) expressed on isolated rat cardiac fibroblasts suggested these receptors to be of lesser functional importance than the A_2ARs (Chen et al., 2004). The A_3AR was investigated for its involvement in protecting against maladaptive cardiac hypertrophy and fibrosis on the basis that ecto-5′-nucleotidase (CD73; catalyzes the conversion of extracellular AMP to adenosine) deficiency exacerbated myocardial hypertrophy and heart failure in TAC mice (Xu et al., 2008). Contrary to hypothesis, A_3AR knock-out mice actually had reduced left ventricular hypertrophy, fibrosis and dysfunction after 5 weeks of TAC compared to wild-type animals. There was no effect of A_3AR deletion on parameters in the unstressed heart, suggesting the A_3AR has a deleterious role in cardiac fibrosis only in response to chronic pressure overload (Lu et al., 2008). In agreement, a recent study using a uninephrectomy and high salt-induced model of hypertension in mice, demonstrated that genetic abrogation of the A_3AR resulted in significantly less cardiac hypertrophy and fibrosis compared to wild-type animals (Yang et al., 2016). These studies suggest A_3AR antagonism may be a valid therapeutic approach to prevent chronic pressure overload-hypertrophy and fibrosis, however, further studies are warranted.

CONCLUSION AND FUTURE DIRECTIONS

Cardiac fibrosis is an important determinant of left ventricular dysfunction and remodeling following MI and is a hallmark of heart failure pathology, which is associated with an extremely high rate of mortality (See et al., 2005; Segura et al., 2012). It is therefore crucial to find new therapeutic approaches to prevent and ideally reverse underlying cardiac fibrosis in order

to modify the disease progression of heart failure. Purinergic signaling downstream of AR activation represents one such novel strategy to influence fibrosis homeostasis, however, much work is still needed to clarify the exact role of the receptor subtypes involved. A central question that remains is how the same receptor subtype can have both pro- and anti-fibrotic activity. The opposing effects as outlined in this review, may reflect differences in underlying disease pathology due to the type and duration of cardiac insult; whereby AR activation appears to be largely anti-fibrotic in acute ischaemic events but potentially pro-fibrotic under conditions of chronic myocardial stress. This supposition is supported by studies of adenosine's involvement in fibrosis of other organ systems (Karmouty-Quintana et al., 2013). In the lung, A_{2B}AR stimulation is protective in acute-bleomycin-induced lung injury but actually promotes fibrosis in chronic models of lung disease (Zhou et al., 2009, 2011). Similarly in the kidney, A_{2B}AR activation is beneficial in attenuating acute kidney injury (Grenz et al., 2012) but prolonged A_{2B}AR signaling increases interstitial fibrosis and collagen deposition in renal tissue (Roberts et al., 2014a,b). The exact mechanism behind these paradoxical effects requires further elucidation, but may reflect changes in differential receptor coupling with changes in cellular background as the disease progresses. Certainly, this idea is readily foreseeable for the A_{2B}AR with its high degree of plasticity and ability to couple to multiple G proteins and intracellular signaling cascades (**Figure 1**) (Cohen et al., 2010). In addition, it should be noted a great deal of our understanding of adenosine's role in cardiac fibrosis, in particular downstream of A_{2B}AR, has come from *in vitro* studies. This may not reflect the true course of disease progression *in vivo* due to the exclusion of the inflammatory response and loss of organ complexity including cross-talk with other cell types. Therefore, while AR signaling appears to be a promising target in cardiac fibrosis, further studies are needed to fully appreciate the potential of AR therapeutics in heart failure and underlying fibrosis.

AUTHOR CONTRIBUTIONS

EV drafted the manuscript. PW and LM made substantial contribution to the writing. EV, PW, and LM provided critical revision of the manuscript and approved it for publication.

FUNDING

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia (Project Grant ID APP1084487) and the Australian Research Council (ARC; ID DE130100117). EAV holds an Australian Government Research Training Program Scholarship and an Australian Cancer Therapeutics scholarship.

REFERENCES

Asakura, M., Asanuma, H., Kim, J., Liao, Y., Nakamaru, K., Fujita, M., et al. (2007). Impact of adenosine receptor signaling and metabolism on pathophysiology in patients with chronic heart failure. *Hypertens. Res.* 30, 781–787. doi: 10.1291/hypres.30.781

Auchampach, J. A., and Bolli, R. (1999). Adenosine receptor subtypes in the heart: therapeutic opportunities and challenges. *Am. J. Physiol.* 276, H1113–H1116.

Camelliti, P., Green, C. R., LeGrice, I., and Kohl, P. (2004). Fibroblast network in rabbit sinoatrial node: structural and functional identification of homogeneous and heterogeneous cell coupling. *Circ. Res.* 94, 828–835. doi: 10.1161/01.RES.0000122382.19400.14

Chan, E. S. L., and Cronstein, B. N. (2009). Adenosine in fibrosis. *Mod. Rheumatol.* 20, 114–122. doi: 10.1007/s10165-009-0251-4

Chan, E. S. L., Fernandez, P., Merchant, A. A., Montesinos, M. C., Trzaska, S., Desai, A., et al. (2006a). Adenosine A2A receptors in diffuse dermal fibrosis: pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. *Arthritis Rheum.* 54, 2632–2642. doi: 10.1002/art.21974

Chan, E. S. L., Montesinos, M. C., Fernandez, P., Desai, A., Delano, D. L., Yee, H., et al. (2006b). Adenosine A2A receptors play a role in the pathogenesis of hepatic cirrhosis. *Br. J. Pharmacol.* 148, 1144–1155. doi: 10.1038/sj.bjp.0706812

Chen, W., and Frangogiannis, N. G. (2013). Fibroblasts in post-infarction inflammation and cardiac repair. *Biochim. Biophys. Acta* 1833, 945–953. doi: 10.1016/j.bbamcr.2012.08.023

Chen, Y., Epperson, S., Makhsudova, L., Ito, B., Suarez, J., Dillmann, W., et al. (2004). Functional effects of enhancing or silencing adenosine A2B receptors in cardiac fibroblasts. *Am. J. Physiol. Heart Circ. Physiol.* 287, H2478–H2486. doi: 10.1152/ajpheart.00217.2004

Chuo, C. H., Devine, S. M., Scammells, P. J., Krum, H., Christopoulos, A., May, L. T., et al. (2016). VCP746, a novel A1 adenosine receptor biased agonist, reduces hypertrophy in a rat neonatal cardiac myocyte model. *Clin. Exp. Pharmacol. Physiol.* 43, 976–982. doi: 10.1111/1440-1681.12616

Cohen, M. V., Yang, X., and Downey, J. M. (2010). A2B adenosine receptors can change their spots. *Br. J. Pharmacol.* 159, 1595–1597. doi: 10.1111/j.1476-5381.2010.00668.x

Cronstein, B. N. (2011). Adenosine receptors and fibrosis: a translational review. *F1000 Biol. Rep.* 3:21. doi: 10.3410/B3-21

Dubey, R. K., Gillespie, D. G., and Jackson, E. K. (1998). Adenosine inhibits collagen and protein synthesis in cardiac fibroblasts: role of A2B receptors. *Hypertension* 31, 943–948. doi: 10.1161/01.HYP.31.4.943

Dubey, R. K., Gillespie, D. G., Mi, Z., and Jackson, E. K. (1997). Exogenous and endogenous adenosine inhibits fetal calf serum–induced growth of rat cardiac fibroblasts: role of A2B receptors. *Circulation* 96, 2656–2666. doi: 10.1161/01.CIR.96.8.2656

Dubey, R. K., Gillespie, D. G., Mi, Z., and Jackson, E. K. (2001a). Endogenous cyclic AMP-adenosine pathway regulates cardiac fibroblast growth. *Hypertension* 37, 1095–1100. doi: 10.1161/01.HYP.37.4.1095

Dubey, R. K., Gillespie, D. G., Zacharia, L. C., Mi, Z., and Jackson, E. K. (2001b). A2B receptors mediate the antimitogenic effects of adenosine in cardiac fibroblasts. *Hypertension* 37, 716–721. doi: 10.1161/01.HYP.37.2.716

Epperson, S. A., Brunton, L. L., Ramirez-Sanchez, I., and Villarreal, F. (2009). Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts. *Am. J. Physiol. Cell Physiol.* 296, C1171–C1177. doi: 10.1152/ajpcell.00290.2008

Feng, W., Song, Y., Chen, C., Lu, Z. Z., and Zhang, Y. (2009). Stimulation of adenosine A2B receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-δ-P38 signalling pathway. *Br. J. Pharmacol.* 159, 1598–1607. doi: 10.1111/j.1476-5381.2009.00558.x

Feoktistov, I., and Biaggioni, I. (1997). Adenosine A2B receptors. *Pharmacol. Rev.* 49, 381–402.

Ferrari, D., Gambari, R., Idzko, M., Muller, T., Albanesi, C., Pastore, S., et al. (2016). Purinergic signaling in scarring. *FASEB J.* 30, 3–12. doi: 10.1096/fj.15-274563

Forman, M. B., Stone, G. W., and Jackson, E. K. (2006). Role of adenosine as adjunctive therapy in acute myocardial infarction. *Cardiovasc. Drug Rev.* 24, 116–147. doi: 10.1111/j.1527-3466.2006.00116.x

Fredholm, B. B., IJzerman, A. P., Jacobson, K. A., Klotz, K. N., and Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 53, 527–552.

Funakoshi, H., Chan, T. O., Good, J. C., Libonati, J. R., Piuhola, J., Chen, X., et al. (2006). Regulated overexpression of the A1-adenosine receptor in mice results in adverse but reversible changes in cardiac morphology and function. *Circulation* 114, 2240–2250. doi: 10.1161/CIRCULATIONAHA.106.620211

Grenz, A., Kim, J. H., Bauerle, J. D., Tak, E., Eltzschig, H. K., and Clambey, E. T. (2012). Adora2B adenosine receptor signaling protects during acute kidney injury via inhibition of neutrophil-dependent TNF-α release. *J. Immunol.* 189, 4566–4573. doi: 10.4049/jimmunol.1201651

Ham, J., and Rees, D. A. (2008). The adenosine A2B receptor: its role in inflammation. *Endocr. Metab. Immune Disord. Drug Targets* 8, 244–254. doi: 10.2174/187153008786848303

Hamad, E. A., Zhu, W., Chan, T. O., Myers, V., Gao, E., Li, X., et al. (2012). Cardioprotection of controlled and cardiac-specific over-expression of A2A-adenosine receptor in the pressure overload. *PLoS ONE* 7:e39919. doi: 10.1371/journal.pone.0039919.t002

Haskó, G., Linden, J., Cronstein, B., and Pacher, P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat. Rev. Drug Discov.* 7, 759–770. doi: 10.1038/nrd2638

Headrick, J. P., Ashton, K. J., Rose'Meyer, R. B., and Peart, J. N. (2013). Cardiovascular adenosine receptors: expression, actions and interactions. *Pharmacol. Ther.* 140, 92–111. doi: 10.1016/j.pharmthera.2013.06.002

Kalk, P., Eggert, B., Relle, K., Godes, M., Heiden, S., Sharkovska, Y., et al. (2007). The adenosine A1 receptor antagonist SLV320 reduces myocardial fibrosis in rats with 5/6 nephrectomy without affecting blood pressure. *Br. J. Pharmacol.* 151, 1025–1032. doi: 10.1038/sj.bjp.0707319

Karmouty-Quintana, H., Xia, Y., and Blackburn, M. R. (2013). Adenosine signaling during acute and chronic disease states. *J Mol. Med.* 91, 173–181. doi: 10.1007/s00109-013-0997-1

Kong, P., Christia, P., and Frangogiannis, N. G. (2013). The pathogenesis of cardiac fibrosis. *Cell. Mol. Life Sci.* 71, 549–574. doi: 10.1007/s00018-013-1349-6

Kopecky, S. L., Aviles, R. J., Bell, M. R., Lobl, J. K., Tipping, D., Frommell, G., et al. (2003). A randomized, double-blinded, placebo-controlled, dose-ranging study measuring the effect of an adenosine agonist on infarct size reduction in patients undergoing primary percutaneous transluminal coronary angioplasty: the ADMIRE (AmP579 delivery for myocardial infarction REduction) study. *Am. Heart J.* 146, 146–152. doi: 10.1016/S0002-8703(03)00172-8

Leask, A. (2007). TGFβ, cardiac fibroblasts, and the fibrotic response. *Cardiovasc. Res.* 74, 207–212. doi: 10.1016/j.cardiores.2006.07.012

Liao, Y., Liao, Y., Takashima, S., Asano, Y., Asakura, M., Ogai, A., et al. (2003). Activation of adenosine A1 receptor attenuates cardiac hypertrophy and prevents heart failure in murine left ventricular pressure-overload model. *Circ. Res.* 93, 759–766. doi: 10.1161/01.RES.0000094744.88220.62

Linden, J. (2001). Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu. Rev. Pharmacol. Toxicol.* 41, 775–787. doi: 10.1146/annurev.pharmtox.41.1.775

Linden, J., Thai, T., Figler, H., Jin, X., and Robeva, A. S. (1999). Characterization of human A2B adenosine receptors: radioligand binding, western blotting, and coupling to Gq in human embryonic kidney 293 cells and HMC-1 mast cells. *Mol. Pharmacol.* 56, 705–713.

Lu, D., Aroonsakool, N., Yokoyama, U., Patel, H. H., and Insel, P. A. (2013). Increase in cellular cyclic AMP concentrations reverses the profibrogenic phenotype of cardiac myofibroblasts: a novel therapeutic approach for cardiac fibrosis. *Mol. Pharmacol.* 84, 787–793. doi: 10.1124/mol.113.087742

Lu, D., and Insel, P. A. (2014). Cellular mechanisms of tissue fibrosis. 6. Purinergic signaling and response in fibroblasts and tissue fibrosis. *Am. J. Physiol. Cell Physiol.* 306, C779–C788. doi: 10.1152/ajpcell.00381.2013

Lu, Z., Fassett, J., Xu, X., Hu, X., Zhu, G., French, J., et al. (2008). Adenosine A3 receptor deficiency exerts unanticipated protective effects on the pressure-overloaded left ventricle. *Circulation* 118, 1713–1721. doi: 10.1161/CIRCULATIONAHA.108.788307

Maas, J. E., Koupenova, M., Ravid, K., and Auchampach, J. A. (2008). Abstract 4831: the A2B adenosine receptor contributes to post-infarction heart failure. *Circulation* 118, S_946.

McIntosh, V. J., and Lasley, R. D. (2012). Adenosine receptor-mediated cardioprotection: are all 4 subtypes required or redundant? *J. Cardiovasc. Pharmacol. Ther.* 17, 21–33. doi: 10.1177/1074248410396877

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., et al. (2016). Heart disease and stroke statistics-2016 update: A report from the American Heart Association. *Circulation* 133, e38–360. doi: 10.1161/CIR.0000000000000350

Mundell, S. J., and Benovic, J. L. (2000). Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. *J. Biol. Chem.* 275, 12900–12908. doi: 10.1074/jbc.275.17.12900

Newby, A. C. (1984). Adenosine and the concept of "retaliatory metabolites.". *Trends Biochem. Sci.* 9, 42–44. doi: 10.1016/0968-0004(84)90176-2

Novitskaya, T., Chepurko, E., Covarrubias, R., Novitskiy, S., Ryzhov, S. V., Feoktistov, I., et al. (2016). Extracellular nucleotide regulation and signaling in cardiac fibrosis. *J. Mol. Cell. Cardiol.* 93, 47–56. doi: 10.1016/j.yjmcc.2016.02.010

Paolocci, N., Tavazzi, B., Biondi, R., Gluzband, Y. A., Amorini, A. M., Tocchetti, C. G., et al. (2006). Metalloproteinase inhibitor counters high-energy phosphate depletion and AMP deaminase activity enhancing ventricular diastolic compliance in subacute heart failure. *J. Pharmacol. Exp. Ther.* 317, 506–513. doi: 10.1124/jpet.105.099168

Peart, J. N., and Headrick, J. P. (2007). Adenosinergic cardioprotection: multiple receptors, multiple pathways. *Pharmacol. Ther.* 114, 208–221. doi: 10.1016/j.pharmthera.2007.02.004

Perez-Aso, M., Fernandez, P., Mediero, A., Chan, E. S., and Cronstein, B. N. (2014). Adenosine 2A receptor promotes collagen production by human fibroblasts via pathways involving cyclic AMP and AKT but independent of Smad2/3. *FASEB J.* 28, 802–812. doi: 10.1096/fj.13-241646

Petrov, V. V., Fagard, R. H., and Lijnen, P. J. (2002). Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension* 39, 258–263. doi: 10.1161/hy0202.103268

Porter, K. E., and Turner, N. A. (2009). Cardiac fibroblasts: at the heart of myocardial remodeling. *Pharmacol. Ther.* 123, 255–278. doi: 10.1016/j.pharmthera.2009.05.002

Puhl, S.-L., Kazakov, A., Müller, A., Fries, P., Wagner, D. R., Böhm, M., et al. (2016). A1 receptor activation attenuates cardiac hypertrophy and fibrosis in response to α1-adrenergic stimulation in vivo. *Br. J. Pharmacol.* 173, 88–102. doi: 10.1111/bph.13339

Roberts, V. S., Cowan, P. J., Alexander, S. I., Robson, S. C., and Dwyer, K. M. (2014a). The role of adenosine receptors A2A and A2B signaling in renal fibrosis. *Kidney Int.* 86, 685–692. doi: 10.1038/ki.2014.244

Roberts, V., Lu, B., Dwyer, K. M., and Cowan, P. J. (2014b). Adenosine receptor expression in the development of renal fibrosis following ischemic injury. *Transplant. Proc.* 46, 3257–3261. doi: 10.1016/j.transproceed.2014.09.151

Ross, A. M., Gibbons, R. J., Stone, G. W., Kloner, R. A., and Alexander, R. W. (2005). A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *J. Am. Coll. Cardiol.* 45, 1775–1780. doi: 10.1016/j.jacc.2005.02.061

Sabbah, H. N., Gupta, R. C., Kohli, S., Wang, M., Rastogi, S., Zhang, K., et al. (2013). Chronic therapy with a partial adenosine A1-receptor agonist improves left ventricular function and remodeling in dogs with advanced heart failure. *Circ. Heart Fail.* 6, 563–571. doi: 10.1161/CIRCHEARTFAILURE.112.000208

Sassi, Y., Ahles, A., Truong, D.-J. J., Baqi, Y., Lee, S.-Y., Husse, B., et al. (2014). Cardiac myocyte-secreted cAMP exerts paracrine action via adenosine receptor activation. *J. Clin. Invest.* 124, 5385–5397. doi: 10.1172/JCI74349DS1

Schnee, J. M., and Hsueh, W. A. (2000). Angiotensin II, adhesion, and cardiac fibrosis. *Cardiovasc. Res.* 46, 264–268. doi: 10.1016/S0008-6363(00)00044-4

See, F., Kompa, A., Martin, J., Lewis, D. A., and Krum, H. (2005). Fibrosis as a therapeutic target post-myocardial infarction. *Curr. Pharm. Des.* 11, 477–487. doi: 10.2174/1381612053382098

Segura, A. M., Frazier, O. H., and Buja, L. M. (2012). Fibrosis and heart failure. *Heart Fail. Rev.* 19, 173–185. doi: 10.1007/s10741-012-9365-4

Shyrock, J. C., and Belardinelli, L. (1997). Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am. J. Cardiol.* 79, 2–10. doi: 10.1016/S0002-9149(97)00256-7

Souders, C. A., Bowers, S. L. K., and Baudino, T. A. (2009). Cardiac fibroblast: the renaissance cell. *Circ. Res.* 105, 1164–1176. doi: 10.1161/CIRCRESAHA.109.209809

Stuart, S. D. F., De Jesus, N. M., Lindsey, M. L., and Ripplinger, C. M. (2016). The crossroads of inflammation, fibrosis, and arrhythmia following myocardial infarction. *J. Mol. Cell. Cardiol.* 91, 114–122. doi: 10.1016/j.yjmcc.2015.12.024

Sun, C. X. (2006). Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *J. Clin. Invest.* 116, 2173–2182. doi: 10.1172/JCI27303

Sun, Y., and Huang, P. (2016). Adenosine A2B receptor: from cell biology to human diseases. *Front. Chem.* 4:1329. doi: 10.4049/jimmunol.0900515

Swaney, J. S., Roth, D. M., Olson, E. R., Naugle, J. E., Meszaros, J. G., and Insel, P. A. (2005). Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.* 102, 437–442. doi: 10.1073/pnas.0408704102

Toldo, S., Zhong, H., Mezzaroma, E., Van Tassell, B. W., Kannan, H., Zeng, D., et al. (2012). GS-6201, a selective blocker of the A2B adenosine receptor, attenuates cardiac remodeling after acute myocardial infarction in the mouse. *J. Pharmacol. Exp. Ther.* 343, 587–595. doi: 10.1124/jpet.111.191288

Vecchio, E. A., Chuo, C. H., Baltos, J.-A., Ford, L., Scammells, P. J., Wang, B. H., et al. (2016). The hybrid molecule, VCP746, is a potent adenosine A2B receptor agonist that stimulates anti-fibrotic signalling. *Biochem. Pharmacol.* 117, 46–56. doi: 10.1016/j.bcp.2016.08.007

Villarreal, F., Epperson, S. A., Ramirez-Sanchez, I., Yamazaki, K. G., and Brunton, L. L. (2009). Regulation of cardiac fibroblast collagen synthesis by adenosine: roles for Epac and PI3K. *Am. J. Physiol. Cell Physiol.* 296, C1178–C1184. doi: 10.1152/ajpcell.00291.2008

Wakeno, M., Minamino, T., Seguchi, O., Okazaki, H., Tsukamoto, O., Okada, K. I., et al. (2006). Long-term stimulation of adenosine A2B receptors begun after myocardial infarction prevents cardiac remodeling in rats. *Circulation* 114, 1923–1932. doi: 10.1161/CIRCULATIONAHA.106.630087

Xu, X., Fassett, J., Hu, X., Zhu, G., Lu, Z., Li, Y., et al. (2008). Ecto-5′-nucleotidase deficiency exacerbates pressure-overload-induced left ventricular hypertrophy and dysfunction. *Hypertension* 51, 1557–1564. doi: 10.1161/HYPERTENSIONAHA.108.110833

Yang, T., Zollbrecht, C., Winerdal, M. E., Zhuge, Z., Zhang, X. M., Terrando, N., et al. (2016). Genetic abrogation of adenosine A3 receptor prevents uninephrectomy and high salt–induced hypertension. *J. Am. Heart Assoc.* 5, e003868. doi: 10.1161/JAHA.116.003868

Yang, Z., Cerniway, R. J., Byford, A. M., Berr, S. S., French, B. A., and Matherne, G. P. (2002). Cardiac overexpression of A1-adenosine receptor protects intact mice against myocardial infarction. *Am. J. Physiol. Heart Circ. Physiol.* 282, H949–H955. doi: 10.1152/ajpheart.00741.2001

Zhang, H., Zhong, H., Everett, T. H., Wilson, E., Chang, R., Zeng, D., et al. (2014). Blockade of A2B adenosine receptor reduces left ventricular dysfunction and ventricular arrhythmias 1 week after myocardial infarction in the rat model. *Heart Rhythm* 11, 101–109. doi: 10.1016/j.hrthm.2013.10.023

Zhou, Y., Schneider, D. J., and Blackburn, M. R. (2009). Adenosine signaling and the regulation of chronic lung disease. *Pharmacol. Ther.* 123, 105–116. doi: 10.1016/j.pharmthera.2009.04.003

Zhou, Y., Schneider, D. J., Morschl, E., Song, L., Pedroza, M., Karmouty-Quintana, H., et al. (2011). Distinct roles for the A2B adenosine receptor in acute and chronic stages of bleomycin-induced lung injury. *J. Immunol.* 186, 1097–1106. doi: 10.4049/jimmunol.1002907

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix 2:

Chapter 6 Supplemental Information

Imaging neonatal cardiac fibroblasts and myofibroblasts:

In order to assess our culturing protocol for NVCFs and to check for the conversion to a myofibroblast phenotype we employed immunofluorescent imaging.

Materials:

Primary and secondary antibodies were purchased from Abcam (Cambridge, UK). All other reagents were of analytical quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methods:

NVCFs grown in 96-well 1% gelatin-coated plates were maintained in serum free DMEM in the presence or absence of TGF-β1 (10 ng/mL) for 48 h prior to imaging. Media was removed and cells were twice washed with warm PBS. Cells were fixed with 4% PFA in PBS and incubated for 15 min at room temperature. Fixing media was aspirated and cells were washed with room temperature PBS three times prior to the addition of the blocking buffer containing PBS/0.1% sodium azide, 5% normal horse serum and 0.1% saponin and allowed to incubate for 1 h at room temperature. Primary antibodies against α-SMA (myofibroblast marker) and vimentin (fibroblast marker) were diluted (1:200) in blocking buffer and added to the cells for 1 h at room temperature. Primary antibodies were aspirated and cells were washed three times with room temperature PBS, allowing cells to stand in PBS for 10 min in between each wash. Secondary antibodies were diluted (1:500) in PBS/0.1% sodium azide and incubated for 1 h at room temperature in diminished light conditions. Cells were washed with PBS/0.1% sodium azide prior to addition of Hoechst stain (1:1000 dilution) and incubation for a further 10 min. Cells were subsequently twice washed with PBS/0.1% sodium azide and imaged on the Operetta High-Content Imaging System (PerkinElmer; Waltham, MA) with analysis of percentage α-SMA and vimentin double positive stained cells.

Results:

The fibroblast marker, vimentin stained up the majority of cells indicating a low percentage of cross contamination with non-fibroblast cells such as cardiomyocytes (Fig. 1A & D).The specific myofibroblast marker, α-SMA stained up close to 100% of cells in both untreated and +TGF-β1 conditions (Fig. 1B & E). Merged images showed that even cells not stained for vimentin were stained for α-SMA, which possibly suggests non-specific labelling (Fig. 1C & F). Optimised culturing conditions, which used cells at passage zero, plating onto pre-coated gelatin plates and serum starving for 48 h in order to minimise the spontaneous conversion to myofibroblasts, were unable to reduce α-SMA staining. Future studies should investigate phenotypic changes such as alterations in cell size and fibre arrangement, different dilutions and brand of antibody and confirmation of α-SMA content by RT-PCR.

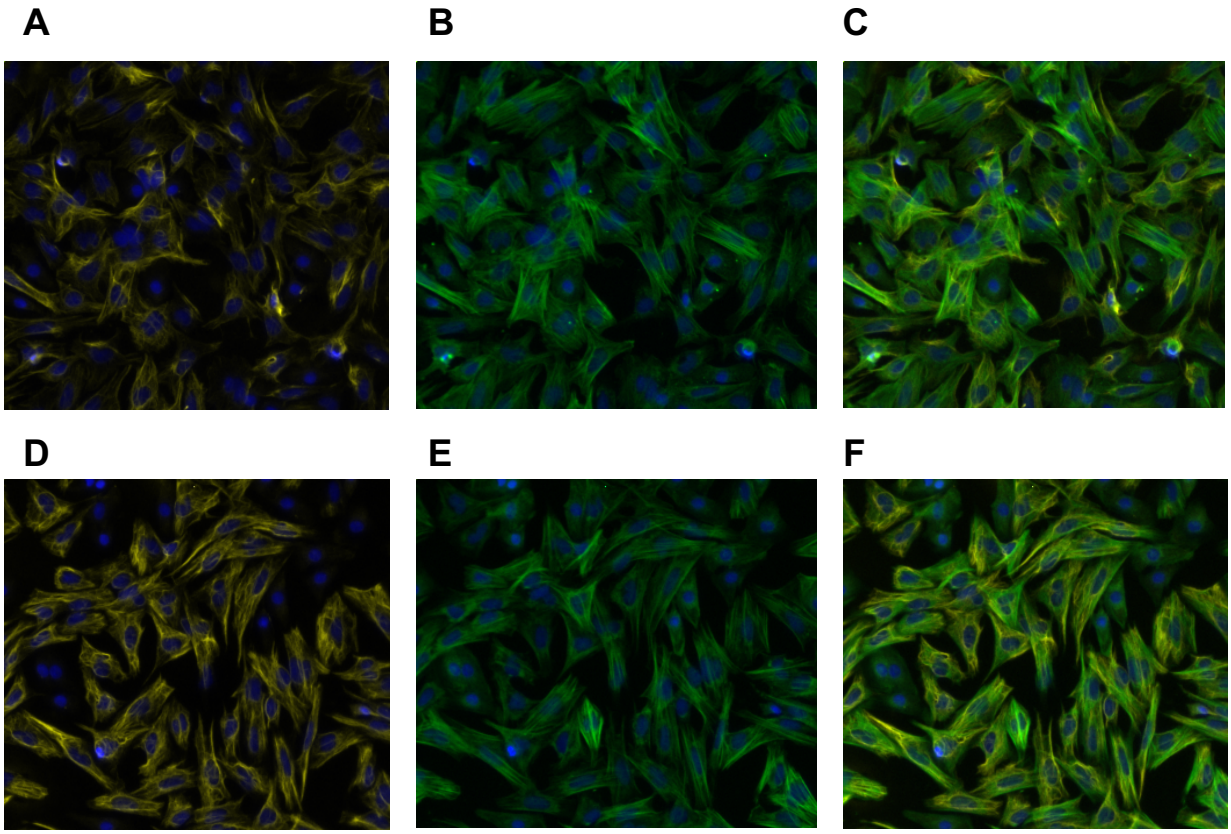

Fig. 1. Representative images of NVCFs in the absence (A,B,C) or presence of TGF-β1 (10 ng/mL for 48 h; D,E,F). Nuclear satin (Hoechst; blue), fibroblast marker (vimentin; yellow), myofibroblast marker (α-SMA; green). C & F are merged images.