# Proprotein convertase 6: role in embryo implantation and clinical implications

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# Summary

To enable embryo implantation for the establishment of a pregnancy, the uterus must acquire endometrial epithelial receptivity and undergo stromal differentiation known as decidualisation. Proprotein convertase 6 (PC6) is a member of the PC family of proteases that are critical in controlling the post-translational activation of a multitude of precursor proteins through limited proteolysis. Our group has established that uterine PC6 is critical for embryo implantation, as it is essential for endometrial epithelial receptivity (in women) and decidualisation (in both women and mice). In addition, PCs including PC6 are also involved in HIV infectivity, and inhibition of PC activity inhibits HIV infections in cells.

We hypothesised that targeting PC6 in the female reproductive tract (vagina, cervix and uterus) could provide a novel approach to the development of non-hormonal and women-controlled contraceptives that could also protect women from HIV infections. The vagina is the entry site of sexually transmitted HIV in women, and transmission can be stopped by vaginal application of anti-HIV drugs. Therefore, vaginal delivery of PC6 inhibitors presents the ideal route of administration to achieve this dual protection. A key requirement to prove this concept is to develop potent and vaginally deliverable PC6 inhibitors, and to test their efficacy in appropriate animal models. This thesis examined the contraceptive potential of two types of PC6 inhibitors, (i) peptide-based and (ii) small molecule compounds.

Work described in Chapter 2 examined Poly R (one of the most potent peptidic PC inhibitors) and its PEGylated derivatives. The study revealed that C-terminally PEGylated Poly Rs had comparable potency to the parental Poly R, but with enhanced vaginal absorption and penetration across the vaginal epithelium. However, only one of the C-PEGylated Poly R derivatives, C-30k-PEG Poly R could reach the uterus at a relatively low level following vaginal administration in mice. This was contradictory to expectation, because it has been widely

demonstrated in women that vaginally administered drugs preferentially localise to the uterus, a phenomenon called the “first uterine pass effect”. In the absence of a better animal model, the study continued to test C-30k-PEG Poly R in mice for inhibition of embryo implantation following vaginal administration (Chapter 3). Overall, inhibition of implantation was achieved in 71% of mice (24% complete and 47% partial inhibitions). These encouraging results strongly suggest that inhibition could be much higher if efficacy of inhibitor delivery could be improved in a more appropriate animal model.

To assist my research and to fill a knowledge gap in the field, a simple, reliable and high-throughput *in vitro* model for human embryo attachment was established (Chapter 4). This model was proven to be effective and reproducible for the study of the attachment of human trophoblast spheroids to endometrial epithelial cells. This publication has attracted attention from other researchers, highlighting its significant contribution to the field.

Chapter 5 described studies on a class of five small molecule compounds that were previously reported as potent inhibitors of furin (another PC member). The work revealed that all five compounds potently inhibited PC6 activity *in vitro*. However, only one (compound 1o) was able to inhibit PC6-dependent cellular processes (decidualisation and epithelial receptivity) in human cell-based models. Compound 1o is unique among the five compounds as it is the most lipophilic. These findings identified compound 1o as a potent small molecule PC6 inhibitor with pharmaceutical potential to inhibit embryo implantation. Future *in vivo* studies need to evaluate whether compound 1o is superior to C-PEGylated Poly Rs as a vaginally deliverable PC6 inhibitor for the dual protection.

In summary, this thesis established an important foundation for future studies to prove the hypothesis that inhibiting PC6 is a viable strategy for the development of non-hormonal and women-controlled contraceptives.

# General declaration

**Monash University, Monash Research Graduate School**

**Declaration for thesis based or partially based on conjointly published or unpublished work**

In accordance with Monash University Regulation 17/Doctor of Philosophy and Master of Philosophy (Mphill) regulations the following declarations are made:

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 2 original papers published in peer-reviewed journals and 1 submitted manuscript. The core theme of the thesis is the potential of proprotein convertase 6 inhibitors as contraceptives. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Prince Henry's Institute of Medical Research through the Department of Biochemistry and Molecular Biology under the supervision of Associate Professor Guiying Nie.

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, my contribution to the work involved the following:

| Thesis chapter | Publication title | Publication status | Nature and extend of candidate's contribution |
|---|---|---|---|
| 2 | PEGylation of a proprotein convertase peptide inhibitor for vaginal route of drug delivery: *in vitro* bioactivity, stability and *in vivo* pharmacokinetics | Published | (75% contribution)<br>Performed biochemical assays, stability test and all mice work, conducted interpretation of all data, statistical analysis and manuscript writing. |
| 4 | A high-throughput *in vitro* model of human embryo attachment | Published | (85% contribution)<br>Performed all experiments, conducted optimisation, interpretation of all data, statistical analysis and manuscript writing. |
| 5 | Small molecule proprotein convertase inhibitors for inhibition of embryo implantation | Submitted | (70% contribution)<br>Performed biochemical assays, spheroid attachment assay, conducted interpretation of data, statistical analysis and manuscript writing. |

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: …….

Date: …….

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# Publications arising from this thesis

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**Huiting Ho**, Harmeet Singh, Mohamad M. Aljofan and Guiying Nie (2012). **A high-throughput *in vitro* model of human embryo attachment**. *Fertil Steril,* 97, 974-8.

**Huiting Ho**, Harmeet Singh, Sophea Heng, Tracy L. Nero, Michael W. Parker, Alan T. Johnson, Guan-Sheng Jiao and Guiying Nie (2013). **Small molecule proprotein convertase inhibitiors for inhibition of embryo implantation.** *Submitted to PLoSONE.*

# Abstracts to scientific meetings

**Huiting Ho**, Tracy L. Nero, Harmeet Singh, Michael W. Parker and Guiying Nie (2012). **Modification of a peptide inhibitor of proprotein convertase for use as a vaginally-deliverable contraceptive with added benefits.** *45th Annual Meeting of The Society for the Study of Reproduction (SSR) (Pennsylvania, USA).* Oral presentation. Enabled by Harold Mitchell Foundation Postgraduate Travel Fellowship Award.

**Huiting Ho**, Harmeet Singh, Mohamad M. Aljofan and Guiying Nie (2012). **A high throughput *in vitro* model of human embryo attachment.** *The Annual Endocrine Society of Australia (ESA) Seminar Meeting (Victoria, Australia).* Oral presentation.

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**Huiting Ho**, Harmeet Singh, Mohamad M. Aljofan and Guiying Nie (2011). **A reliable and high throughput *in vitro* model of human implantation using trophoblast spheroid attachment to endometrial epithelial cells.** *The Society for Reproductive Biology (SRB) (Cairns, Australia)*. Poster.

**Huiting Ho**, Tracy L. Nero, Harmeet Singh, Michael W. Parker and Guiying Nie (2011). **Modifications to a Proprotein Convertase 6 inhibitor to improve pharmacokinetic properties for *in vivo* use.** *World Congress on Reproductive Biology (Cairns, Australia).* Poster.

**Huiting Ho**, Tracy L. Nero, Harmeet Singh, Michael W. Parker and Guiying Nie (2011). **Modifications to a Proprotein Convertase 6 inhibitor to improve pharmacokinetic properties for *in vivo* use.** *Southern Health Research Week (Melbourne, Australia).* Poster.

**Huiting Ho**, Guiying Nie (2010). **Development of potent and stable PC6 inhibitors to block embryo implantation for female contraception and prevention of HIV.** *The Society for Reproductive Biology (SRB) (Sydney, Australia). Oral presentation – Australia and New Zealand Placental Research Association (ANZPRA) Emerging Investigator award finalist.*

**Huiting Ho**, Guiying Nie (2010). **Targeting PC6: A new strategy for female contraception and preventing HIV**. *Australian Society for Medical Research (ASMR) 1st Victorian Student Research Symposium (Melbourne, Australia).* Oral Presentation.

# List of abbreviations

| | |
|---|---|
| AMC | 7-amino-4-methylcoumarin |
| ATCC | American Type Culture Collection |
| AUC | area under the curve |
| AZA | 5-Aza-2′-deoxycytidine |
| BLT | bone marrow, liver, thymus |
| BMP2 | bone marphogenetic protein 2 |
| BSA | bovine serum albumin |
| cAMP | 8-bromoadenosine 3′:5′ cyclic monophosphate |
| CL | corpora lutea |
| $C_{max}$ | maximum concentration |
| CPPs | cell penetrating peptides |
| cs-FCS | charcoal stripped fetal calf serum |
| DAPI | 4′,6-diamidino-2-phenylindole |
| dec-RVKR-cmk | decanoyl-Arg-Val-Lys-Arg-chloromethylketone |
| DMEM | Dulbecco's Modified Eagle Medium |
| E2 | estradiol |
| EBP50 | ezrin-radixinmoesin binding phosphoprotein 50 |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immuno sorbent assay |
| Em | emission monochromator |
| Ex | excitation monochromator |

| | |
|---|---|
| F12 | Ham's F- 12 |
| FAM | 5(6)-carboxyfluoresceine |
| FCS | fetal calf serum |
| FDA | Food and Drug Administration |
| FSH | follicle-stimulating hormone |
| GRF | growth hormone-releasing factor |
| HE | haematoxylin and eosin |
| HESC | human endometrial stromal cell |
| HIV | human immunodeficiency virus |
| HPV | human papillomavirus |
| HRP | horseradish peroxidase |
| HSPG | heparan sulfate proteoglycan |
| i.m. | intramuscular |
| IUD | intrauterine devices |
| IVF | *in vitro* fertilisation |
| IVR | intravaginal rings |
| Ki | inhibition constant |
| LIF | leukemia inhibitory factor |
| LPC | lymphoma proprotein convertase |
| LPC | lysophosphatidylcholine |
| LPG | lysophosphatidyglycerol |
| LPH | lipotropin hormone |
| MEM | minimal essential medium |

| | |
|---|---|
| MPA | medroxy-progesterone acetate |
| MW | molecular weight |
| NARC | neural apoptosis regulated convertase |
| NEC | neuroendocrine convertase |
| O.C.T | optimum cutting temperature |
| PACE | paired basic amino acid residue cleaving enzyme |
| PBS | phosphate Buffered Saline |
| PC | proprotein convertase |
| PCC | palmitoylcarnitine chloride |
| PEG | polyethylene glycol |
| PEGLA | PEGylated LIF antagonist |
| PRL | prolactin |
| PS-SPCLs | positional scanning of synthetic peptide combinatorial libraries |
| PVP | polyvinylpyrrolidone |
| rhPC6 | recombinant human proprotein convertase 6 |
| rpm | revolutions per minute |
| RT | room temperature |
| S1P | site-1 protease |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | standard error of the mean |
| SKI-1 | subtilisin kexin isozyme-1 |
| SPC | subtilisin-like proprotein convertase |
| STHDF | sodium taurodihydrofusidate |

| | |
|---|---|
| TBS | tris-buffered saline |
| TCA | trichloroacetic acid |
| TGN | trans-Golgi network |
| $T_{max}$ | time to reach $C_{max}$ |
| v/v | volume/volume |
| Vpr | viral protein R |

## Amino acids

| | |
|---|---|
| Arg | Arginine |
| Asp | Aspartic acid |
| Glu | Glutamic acid |
| His | Histidine |
| Leu | Leucine |
| Lys | Lysine |
| Ser | Serine |
| Trp | Tryptophan |
| Val | Valine |
| D | Aspartic acid |
| E | Glutamic acid |
| H | Histidine |
| K | Lysine |
| L | Leucine |
| R | Arginine |
| S | Serine |
| W | Tryptophan |

# Chapter 1 –

# Literature review

# 1. Proprotein convertase (PC) and PC6

## 1.1 Background on PCs

In 1967, two research groups expressed the notion that biologically active proteins and peptides are generated by proteolysis of their inactive precursors. Using pulse-chase experiments, Steiner et al. (1967) showed that active insulin is produced through cleavage of a larger precursor protein. Chretien and Li (1967) compared the amino acid sequences of sheep pituitary β-lipotropin hormone (β-LPH), γ-LPH and β-melanocyte-stimulating hormone and noted the presence of the latter two within the first. They drew a similar conclusion that active hormones were generated from their precursors. Subsequent research revealed that bioactive products resulting from such proteolysis include most peptide hormones, neuropeptides, and also transcription factors, growth factors, membrane receptors, adhesion molecules, extracellular matrix proteins, viral glycoproteins and bacterial toxins (Nakayama, 1997). Therefore, the generations of biologically active proteins and peptides have two major requirements: (i) the initially synthesised non-active precursors; and (ii) the proteolytic enzyme(s) that convert the precursors into their final bioactive products. One family of the enzymes responsible for this conversion are named proprotein convertases (PCs) and regarded as "master switches" because of their importance in regulating cellular and physiological functions.

The key to the identification of mammalian PCs had come from studies of kexin gene in yeast *Saccharomyces cerevisiae* (Julius et al., 1984). The role of kexin as an endoprotease in precursor processing was revealed by genetic-complementation analysis of kexin mutant strains defective in the production of active α-mating factor and killer toxin, both of which are

generated through cleavage of their precursors (Fuller et al., 1988). Kexin was later shown to be able to cleave mammalian precursors, proalbumin (Bathurst et al., 1987) and opiomelanocortin (Thomas et al., 1988). These findings implied that kexin was an unequivocal example of mammalian PCs. The catalytic domain of kexin is homologous to bacterial calcium-dependent serine proteases of the subtilisin family. Based on the homology of catalytic domains, Leunissen and Siezen (1997) classified subtilisin into different clans. To date, nine mammalian PCs have been identified in mammals and named as furin, PC1, PC2, PC4, PC5/6 (referred to PC6 in this thesis), PC7, PACE4, SKI-1 and PC9 (Bassi et al., 2005). Table 1 summarises the nomenclatures and human gene symbols of these nine PCs. The first seven PCs belong to the kexin-like clan, which cleave at basic residues within the general motif $(R/K)(X)_n$-(R/K)↓, where the arrow indicates the cleavage site; n is 0, 2, 4 or 6 (Seidah and Prat, 2012). The eighth member, PCSK8, cleaves non-basic residues and the last member, PC9 cleaves itself at its interval sequence (Seidah and Prat, 2012).

Table 1. Nomenclature of PC enzymes

| Name (and alternatives) | Human Gene Symbol |
|---|---|
| Furin = SPC1 = PACE | PCSK3 (FURIN) |
| PC1 = PC3 = SPC3 = NEC1 | PCSK1 |
| PC2 = SPC2 = NEC2 | PCSK2 |
| PC4 = SPC5 | PCSK4 |
| PC5 = PC6 = SPC6 | PCSK5 |
| PACE4 = SPC4 | PCSK6 |
| PC7 = PC8 =LPC = SPC7 | PCSK7 |
| PCSK8 = SKI-1 = S1P | MBTPS1 |
| PC9 = NARC-1 | PCSK9 |

SPC, subtilisin-like proprotein convertase; PACE, paired basic amino acid residue cleaving enzyme; NEC, neuroendocrine convertase; LPC, lymphoma proprotein convertase; NARC, neural apoptosis regulated convertase; SKI-1, subtilisin kexin isozyme-1; S1P, site-1 protease.

A schematic representation of the protein structures of the nine PCs is shown in Figure 1. Structurally, all PC members contain five characteristic domains: (i) the signal peptide at the N-terminal, responsible for directing proteins into the secretory pathway; (ii) the prodomain, acting as a chaperone to assist folding and regulation of enzymatic activity; (iii) the catalytic domain containing the catalytic triad composed of an Asp, His and Ser, highly homologous among the PC family, responsible for the interaction and the cleavage of specific substrates; (iv) the P domain, essential for enzyme stability, calcium and pH dependence; and (v) the C-terminal domain that varies and unique to each PC, and can contain transmembrane, cytoplasmic, amphipathic, serine/theonine or cysteine rich domains (Couture et al., 2011).

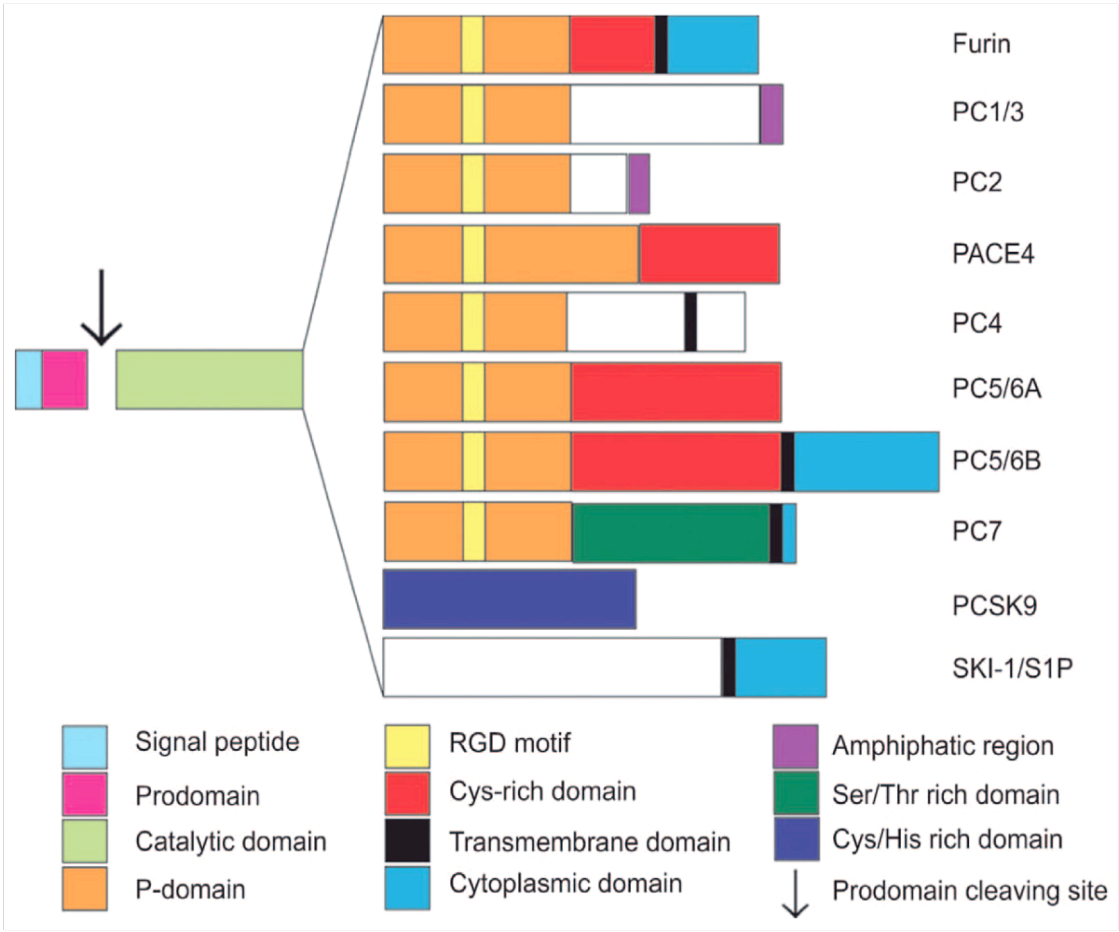


Figure 1. Structural domains of proprotein convertases

See text for details (Image source: Conture et al., 2011).

## 1.2 Proprotein convertase in health and disease

It is clear that PCs play vital roles in a variety of physiological processes and are important in the regulation of critical functions. Several important pathologies have been linked with PC activities, including infertility, developmental defects, metabolic dysfunctions, cancer, cardiovascular disease, osteoarthritis and viral infections (Chretien et al., 2008, Couture et al., 2011, Taylor et al., 2003).

Table 2. Summary of phenotypes of PC knockout mice and patients with null PC1 and PCSK9

| PC | Null phenotype | Reference |
|---|---|---|
| Furin | Embryonic lethality (e10.5-11.5).<br>Ventral closure defects.<br>Failure to undergo axial rotation. | (Roebroek et al., 1998) |
| PC1 | Pre- and postnatal lethality.<br>Dwarfism.<br>Chronic diarrheal. | (Zhu et al., 2002, Villeneuve et al., 2002) |
| PC1 (human) | Impaired glucose homeostasis and gastrointestinal function.<br>Hypogonadotropic hypogonadism.<br>Hypocortisolism. | (Jackson et al., 1997, O'Rahilly et al., 1995) |
| PC2 | Retarded growth.<br>Hypoglycaemia. | (Furuta et al., 1997, Furuta et al., 2001) |
| PC4 | Impaired male fertility. | (Mbikay et al., 1997, Li et al., 2000a, Li et al., 2000b, Li et al., 1998) |
| PACE4 | Embryonic lethality.<br>Bone morphogenetic defects.<br>Craniofacial malformations. | (Constam and Robertson, 2000) |
| PC6 | Embryonic lethality. | (Essalmani et al., 2006) |
| PC7 | Loss of anxiety | (Seidah and Prat, 2012) |
| SKI-1 | Embryonic lethality.<br>Reduced cholesterol and fatty acid synthesis | (Constam and Robertson, 2000, Ye et al., 2000) |
| PC9 | Increased cholesterol uptake by the liver | (Allard et al., 2005, Rashid et al., 2005) |
| PC9 (human) | Hypercholesterolemia | (Abifadel et al., 2003) |

### 1.2.1 Physiological importance of PCs: evidence from knockouts

The production of knockout mice and observations of human cases with specific PC deficiency have allowed the assessment of the physiological importance of a number of PCs. Table 2 summarises the phenotypes of PC knockout mice and patients with null PC1 and PCSK9.

### 1.2.2 PCs and viral infections

For many viruses, proteolytic activation of their envelope glycoproteins is facilitated by the host PCs, enabling entry into host cells and hence, the production of infectious virions. The human immunodeficiency virus (HIV) envelope protein gp160 was the first identified viral protein that requires PC cleavage, producing the functionally active gp120 and gp41 subunits for infectivity (Hallenberger et al., 1992). Furin, PC6, PC1, PACE4 and PC7 were shown to have the ability to cleave gp160 at the highly conserve site R-G-K-R ↓ A-V in cell lines (Decroly et al., 1996, Hallenberger et al., 1992, Vollenweider et al., 1996). To identify the relevant PC in gp160 processing, Hallenberger et al. (1992) screened the level of PCs expressed in human T cells, the most common HIV target, and found furin, PC6 and PC7 to be the major PCs for gp160 processing. A more complex study (Miranda et al., 1996) excluded the possibility of furin action since furin expression levels did not correlate with the efficiency of gp160 processing in T cells. With evidence of PC6 expression in T cells, they suggested that **PC6 represented the most likely host PC responsible for gp160 processing** (Miranda et al., 1996). Extracellular PCs have also been linked to the processing of HIV viral protein R (Vpr), which plays a critical role in viral infection (Xiao et al., 2008). **The study suggested that PC6 and PACE4 are the most likely candidates for Vpr processing** based on the observation of transient co-expression of these PCs with Vpr cleavage (Xiao et al., 2008).

Human papillomaviruses (HPV) is another medically important virus since infection with oncogenic HPV types is the causative agent of cervical carcinoma (Richards et al., 2006). Indeed, processing of the minor capsid protein L2 by PC activity is necessary for infectious entry of these viruses (Richards et al., 2006). A study using a murine challenge model indicated that **both furin and PC6 are abundant at sites of disruption in the murine cervicovaginal tract,** thus representing the major PCs that contribute to L2 cleavage (Kines et al., 2009).

Table 3. Therapeutic potentials of PC inhibitors

| **Inhibitors of:** | **Therapeutic potential** | **References** |
|---|---|---|
| Furin, PACE4 | Cancer and/or metastasis | (Senzer et al., 2012, Coppola et al., 2008) |
| | Arthritis | (Byun et al., 2010) |
| | Viral and pathogenic infections | (Komiyama et al., 2009, Jiao et al., 2006, Becker et al., 2010) |
| PC2 | Diabetes (PC2 inhibitors that simulate PC1) | (Vivoli et al., 2012) |
| PC4 | Male contraception | (Majumdar et al., 2010) |
| PC6 | Female contraception with potential to reduce sexually transmitted HIV infections | Research hypothesis of this thesis |
| PC7 | Anxiety | (Seidah and Prat, 2012) |
| SKI-1 | Viral infections (Arenaviruses including Lassa virus, Crimean-Congo haemorrhagic fever virus, lymphocytic choriomeningitis virus and hepatitis C virus) | (Pullikotil et al., 2004, Urata et al., 2011, Olmstead et al., 2012) |
| | Dyslipidemia | (Hawkins et al., 2008) |
| | Malaria parasite | (Bastianelli et al., 2011) |
| PCSK9 | Hypercholesterolaemia/Cardiovascular disease | (Zhang et al., 2012, Crunkhorn, 2012, Ni et al., 2011, Chan et al., 2009, Liang et al., 2012, Ni et al., 2010, Frank-Kamenetsky et al., 2008, Gupta et al., 2010, Lindholm et al., 2012) |

## 1.3 Inhibition of PCs as a powerful therapeutic strategy for a number of conditions

The involvement of PCs in a range of pathological processes makes them promising targets for the treatment of a number of diseases. Therefore, substantial efforts have been made to develop potent and specific PC inhibitors. Table 3 summaries the therapeutic potential of PC inhibitors.

Inhibitors of six of the nine PCs are potentially applicable to treatments of specified clinical conditions [Table 3, modified from (Seidah and Prat, 2012)]. The most promising approaches to date are inhibitors against furin in the treatment of cancer and/or metastasis (Bassi et al., 2005, Scamuffa et al., 2008), as well as viral and pathogenic infections (Komiyama et al., 2005, Ozden et al., 2008); PACE4 inhibitors in cancer (D'Anjou et al., 2011, Bassi et al., 2005) and arthritis (Malfait et al., 2008, Wylie et al., 2012); PC4 inhibitors as male contraceptives (Gyamera-Acheampong et al., 2006); SKI-1 inhibitors in arenavirus (Pasquato et al., 2012) and hepatitis infections (Olmstead et al., 2012); and PC9 inhibitors in hypercholesterolemia to reduce the incidence of coronary artery diseases and associated atherosclerosis (Zhang et al., 2003, Crunkhorn, 2012, Ni et al., 2011). However, none of the PC inhibitors is highly specific to a single PC member due to the high homology of the PC catalytic domains. In this thesis, I proposed that PC6 inhibitors are potential female contraceptives that could also reduce sexually transmitted HIV infections (see Section 5).

## 1.4 Known characteristics of PC6

Nakagawa et al. (1993) first identified PC6. It has two isoforms (PC6 A and B) that are generated via alternative splicing (Nakagawa et al., 1993a, Nakagawa et al., 1993b). PC6 is expressed in the trans-Golgi network (TGN) and PC6A is associated with the regulated

secretory pathway; whereas PC6B is associated with the plasma membrane (Bergeron et al., 2000) (Figure 2). Furthermore, PC6A can also be localised and active at the cell surface through association with heparan sulfate proteoglycans (HSPGs) (Nour et al., 2005) (Figure 2).

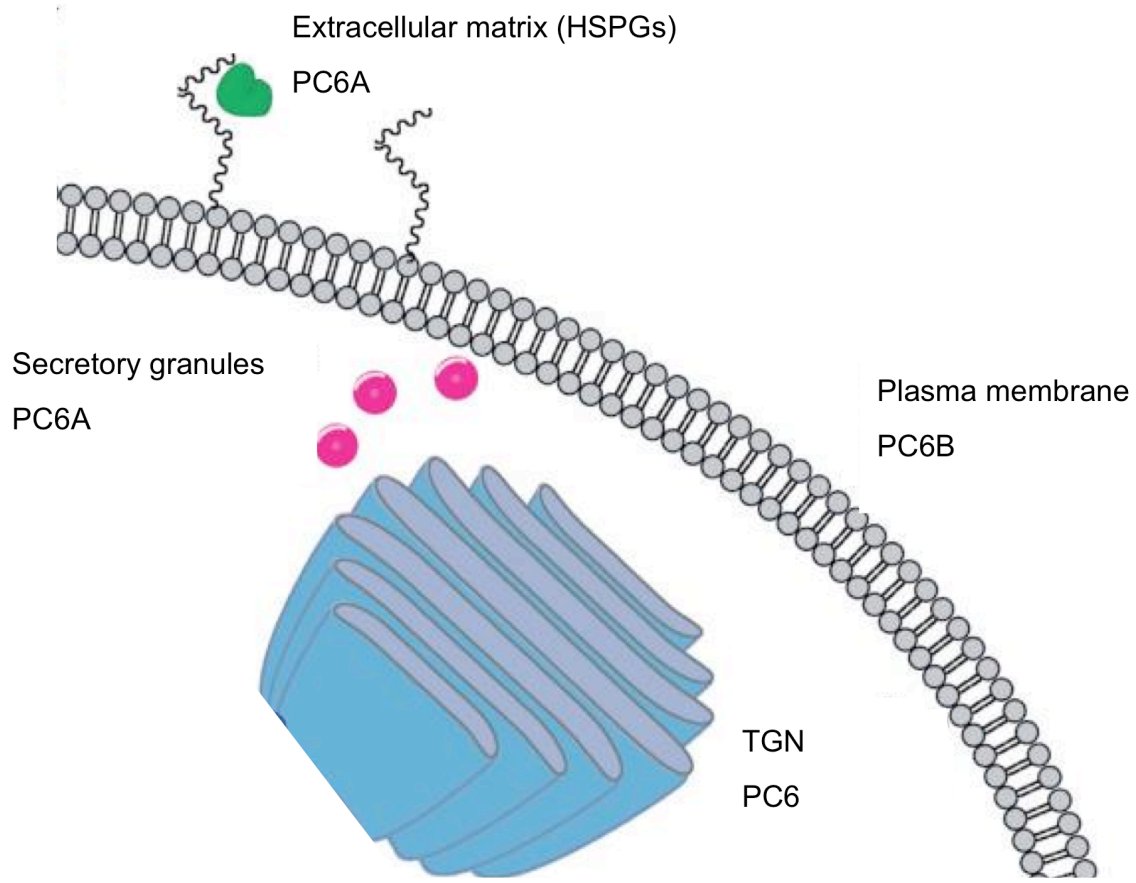


Figure 2. Cellular localisation of PC6

PC6 is expressed in TGN and is found in two isoforms; PC6A and PC6B. PC6A is associated with the regulated secretory pathway, while PC6B is associated with the plasma membrane by its transmembrane domain (Bergeron et al., 2000). PC6A is also at the cell surface, associated with heparan sulfate proteoglycans (HSPGs) (Nour et al., 2005). (Figure adapted from Conture et al. 2011)

Documentation of PC6 mRNA expression in mice, human and cell lines revealed that its distribution is richest in the adrenal cortex, small intestine, kidney, ovary, uterus, lung, aorta and brain cortex (Essalmani et al., 2006). The widespread distribution of PC6 suggests its involvement in relevant biological processes in those organs. However, the key factors that determine the function of PC6 in an organ depend on its cellular expression level, activity and intraorganellar localisation. In 2003, Nie et al. detected specific and transient up-regulation of PC6A mRNA at the site of embryo implantation in mice (Nie et al., 2003). Functional studies using human cells and mice suggested that PC6 plays an important role in embryo implantation (Nie et al., 2005b, Okada et al., 2005), and prompted further research into PC6 in relation to embryo implantation in different animal models and in women.

# 2. PC6 and embryo implantation

Embryo implantation is a complex and critical step in the establishment of a successful pregnancy. It is the process by which the embryo and the lining of the uterus (the endometrium) establish a close physical and physiological contact (Carson et al., 2000, Lee and DeMayo, 2004).

## 2.1 Mechanisms of implantation

In mammals, once an embryo has developed into a blastocyst, it must implant into the uterus for its subsequent development. The process of implantation is categorized into three phases: apposition, attachment (adhesion) and invasion (penetration) (Guzeloglu-Kayisli et al., 2007) (Figure 3). These require transformation of the epithelium (increased secretion, alterations in adhesiveness and functional complexes). In addition, the endometrium has to undergo an irreversible differentiation process of stromal fibroblasts into morphologically

distinct decidual cells (decidualisation) to provide an environment that is conducive to implantation of a blastocyst (Salamonsen et al., 2009). Apposition is the loose adhesion between the trophoblast of the blastocyst and the endometrial surface epithelium, when the trophoblast becomes closely apposed to the luminal epithelium (Tabibzadeh and Babaknia, 1995). This is followed by the attachment phase, a stronger adhesion triggered by local paracrine signalling between the embryo and endometrium (Sharkey and Smith, 2003). Invasion involves penetration of the embryo through the luminal epithelium and its basal lamina, coming into contact with the underlying stroma and fibroblasts, which undergoes decidualisation (Figure 3).

In addition to embryo quality, uterine receptivity and a synchronized dialog between maternal and embryonic tissues are of utmost importance for implantation. For implantation to succeed, timely completion of embryo attachment, invasion and decidualisation are critical. Implantation will fail if the endometrium is not competent to participate in all of the different implantation phases.

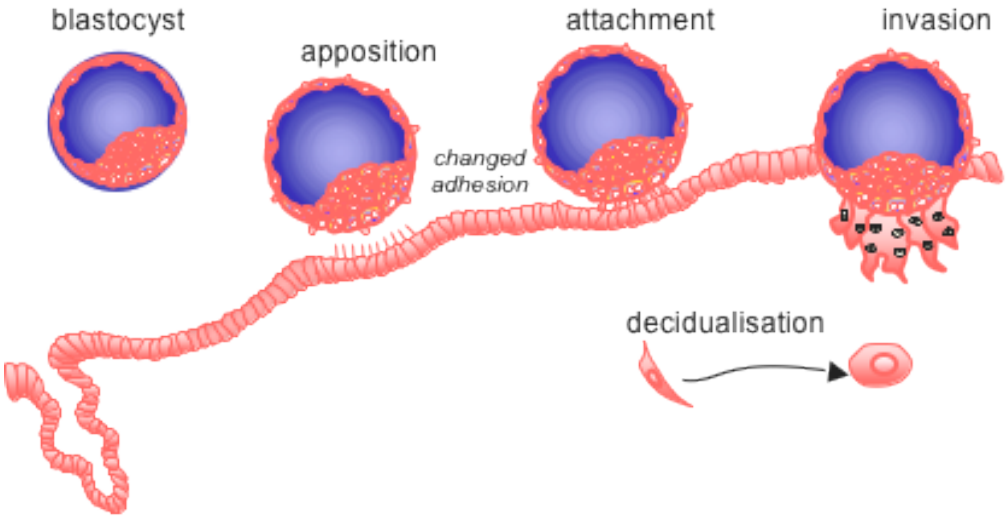


Figure 3. Events of embryo implantation in the human

See text for details. (Figure adapted from Salamonsen et al., 2009).

## 2.2 Different models of implantation

Despite the common steps and the eventual interaction of trophoblast with maternal blood, this is achieved differently in different species. Accordingly, implantation can be classified into three broad categories: centric, eccentric and interstitial (Lee and DeMayo, 2004, Carson et al., 2000). In centric implantation, blastocysts adhere to the apices of epithelial cells and fuse with them prior to penetration into stroma. Rabbits, dogs, many marsupials and domestic animals including cows, pigs and sheep have centric implantation. Eccentric implantation occurs in mice, rats and hamsters where trophoblast is surrounded by luminal epithelium. Penetration is aided by apoptosis of the epithelial cells and the luminal epithelial basal lamina. Implantation in humans and guinea pigs is classified as interstitial; the trophoblast penetrates between the luminal epithelial cells followed by the invasion of the whole blastocyst into the endometrial stroma.

For both practical and ethical reasons, it is not possible to study implantation *in vivo* in humans. Therefore, animal models are essential to decipher the biology and understand the molecular mechanism of this complex process. As implantation differs between animal models, different animals represent more suitable models for studying particular steps of human implantation. Mice and rabbits serve as a good model to study decidualisation and attachment respectively (Carson et al., 2000, Lee and DeMayo, 2004).

## 2.3 PC6 and implantation in mice, rabbits and women

PC6 is the only PC member that is associated with embryo implantation (Freyer et al., 2007, Tang et al., 2005). In mice, PC6 is up-regulated specifically in decidualising stromal cells adjacent to the implanting embryo at the time of attachment (Nie et al., 2003). In rabbits, PC6 in the uterine epithelium dramatically increases prior to embryo attachment and intensifies in

decidual cells at the site of embryo implantation (Nicholls et al., 2011). In women, PC6 is expressed in the uterine epithelium across the menstrual cycle, peaking during the receptive phase, approximately days 20-24 of a 28-day menstrual cycle: that is, during the mid-secretory phase (Nie et al., 2005a) (Figure 4). Decidualisation is initiated after day 22 in each menstrual cycle and continues into pregnancy (Salamonsen et al., 2009) (Figure 4). PC6 is specifically up-regulated in decidual stromal cells in both mice and women (Nie et al., 2005b). Functional studies in human cells in culture and mice *in vivo* have demonstrated the critical role of PC6 in stromal cell decidualisation (Nie et al., 2005b, Okada et al., 2005). Recently, PC6 was also shown to be essential in the establishment of endometrial epithelial receptivity in women (Heng et al., 2011a, Heng et al., 2011b).

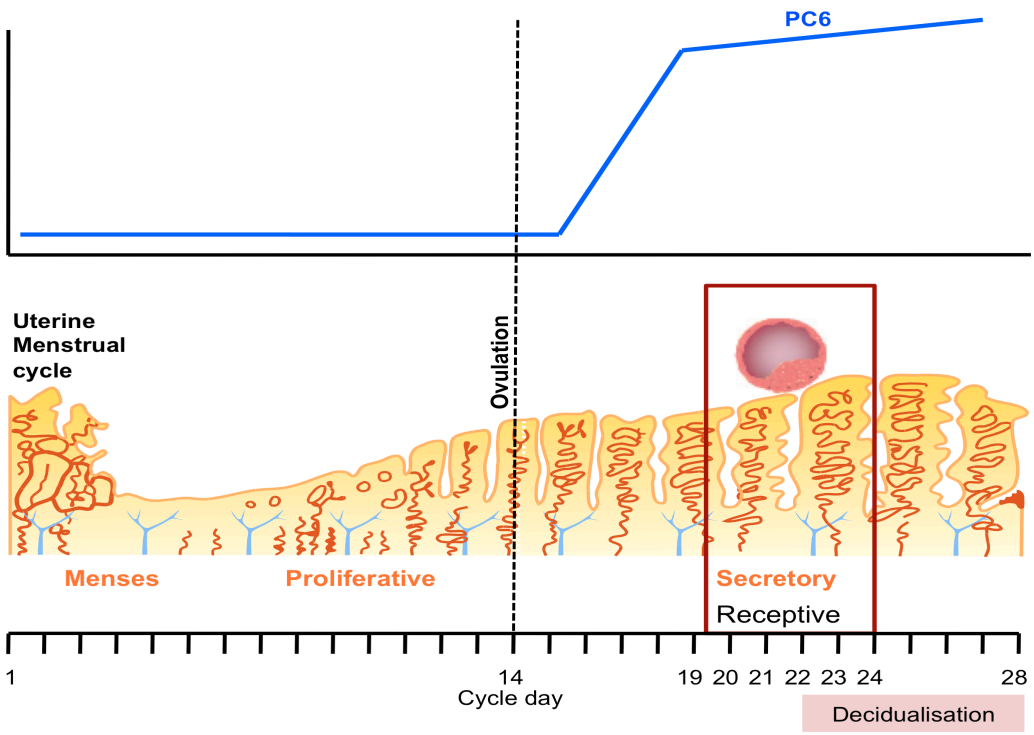


Figure 4. Pattern of PC6 expression during the menstrual cycle

The PC6 level starts to rise in the early secretory phase and reaches a peak during the mid-secretary phase, at the receptive phase (window of implantation) and decidualisation (Figure adapted from Salamonsen et al., 2009).

# 3. PC6 and decidualisation

## 3.1 Decidualisation

Decidualisation is defined as the differentiation process by which the endometrium transforms into morphologically and functionally distinct decidual tissue required to establish pregnancy (Salamonsen et al., 2009). Morphologically, it is characterised by the transformation of the elongated fibroblast-like endometrial stromal cells close to the spiral arterioles into enlarged, rounded cells with specific ultrastructural modifications (Garrido-Gomez et al., 2011) (Figure 3). In a more comprehensive sense, decidualisation also involves remodelling of the vasculature and extracellular matrix, as well as the influx of specific subsets of macrophages and uterine natural killer cells into the tissue (Salamonsen et al., 2009).

Although it has been well established that decidualisation is critical for embryo implantation and successful pregnancy, its specific functions are not well understood. Several studies revealed a few possible explanations: (1) limits excessive invasion of trophoblast by release of antiproteases such as tissue inhibitors of metalloproteinases (TIMPs) and cystatin C (Zhang and Salamonsen, 1997, Afonso et al., 1997); (2) forms incomplete tight junctions as a barrier to prevent the passage of large molecules (Wang et al., 2004); (3) ensures increased protein synthesis to supply nutrients to the developing embryo before the establishment of the placenta (Tranguch et al., 2005); (4) regulates differentiation and invasion of trophoblast for placentation (Hess et al., 2007), and (5) becomes biosensor of embryo quality (Salker et al., 2010, Teklenburg et al., 2010).

## 3.2 PC6 in human decidualisation in endometrial tissues and human cell models to study decidualisation

In women, decidualisation occurs spontaneously during the mid-secretory phase: that is, after day 22 of each menstrual cycle, and the process continues throughout pregnancy (Gellersen et al., 2007) (Figure 4). Impaired decidualisation predisposes women to recurrent pregnancy loss by disabling natural embryo selection and by disrupting the maternal responses to embryonic signals, indicating the importance of decidualisation for the establishment of successful pregnancy (Salker et al., 2010, Teklenburg et al., 2010).

PC6 is closely associated with decidualisation in the human endometrium; it increases dramatically during decidualisation prior to implantation and intensifies with implantation establishment (Nie et al., 2005b). To study the role of PC6 in decidualisation in women, *in vitro* models using a primary human endometrial stromal cell culture system that can reproduce the decidualisation process, have proven to be powerful. The primary human endometrial stromal cells (HESCs) can be isolated relatively easily from curettage materials and decidualised in culture in the presence of decidualising stimuli. With this approach, it was shown that specific inhibition of PC6 production in HESCs by morpholino antisense oligonucleotides, significantly attenuated decidualisation (Okada et al., 2005). This finding strongly indicates the importance of PC6 in decidualisation in women.

## 3.3 PC6 in mouse decidualisation in endometrial tissues and mouse models to study decidualisation

In mice, decidualisation occurs naturally only in the presence of a conceptus (Abrahamsohn and Zorn, 1993). The importance of decidualisation for embryo implantation is evidenced in infertile mice with impaired decidualisation (Abrahamsohn and Zorn, 1993, Lim et

al., 1997, Bhatt et al., 1991, Lydon et al., 1995, Xu et al., 1998, Bagot et al., 2000, Gendron et al., 1997, Bilinski et al., 1998, Robb et al., 1998, Ashkar et al., 2000). The decidual reaction in mice can be artificially stimulated by trauma and decidualised regions in pregnant mice are easily discernable, thereby making mice a good model to study the mechanism of decidualisation (Lee and DeMayo, 2004). PC6 is up-regulated specifically in decidualising stromal cells adjacent to the implanting embryo in the uterus (Nie et al., 2003). Specific inhibition of PC6 production in the mouse uterus *in vivo* by morpholino antisense oligonucleotides inhibited decidualisation and caused implantation failure (Nie et al., 2005b). In this study, morpholino antisense oligonucleotides were injected directly into mouse uterine lumen through surgery. This data directly reveals the critical role of PC6 in decidualisation and successful implantation in mice.

## 3.4 Newly established mechanisms of PC6 action in decidualisation in human cells

PC6 is predicted to act as a "master switch" owing to its proteolytic actions. It has been envisaged that PC6 activates a number of important molecules (Figure 5), including growth factors, cytokines and proteins associated with reorganisation of the cytoskeleton, as required for implantation (Salamonsen et al., 2009).

To understand the molecular mechanisms of PC6 action in the uterus for decidualisation, Nie's group has sought to identify the physiological substrate of PC6 using proteomic approaches. Caldesmon and bone morphogenetic protein 2 (BMP2) are identified as PC6 substrates (Kilpatrick et al., 2009, Heng et al., 2010). Caldesmon is a structural protein involved in actin filament reorganisation and cytoskeletal structure remodelling (Sobue et al., 1999). As decidualisation of endometrial stromal cells requires major morphological, structural

and functional changes, in association with cytoskeletal reorganisation, it is not surprising that caldesmon is regulated by PC6 during decidualisation (Kilpatrick et al., 2009). BMP2 is a member of the BMP multifunctional growth factors of the TGF-β superfamily that are known to require major posttranslational modifications to become biologically active (Heng et al., 2010). BMP2 expression is closely associated with decidualisation and functional studies deleting BMP2 confirmed that BMP2 is critical for decidualisation (Lee et al., 2007, Li et al., 2007, Paria et al., 2001, Stoikos et al., 2008). Furthermore, Heng et al., (2010) demonstrated that the bioactivity of BMP2 is dependent on the proteolytic cleavage of PC6 during decidualisation.

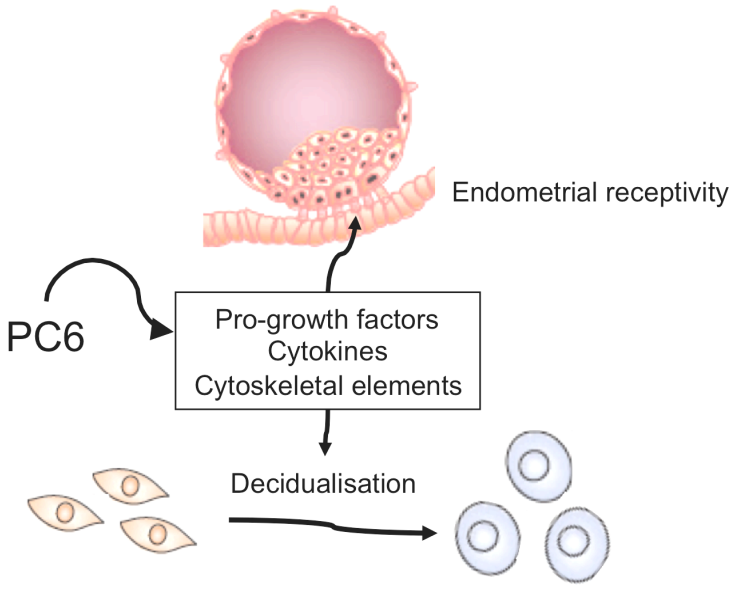


Figure 5. Proposed action of PC6 as a "master switch" in activating key regulatory molecules in the uterus during its preparation for receptivity and decidualisation

# 4. PC6 and endometrial epithelial receptivity

Endometrial epithelial receptivity is pivotal for successful embryo attachment as non-receptive endometrium is causative for implantation failure and some infertility in women. It is established that PC6 plays a critical role in endometrial epithelial receptivity.

## 4.1 PC6 in human and monkey uterus across the menstrual cycle

The endometrium is optimally receptive to implanting blastocyst only for a finite time (D6-10 postovulation) in a menstrual cycle, known as the "window of implantation" (Salamonsen et al., 2009) (Figure 4). Studies in primates, including the rhesus monkey and humans, showed a significant up-regulation of endometrial epithelial PC6 during the window of implantation (Nie et al., 2005b). This suggests that PC6 may be essential in modulating endometrial epithelial receptivity to support embryo attachment and implantation.

## 4.2 Newly established mechanisms of PC6 action in the epithelium for receptivity

During the receptive phase, in preparation for implantation of a blastocyst, the endometrial epithelium undergoes plasma membrane transformation to alter membrane organisation and the membrane-associated cytoskeleton (Murphy, 1995). PC6 was identified to play a critical role in regulating one of these fundamental cellular changes, acting on a key scaffolding protein, ezrin-radixin-moesin binding phosphoprotein 50 (EBP50) (Heng et al., 2011a). PC6 cleaves EBP50, thereby affects EBP50 interaction with its binding protein, ezrin (a key protein bridging actin filaments and plasma membrane), hence controlling the cellular localisation of both EBP50 and ezrin and the cytoskeleton-membrane linkage (Heng et al., 2011a). This mechanism of PC6 action in the epithelium for receptivity was further validated in

the human endometrium *in vivo* in fertile *vs.* infertile women. Immunohistological data showed that PC6 expression was significantly lower in the endometrial epithelium during the receptive window in infertile women, and the localisation of EBP50 and ezrin was abnormal (Heng et al., 2011a).

## 4.3 Models to study epithelial receptivity and embryo attachment

*In vitro* models using human cell culture systems to reproduce critical stages of the implantation process have been developed and used in the study of human implantation (Diedrich et al., 2007). As it is difficult to obtain sufficient epithelial and trophoblast cells from primary tissues, endometrial epithelial and trophoblast cell lines are widely used for *in vitro* studies. Commonly used endometrial epithelial cell lines include ECC-1, Ishikawa, HEC1A and RL-95, and trophoblast cell lines include JAR, BeWo and JEG-3 (Hannan et al., 2010). *In vitro* models of embryo attachment involving coculture of a monolayer of human endometrial epithelial cells (to mimic the uterine epithelium) and trophoblast spheroids (to mimic blastocysts) have been developed (Li et al., 2002, Rahnama et al., 2009, Thie and Denker, 2002, Thie et al., 1997, Thie et al., 1998, Tinel et al., 2000). Mouse blastocysts replace trophoblast spheroids in some studies. These models are useful to study the epithelial receptivity for embryo attachment. The critical importance of PC6 in the establishment of receptivity was evidenced by a significant reduction in the attachment of mouse blastocysts to endometrial epithelial HEC1A cells when PC6 was specifically knocked-down by small interfering RNA (siRNA) (Heng et al., 2011a).

### 4.4 PC6 as a potential biomarker to detect receptivity in women

In women, PC6 is secreted into the uterine cavity and can be detected in uterine lavage. A close association between PC6 levels in uterine lavage and uterine receptivity has been demonstrated with relatively large number of uterine lavage samples (Heng et al., 2011b). The data showed that PC6 levels in the uterine lavage are markedly elevated in the receptive compared with the non-receptive phase of the menstrual cycle in fertile women (Heng et al., 2011b). Furthermore, PC6 levels during the putative receptive phase in a subgroup of women with unexplained infertility were significantly lower than that of fertile women (Heng et al., 2011b). Since uterine function is closely associated with the secretion profile (Ametzazurra et al., 2009), PC6 in the uterine lavage may represent a biomarker for the development of a non-surgical approach for the detection of uterine receptivity in women.

## 5. Inhibition of PC6 for dual-role contraception

Because of the critical role of uterine PC6 in embryo implantation for the establishment of pregnancy, PC6 becomes an attractive and potential target for the development of new non-hormonal contraception for women. Furthermore, PCs including PC6 are also involved in HIV infection (Xiao et al., 2008). Therefore, we hypothesised that inhibiting PC6 in the female reproductive tract (uterus, cervix and vagina) may protect women from both pregnancy and sexually transmitted HIV infections. To achieve such a dual-protection, vaginal delivery of PC6 inhibitor(s) will be the preferred administration route. One of the first requirements to prove this concept is to develop suitable PC6 inhibitors and test them in an animal model.

## 5.1 PC6 inhibitors

To date, a number of promising PC inhibitors have been developed. However, with the relatively high homology among the PC catalytic domains, development of a highly specific inhibitor against PC6 remains a great challenge. Several peptide-based or small molecules PC inhibitors with different inhibitory potency (Ki) against PC6 have been reported (Table 4). Recently, more selective peptidic inhibitors were developed, based on positional scanning of synthetic peptide combinatorial libraries (PS-SPCLs). Using this approach, Fugere et al. (2007) accessed the preferred residues of each sub-site of the catalytic pockets of PC6 and PC7, and developed nona-D-arginine as a potent and more selective inhibitor of PC6 and PC7. Nona-D-arginine also inhibits PCs involved in processing gp160 and diminishes HIV infectivity in cell culture (Kibler et al., 2004).

Table 4. Published PC6 inhibitors

| **Molecules** | **Type** | **Ki (nM)** | **Reference** |
|---|---|---|---|
| TPRARRRKKRT-$NH_2$ | Peptide | 232 | (Shiryaev et al., 2007) |
| 8-Amino-octanoyl-RARRRKKRT-$NH_2$ | Tailed peptide | 3 | (Remacle et al., 2010) |
| Hexa-D-Arginine | Peptide | 206 | (Fugere et al., 2007, Cameron et al., 2000) |
| Nona-D-arginine | Peptide | 19 | (Karicherla and Hobden, 2009, Karicherla and Hobden, 2010, Fugere et al., 2007) |
| Decanoyl-RVKR-CMK | CMK-peptide | 0.12 | (Angliker et al., 1993, Jean et al., 1998, Fugere et al., 2002) |
| Guanidinylated Aryl 2,5-dideoxystreptamine derivatives | Small molecules | 4 | (Jiao et al., 2006) |
| Dicoumarol derivatives | Small molecules | 16000 | (Komiyama et al., 2009) |
| Phenylacetyl-RVR-4-amidino-benzylamide | Peptidomimetic | 1.6 | (Becker et al., 2010) |

Although these PC6 inhibitors have shown promising inhibitory activity *in vitro*, there are concerns regarding their use as potential therapeutic agents, particularly possible side effects due to their lack of selectivity. However, they could serve as a foundation for the development of inhibitors with enhanced and optimised properties, such as *in vivo* stability and beneficial pharmacokinetic properties that are essential for potent drugs. In addition, the ability to deliver these inhibitors specifically to the targeted organs and cells will be another crucial determinant for their therapeutic applications. To prove our hypothesis, PC6 inhibitors need to be effectively delivered through the vagina to inhibit sexually transmitted HIV. According to the "first uterine pass effect" (details in Section 6.2.1), these inhibitors should reach the uterus selectively to inhibit embryo implantation.

# 6. Vaginal route of drug delivery

Vaginal administration of drugs provides new opportunities for female health conditions, including hormone replacement therapy, contraception, and prevention and treatment of sexually transmitted diseases. This route of drug delivery takes advantage of a direct local effect and consequently minimises the systemic side effects.

## 6.1 Vagina as a drug absorbing organ

Historical records show that women have been administering drugs to the vagina for thousands of years. However, the concept of the vagina as an organ capable of absorbing drugs was not formalised until 1918 (Macht, 1918). Since then, the vagina has been studied as a favourable site for local and systemic delivery of drugs for treatment or prevention of female-related conditions (Vermani and Garg, 2000). For example, Yamazaki (1984) demonstrated that luteinizing-hormone-releasing hormone agonist administered via the

vagina is more effective in pregnancy-termination than subcutaneous, rectal or nasal administration.

The vaginal route of drug administration presents a number of advantages: (1) avoidance of hepatic first-pass metabolism. This advantage is particularly important for compounds that are highly metabolized by the liver. For example, the bioavailability of propranolol is greatly retained after vaginal administration but mostly lost following oral delivery (Patel et al., 1983); (2) avoidance of undesirable gastrointestinal effects as observed in oral bromocriptine treatment of hyperprolactinemia (Vermesh et al., 1988); (3) avoidance of hepatic side effects of steroids used in contraception or hormone replacement therapy (Cedars and Judd, 1987, Arafat et al., 1988); (4) avoidance of local pain, tissue damage and possible infection by other parenteral routes.

### 6.1.1 Contraception via vaginal delivery of drugs

Vaginal delivery has been studied as an ideal route for contraceptive administration to achieve local delivery and to reduce the complications of the prolonged use of oral contraception. Over the past decades, numerous studies have confirmed the efficacy of vaginal delivery of contraceptive hormones in preventing pregnancy (Sitruk-Ware, 2005). Currently, contraceptive hormones formulated in vaginal rings for sustained delivery, including Nesterone (progesterone) (Kumar et al., 2000) and Nuvaring (etonogestrel and ethinyl oestradiol) (Killick, 2002) have been approved and released to market.

### 6.1.2 Vaginal delivery system for viral infections

Chemical substances called vaginal microbicides have been developed for vaginal application before intercourse to inhibit sexual transmission of HIV. The mechanism of these substances in preventing HIV or STIs can be through creating a barrier between the pathogen

and vaginal epithelium, inactivation of pathogens, inhibition of pathogen replication and entry into cells, and enhancement of natural vaginal defence (Keller et al., 2005).

## 6.2 Uterus as drug absorbing organ

The administration of drugs to the uterus can be achieved by the application of a formulated drug to the vagina. This route of administration is particularly favourable for drugs destined to exert their primary effect on the uterus itself. The evidence for direct transport of drugs from the vagina to the uterus has accumulated since 1986, where progesterone administered to the vagina was distributed selectively to the uterus (De Ziegler et al., 1997). This phenomenon has been demonstrated to be the result of a preferential transport mechanism called the "first uterine pass effect". So far, this phenomenon has only been reported in women but not other mammals.

### 6.2.1 The first uterine pass effect

During the course of experiments with vaginal progesterone, evidence supported a preferential delivery of vaginally administered drugs to the uterus, although systemic absorption was observed to be low (Cicinelli et al., 1998, Cicinelli and de Ziegler, 1999, Cicinelli et al., 2000, Ficicioglu et al., 2004, Miles et al., 1994). A number of clinical studies provide clear verification of selective transport of progesterone from the vagina to the uterus. When comparing serum and endometrial tissue concentrations of progesterone after vaginal and intramuscular (i.m.) administration, ratios of endometrial to serum concentrations of progesterone were higher after vaginal administration than i.m. injections (Cicinelli et al., 2000, Miles et al., 1994, Ficicioglu et al., 2004). Fanchin et al (1997) further confirmed that a higher concentration of progesterone was achieved in endometrial tissue despite a relatively low serum progesterone concentration after vaginal administration. Furthermore, vaginal

progesterone was shown to be more effective than oral and i.m. progesterone in improving the ongoing pregnancy and implantation rate in women (Bourgain et al., 1990, Smitz et al., 1992). The existence of the "first uterine pass effect" was further proven, as plasma levels of progesterone were significantly higher in the uterine artery than in the radial artery following vaginal administration (Cicinelli et al., 1998, Ficicioglu et al., 2004).

The first uterine pass effect has also been observed with substances other than progesterone, namely terbutaline (Bulletti et al., 2001, Kullander and Svanberg, 1985) and danazol (Mizutani et al., 1995). Terbulatine, a beta-adrenergic agonist is an uterorelaxing substance for the treatment of spontaneous contractility. In the study of Kullander and Svanberg (1985), vaginally administered terbutaline significantly inhibited contraction in premature labour within 30 minutes, while the level of terbulatine in peripheral venous blood remained low. A similar observation was reported by Mizutani et al. (1995) who compared the uterine and serum concentrations of danazol, a modified testosterone for the treatment of women with uterine fibroids, following vaginal administration (Mizutani et al., 1995). Recently, the first uterine pass effect was demonstrated with human follicle-stimulating hormone (FSH) in women receiving in-vitro fertilization (IVF) treatment (Hsu et al., 2009, Hsu et al., 2008).

### 6.2.2 Mechanisms of action explaining the first uterine pass effect

Limited studies have been done to explain the routes that govern the direct transport of drugs from the vagina to the uterus. Theoretically, at least four different mechanisms can be contemplated to explain the phenomenon of the first uterine pass effect (reviewed in Cicinelli and de Ziegler, 1999): (1) direct (passive) diffusion through tissues; (2) passage through the cervical lumen (aspiration) from the vagina to the uterus; (3) transport through the venous or lymphatic circulatory systems; (4) counter current vascular exchange involving diffusion between utero-vaginal veins and/or lymph vessels and arteries.

The existence of the direct transport of drugs through vagina-to-uterus diffusion has been established using a human *ex-vivo* uterine perfusion model (Bulletti et al., 1997). In this study, [$^{3}$H]-progesterone applied to the vaginal tissue remained attached to the cervix and the uterus after hysterectomy, diffused to the entire uterus and reached a steady state within 5 hours after application. This mechanism of action has also been demonstrated with [$^{3}$H]terbutaline in a similar model (Bulletti et al., 2001). An alternative direct transport mechanism involving aspiration through the cervical lumen into the uterus has also been demonstrated using vaginally administered sperm-sized $^{99m}$Tc-labeled macroaggregates of human serum albumin (Kunz et al., 1996, Wildt et al., 1998). Characteristically, the transport is extremely rapid, which contrasts with the 5-6 hours of the perfusion model. Kunz et al. (1996) observed that the Tc-99 activity migrated to the uterus within minutes of administration. Direct transport through the venous or lymphatic circulatory system has been demonstrated in porcine (Magness and Ford, 1983).

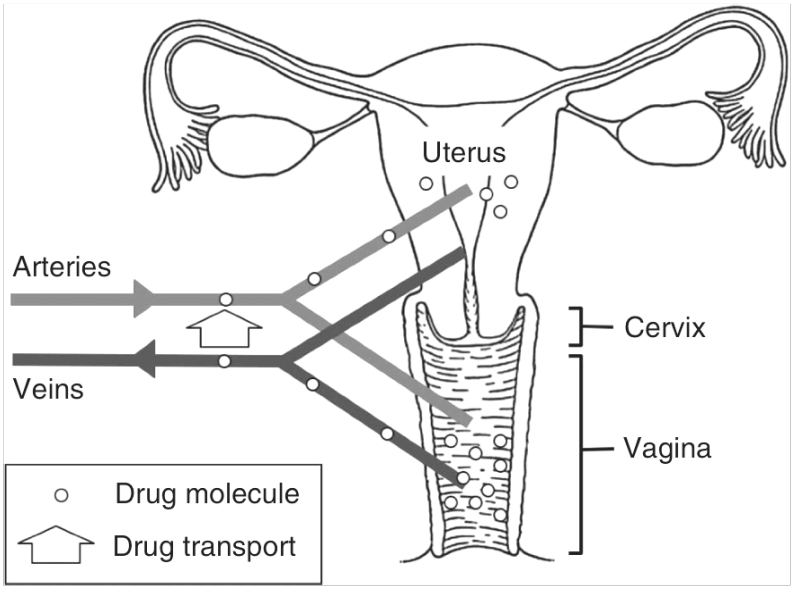


Figure 6. Illustration of counter-current transfer of drug molecule between vaginal vein blood and uterine arterial blood in a woman

(Malcolm et al., 2006).

The counter-current mechanism in which the substances transport from the vagina to the uterus through the counter flow between vaginal vein and uterine artery is considered as an important mechanism of the first uterine pass effect in the female reproductive system (Einer-Jensen, 1988, Einer-Jensen, 1990, Einer-Jensen and Hunter, 2000, Einer-jensen and McCracken, 1977, Einer-Jensen et al., 1989, Einer-Jensen et al., 2001, Einer-Jensen et al., 2002, Einer-Jensen and Hunter, 2005, Einer-Jensen et al., 1993). Counter-current transfer has been defined as a passive transfer of energy such as heat or substances from one solvent to the other (Einer-Jensen and Hunter, 2005). In the first uterine pass effect, it involves local transfer from the vaginal lymph or vein blood to the uterine artery (Figure 6). This phenomenon of counter-current transfer was clearly demonstrated by cooling the vagina, which resulted in rapid cooling of the uterus in women (Einer-Jensen et al., 2001).

## 6.3 Strategies to improve absorption of peptide and protein drugs administered via the vagina

Despite being a favourable route for treatment of female-specific conditions, vaginal delivery must overcome several drawbacks such as residence time, mucosal permeability and leakage, for optimal pharmacological action. There have been several strategies designed to improve the absorption of drugs administered via the vaginal route.

### 6.3.1 Sustained release formulations

Improving and prolonging the contact between the drug and the vaginal epithelium can greatly enhance the drug's absorption. Drugs are formulated into tablets, gels, creams, foams, suppositories, pessaries, intrauterine devices (IUD) and intravaginal rings (IVR) for sustained delivery. Ideally, selections of the most appropriate form of delivery system should be based on the desired therapeutic effects of the drugs in the vagina and/or uterus. Selection

should consider a number of factors: a local or systemic effect, immediate or sustained release, the physiochemical and pharmacological nature of the drugs, the cost and patient tolerability.

### 6.3.2 Bioadhesive delivery systems

Conventional formulations have some limitations, such as leakage, poor distribution in the vagina, and low retention in the vaginal cavity (Valenta, 2005). Bioadhesion is defined as the extended interaction period held between a biological tissue and a biological or synthetic surface (das Neves and Bahia, 2006). It is called mucoadhesion if the interaction is between a synthetic or natural polymer and a mucus coated surface (das Neves and Bahia, 2006), and this concept was applied in drug delivery systems as early as the 1980's (Ahuja et al., 1997). The vagina is highly appropriate for a mucoadhesion drug delivery system as the vaginal epithelium is coated with and bathed by vaginal fluids containing mucus (Valenta, 2005). Ideally, the muocoadhesive vaginal drug delivery system will enhance drug absorption by retaining the formulation at the vaginal surface, and releasing the drug at the site for an extended period of time (Woodley, 2001).

Several theories explain mucoadhesion, including electronic, adsorption, wetting, mechanical, fracture, and diffusion or interpenetration theories (Smart, 2005, Chickerin and Mathiowitz, 1999). Substantial research has focused on the last theory, which is the most accepted. It involves two steps: (1) formation of intimate contact between the drug and the mucosa; (2) interpenetration of the polymer chain and mucin, leading to consolidation of adhesion forces (Smart, 2005).

Of the mucoadhesive vaginal drug formulations: gels, tablets, films and pessaries, the gels are more common, due to their advantage of greater contact area in comparison with other formulations, and their current availability in the market. Of the marketed vaginal gels,

Replens is based on polymer or polycarbophil, and intended to moisturise the vagina; Advantage-S®, Conceptrol ® and Gynol II® are marketed as contraceptives and are based on polycarbophil or sodium carboxymethycellulose; and Crinone is a vaginal progesterone gel that uses polycarbophil to provide prolonged release of progesterone (das Neves and Bahia, 2006, Casanas-Roux et al., 1996, Damario et al., 1999). Overall, the mucoadhesion delivery system has proven to be useful for administration and absorption of vaginal drugs.

Absorption of peptide and protein drugs administered via the vagina can be improved by absorption enhancers that act on the vaginal epithelium to increase permeability. The bioavailability of the administered drug can be improved by co-administration of enzyme inhibitors to prevent peptides and proteins from degradation, and by therapeutic formulations and modification of the peptides and proteins to sustain their delivery.

### 6.3.3 Absorption enhancers

Absorption enhancers improve the vaginal absorption of peptide and protein drugs. Richarson et al. (1989) demonstrated that coadministration of surfactant-type absorption enhancers, lysophosphatidylcholine (LPC), palmitoylcarnitine chloride (PCC) and polyoxyehylene-9-lauryl ether (laureth-9), significantly increased (6-9 fold) the vaginal absorption of gentamicin in rats. Subsequently they showed that these enhancers as well as lysophosphatidyglycerol (LPG) and a bile salt-type enhancer, sodium taurodihydrofusidate (STHDF), also facilitated the systemic absorption of insulin administered through the vagina (Richardson et al., 1992). In addition, immunisation of rats with a synthetic peptide from the HIV envelope glycoprotein through the vagina was enhanced with coadministration of LPG (O'Hagan et al., 1992). It has been suggested that the mechanism of surfactant absorption enhancement may involve disruption of the vaginal epithelium to increase its permeability for drug diffusion (Richardson et al., 1989).

Okada et al. (1982, 1983 and 1984) showed that citric acid, a chelating agent, enhanced the vaginal absorption of a potent luteinising hormone-releasing hormone analog (leuprolide) in rats, as evidenced by its increased ovulation-inducing activity. Citric acid may enhance the vaginal absorption of leuproline by acidification and removal of calcium ions from intercellular tight junctions, to facilitate drug diffusion (Okada et al, 1982).

### 6.3.4 Enzyme inhibitors

Co-administration of enzyme inhibitors to protect the peptides or proteins from degradation may improve vaginal absorption. Nakada et al. (1993) demonstrated an enhanced vaginal absorption of calcitonin in rats by co-administration of the exopeptidase inhibitor, bestatin and endopeptidase inhibitors, leupeptin and pepstatin A. Calcitonin is a polypeptide that lowers blood calcium, and its inhibition from degradation by peptidases in the vaginal mucosal homogenate correlated with its enhanced vaginal absorption, as indicated by decreased plasma calcium levels.

### 6.3.5 Improvement of drug stability and half-life

Improvement of stability to enzymes and subsequently the plasma half-life of peptide and protein drugs are believed to enhance both vaginal and uterine drug absorption. Two strategies to increase stability and thus prolong the half-life of peptides or proteins are PEGylation and cyclisation.

#### 6.3.5.1 *PEGylation*

PEGylation is the modification of peptides and proteins by the attachment of polyethylene glycol (PEG) chains. PEGylation greatly extends the half-life of the conjugates in plasma. This is attributed to the increased molecular size of the peptide/protein by the PEG

chain, which reduces its clearance by glomerular filtration in the kidney and masks it from proteolytic degradation. Davies and Abuchowsky pioneered studies of PEGylation in the late 1970's (Abuchowski et al., 1977a, Abuchowski et al., 1977b). The unique properties of PEG as a non-toxic, non-immunogenic polymer resulted in the Food and Drug Administration (FDA) approval of the first PEGylated protein (bovine adenosine deaminase), Adagen® in 1990. Since then, a multitude of studies have demonstrated the effectiveness of PEGylation in improving drug efficacy, providing another 8 novel drugs to the market (Veronese and Mero, 2008). These drugs have plasma half-life time increased by 3-100 times when compared to their parental drug (Table 5).

Table 5. FDA approved PEGylated drugs and their increase in plasma half-life compared to the parent products.

| Trade-name | Parent drug | PEG size (kDa) | Targeted disease | Year of approval | Increase in plasma half-life | Reference |
|---|---|---|---|---|---|---|
| Adagen® | Adenosine deaminase | 5 | Severe combined immunodeficiency disease (SCID) | 1990 | From <30 second to 48-72 hours | (Hershfield et al., 1987, Levy et al., 1988) |
| Oncaspar® | Asparaginase | 5 | Leukemia | 1994 | 3-fold | (Ettinger et al., 1995, Graham, 2003) |
| Neulasta® | G-CSF | 20 | Neutropenia | 2002 | 10-fold | (Piedmonte and Treuheit, 2008, Molineux, 2004) |
| Pegintron® | Interferon-α-2b | 12 | Hepatitis C | 2000 | 11-fold | (Foster, 2004) |
| Pegasys® | Interferon-α-2b | 40 | Hepatitis C | 2001 | 4-fold | (Foster, 2004) |
| Somavert® | hGH | 5 | Acromagaly | 2003 | 3-fold | (Pradhananga et al., 2002, Clark et al., 1996, Esposito et al., 2003) |
| Macugen® | Anti-VEGF aptamer | 40 | Age-related macular degeneration (AMD) | 2004 | 94-fold | (Ng et al., 2006) |
| Mircera® | Erythroprotein | 40 | Anemia | 2007 | 6 to 7-fold | (Mircera®, 2010, Macdougall et al., 2006) |
| Cimzia | Anti-TNFα Fab' | 40 | Rheumatoid arthritis and Crohn disease | 2008 | 13.5-fold | (Chapman, 2002, Blick and Curran, 2007) |

#### 6.3.5.2 *Cyclisation*

Cyclisation is another successful approach to increase stability of enzymes and plasma half–life of peptides and proteins. Growth hormone-releasing factor (GRF) is a hypothalamic regulatory peptide that regulates the release of hormones from the anterior pituitary. Linear GRF has a half-life of only 13 minutes in plasma. Modifications to linear GRF by replacement of labile amino acids improved the half-life to almost 1 hour. The most significant improvement was observed with cyclic GRF analogs, which had half lives of 4 hours and greater (Su et al., 1991). β-secretase (BACE1) is a molecular target for therapeutic intervention in Alzheimer's disease. Cyclisation of the linear phosphino dipeptide isostere inhibitor of BACE1 enhanced its stability in human serum by three-fold (Huber et al., 2009). Osapay et al. (1997) studied the stability of lanthionine-sandostatin and sandostatin analogues in rat brain homogenates. The cyclic lanthionine-sandostatin analog had a 2.4-fold prolonged half-life compared to sandostatine. In addition, a cyclic disulfide bond of indolicidin, an antimicrobial cationic peptide analogue was 4.5-fold more stable in the presence of trypsin (Rozek et al., 2003).

## 7. Plan for the studies in this thesis

PC6 presents a potential target for the development of new non-hormonal and women-controlled contraceptives. As PCs including PC6 are also involved in HIV infectivity, inhibiting PC6 presents exciting target for the development of dual-function contraceptives in women. Although the currently available PC6 inhibitors have proved the importance of PC6 in embryo implantation and HIV infection *in vitro*, it is important to prove the concept that inhibiting PC6 in the female reproductive tract (uterus, cervix and vagina) will prevent pregnancy and vaginally acquired HIV infections in animal models. The key is to identify/develop a potent PC6 inhibitor with appropriate physiochemical properties for *in vivo*

studies and for therapeutic applications. Vaginal delivery of PC6 inhibitors presents the ideal route of administration to prevent pregnancy, and at the same time, to provide protection from sexually transmitted HIV infections. Given that the World Health Organisation has identified an urgent need for wider contraceptive options and for reducing HIV infections in women (UNAIDS, 2012), the developments of dual-role contraception by inhibiting PC6 in the female reproductive tract would have considerable impact on women's reproductive health worldwide.

At the beginning of my PhD research, the aim was to develop potent PC6 inhibitors for vaginal route of administration. The first approach was to modify a published peptidic PC6 inhibitor (Poly R) to improve its physiochemical properties for vaginal administration without affecting its potency to inhibit PC6-dependent cellular processes (Chapter 2). In this chapter, we reported for the first time, that C-terminal PEGylation of Poly R significantly enhanced its therapeutic properties for vaginal drug delivery. One of the C-PEGylated Poly R inhibitors was then tested in the mouse as an animal model, for its efficacy in inhibiting embryo implantation following vaginal administration (Chapter 3). This chapter provides the proof-of-concept that vaginally administered PC6 inhibitors could block embryo implantation. However, the data from both Chapters 2 and 3 identified that mouse is not the ideal animal model for testing vaginally administered drugs when the uterus is the target organ.

The role of PC6 in the establishment of endometrial epithelial receptivity in women was identified in our laboratory in the middle of my thesis research (Heng et al., 2011a, Heng et al., 2011b). Therefore, it becomes important to demonstrate that potential PC6 inhibitors would also inhibit epithelial receptivity and embryo attachment. To assist my research and to fill a knowledge gap in the field, a simple, reliable and high-throughput *in vitro* model for human embryo attachment was established (Chapter 4). This model was proven to be effective

and reproducible to evaluate potential PC6 inhibitors that should theoretically reduce the endometrial epithelial receptivity to human trophoblast spheroids that mimic human embryos.

Throughout the research, I continued to search for non-peptidic PC6 inhibitors because of the concern that the physiochemical properties of peptide-based inhibitors could limit their usefulness in therapeutic application. Several non-peptidic small molecule compounds, reported as inhibitors of furin (another PC member) were obtained from PanThera Biopharma LLC through collaboration, and these compounds were investigated as potential PC6 inhibitors (Chapter 5). We identified one of the compounds (1o) as a potent small molecule PC6 inhibitor with pharmaceutical potential to inhibit embryo implantation.

To evaluate the potency of the PC6 inhibitors that were investigated in this thesis, they were first tested in biochemical assays, and *in silico* docking (through collaboration) to visualise their predicted binding mode in PC6 catalytic site. The most potent P6 inhibitors were then tested for their actions in inhibiting PC6-dependent cellular processes critical for embryo implantation using relevant human cell models: (1) decidualisation of primary HESCs, the methodology for which was well established in our laboratory; (2) the attachment of human trophoblast spheroids to endometrial epithelial cells using the model for human embryo attachment that I established in Chapter 3.

# Chapter 2 –

# PEGylation of a proprotein convertase peptide inhibitor for vaginal route of drug delivery: *In vitro* bioactivity, stability and *in vivo* pharmacokinetics

# Declaration for Thesis Chapter 2

## Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

| **Nature of Contribution** | **Extent of contribution (%)** |
|---|---|
| Performed experiments including activity assay, stability test and all mice work, conducted interpretation of all data, statistical analysis and manuscript writing. | 75 |

The following co-authors contributed to the work. Co-authors who are students at Monash University have the extent of their contribution indicated in percentage terms:

| **Name** | **Nature of contribution** | **Extent of contribution for <u>student</u> co-authors only (%)** |
|---|---|---|
| Tracy Nero | Conducted structural biology analysis and contributed to manuscript drafting | Not applicable |
| Harmeet Signh | Conducted experiments and data analysis | Not applicable |
| Michael Parker | Contributed to manuscript revision | Not applicable |
| Guiying Nie | Conception of project, contributed to data analysis and supervision | Not applicable |

Candidate's Signature

| | **Date** |
|---|---|

## Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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| | | |
|---|---|---|
| **Signature 1** | [redacted] | **Date** 2/1/2013 |
| **Signature 2** | | 20/05/2013 |
| **Signature 3** | | 2 January 2013 |
| **Signature 4** | | 21/05/2013 |

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# PEGylation of a proprotein convertase peptide inhibitor for vaginal route of drug delivery: *In vitro* bioactivity, stability and *in vivo* pharmacokinetics

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ABSTRACT

Uterine proprotein convertase (PC) 6 is critical for embryo implantation in mice and women. It is also one of the PC family members that play a vital role in HIV infectivity. We hypothesized that inhibiting PC6 in the female reproductive tract (vagina, cervix and uterus), may protect women from both pregnancy and HIV infection. One key requirement to prove this concept in an animal model is a vaginally deliverable PC6 inhibitor. Nona-D-arginine (Poly R) is a potent peptide PC inhibitor and is able to inhibit HIV in cell culture. We modified Poly R by PEGylation with different strategies and determined their biochemical properties *in vitro* and *in vivo*. PEGylation at the C-terminus, regardless of the PEG size (30 kDa or 1239 Da) did not compromise the inhibitory potency of Poly R. In contrast, PEGylation at both termini (1239 Da) dramatically reduced its inhibitory activity. Poly R and C-PEGylated Poly Rs also showed equal potency in inhibiting a PC6-dependent cellular process critical for embryo implantation. Poly R and the equipotent C-PEGylated Poly Rs were further tested for their serum stability *in vitro* and pharmacokinetics *in vivo* following vaginal administration in mice. All Poly Rs were equally stable in mouse serum *in vitro* for 24 h; C-PEGylated Poly Rs showed enhanced vaginal absorption and penetration across the vaginal mucosa/epithelium. This is the first report that C-terminal PEGylation significantly enhances the therapeutic properties of Poly R for vaginal drug delivery. Our findings also provide important insights into future design of Poly R derivatives.



## 1. Introduction

The proprotein convertase (PC) family are important enzymes processing a multitude of precursor proteins at basic residues within the general motif of (Arg/Lys)-(X)n-Arg ↓ ($n$ = 0, 2, 4 or 6 and X is any amino acid) [41]. This processing activates hormones, neuropeptides, growth factors, and even membrane fusion proteins and pro-toxins of some viruses and bacteria from precursors into active forms [42,44]. PC6, one of the PC members, plays a critical role in embryo implantation that is pivotal for the establishment of pregnancy [15]. To enable implantation, the uterus must acquire epithelial receptivity and undergo a process known as decidualization to differentiate stromal fibroblasts into phenotypically and functionally distinct decidual cells [38]. The decidualization process is driven by a complex interplay between numerous bioactive factors, including cytokines, growth factors, hormones and proteases [18,37]. PC6 is up-regulated in the uterus specifically at the time of decidualization (mouse and primate) [30,31]. When uterine PC6 production was blocked locally in mice by anti-PC6 morpholino oligonucleotides, decidualization was inhibited and implantation prevented [29]. Therefore, PC6 is an attractive target for contraceptive purposes. Furthermore, PCs including PC6 also play a critical role in HIV infection [9,28,46], and inhibition of PC activity inhibits HIV infection in cells [1,19]. The vagina is usually the initial site of sexually transmitted HIV infection in women; hence vaginal application of anti-HIV drugs is essential to stop the initial HIV transmission. We hypothesize that PC6 is a potential target for the development of a novel, non-hormonal, female-controlled contraception that could also protect women from HIV infection. Vaginal delivery of inhibitors to block PC6 activity in the reproductive tract presents the ideal route of administration to achieve this dual protection.

One of the most potent PC inhibitors known to date is nona-D-arginine (Poly R), a short polybasic peptide comprising 9 arginine residues in the D-configuration [16]. Poly R inhibits PC6 *in vitro* with

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**Table 1**
List of Poly R and derivatives, modification details and molecular weight.

| Name | Modification[a] | MW (Da) |
|---|---|---|
| Poly R | Parental Poly R | 1781 |
| C-30k-PEG Poly R | PEGylation at the C-terminus with a 30 kDa PEG | 31,912 |
| C-1239-PEG Poly R | PEGylation at the C-terminus with a 1239 Da PEG | 3132 |
| C1239N-PEG Poly R | PEGylation at both C- and N-termini with a 1239 Da PEG | 3936 |

[a] Each contains a FAM fluorescent tag at the N-terminus.

a Ki in the nanomolar range and has been shown to inhibit HIV in cell culture [16,24]. However, there is limited data on Poly R for *in vivo* applications and there has been no study on vaginal administration of this peptide inhibitor. Due to the physicochemical characteristics of polypeptides and potential enzymatic degradation, vaginal absorption of Poly R could be low. One of the strategies to address these problems is to covalently link the polypeptide to a polymer, such as polyethylene glycol (PEG) [33]. PEG is FDA approved, non-toxic, non-immunogenic, non-antigenic and highly soluble in water [33]. These properties can be transferred to the PEGylated peptides, to improve the pharmacokinetics and pharmacodynamic profiles of parental peptides [47].

The absorptive mucosa of the vagina has previously been investigated for delivery of peptide drugs [39]. To prolong the residence time of the drug in the vaginal cavity, mucoadhesive vaginal drug delivery systems have been developed. Mucoadhesion is defined as the interaction between a mucin suface and a synthetic or natural polymer [49]. Bioadhesive vaginal gel is used most widely in the mucoadhesive vaginal drug delivery systems. Ideally, the gel will retain the drug in the vaginal cavity for an extended period of time and the drug will be released close to the absorptive mucosa, with a consequent enhancement of absorption and bioavailability [49]. In women, drugs administered through the vagina are preferentially localized to the uterus, a mechanism referred to as the "first uterine pass effect" [4,8]. This was originally identified by the high levels of vaginally administered progesterone in the uterus, despite relatively low serum progesterone concentrations [3–5,14,27].

In this study, we modified Poly R by PEGylation, and examined the inhibitory potency of the PEGylated derivatives against PC6 activity *in vitro*. A binding mode of Poly R in the active site of human PC6 was predicted and visualized by *in silico* docking. PEGylated Poly Rs, showing similar potency to the parental Poly R, were then tested for their ability to inhibit PC6 in primary human endometrial stromal cells to block decidualization and their stability in mouse serum *in vitro*. Finally, their serum pharmacokinetics and tissue distribution were examined in mice following vaginal administration with a bioadhesive gel.

## 2. Materials and methods

### 2.1. Materials

Nona-D-arginine peptide (Poly R) with its N-terminus conjugated to 5(6)-carboxyfluoresceine (FAM) and C-terminal amidated and Poly R derivative peptides (Table 1), each tagged with FAM at the N-terminus, were synthesized [16] (Mimotopes Pty. Ltd., Australia). C-30k-PEG Poly R and C-1239-PEG Poly R have a 30 kDa or 1239 Da PEG respectively added to the C-terminus of Poly R. C1239N-PEG Poly R was PEGylated at both the C- and N-termini with a 1239 Da PEG. Site-specific PEGylation was achieved by covalent attachment through an L-cysteine introduced to the peptide [45].

### 2.2. Inhibition of PC6 activity by Poly R or PEGylated Poly Rs in vitro

An *in vitro* PC6 activity assay, as described previously [16,20], was used to evaluate PC6 inhibition by Poly R and its derivatives. In brief, 2 units of active recombinant human PC6 (rhPC6) (PhenoSwitch BioScience Inc., Québec, Canada) were incubated with Poly R or its derivatives (1 or 10 μM), in Dulbecco's modified Eagle's medium/Ham's F12 culture medium (DMEM/F12, Sigma, St. Louis, MO) in the presence of 100 μM fluorogenic substrate pERTKR-AMC (Bachem, Torrance, CA) at 37 °C. The real-time kinetic progression of substrate hydrolysis [release of fluorescent 7-amino-4-methylcoumarin (AMC)] was monitored every 5 min at excitation/emission of 355/460 nm (Wallac, Victor 2 spectrophotometer; PerkinElmer, Boston, MA) for 1 h. Inhibition of PC6 activity was expressed as a reduction in the rate of substrate hydrolysis relative to the control (PC6 activity in the absence of inhibitory peptides).

### 2.3. In silico docking of Poly R into the catalytic site of hPC6

PCs consist of two separate domains, a spherical catalytic domain and a barrel-like P domain [21]. The crystal structure of the catalytic and P domains of mouse furin [21], with the inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk) covalently bound in the catalytic domain, was used as the template to construct a homology model of hPC6. The seven known hPCs have overall 50–70% sequence identity and the catalytic domains of mouse furin and hPC6 share 96% sequence similarity, allowing for conservative amino acid substitution. The sequences of all human and mouse PCs were aligned using Muscle 3.6 [11,12]. The hPC6 homology model was constructed using SwissPDB (http://spdbv.vital-it.ch/) using the PC multiple sequence alignment as a guide. The two $Ca^{2+}$ ions and the corresponding $Ca^{2+}$-coordinated water molecules present in the mouse furin crystal structure were incorporated into the hPC6 homology model. The high quality of the hPC6 homology model was confirmed with Verify3D (http://nihserver.mbi.ucla.edu/Verify_3D/) [26] and PROCHECK (www.ebi.ac.uk/thornton-srv/software/PROCHECK/refs.html) [25]. The hPC6 active site is a canyon-like groove lined with clusters of negatively charged groups [17]. These clusters or subpockets have been defined as S1–S6 and S1′. The important residues in each sub-pocket are: S1 – Asp277, Asp325; S2 – Asp173, Glu210; S3 – Leu246, Trp273; S4 – Glu255, Asp283; S5 – Asp276, Asp283; S6 – Asp 249, Asp252; S1′ – Lys212, Arg216, His381 [17]. The hPC6 catalytic triad consists of Asp172, His213 and Ser385 [17]. Poly R with N-terminally labeled FAM, was constructed using standard bond lengths and bond angles in Sybyl-X1.2 (Tripos International, http://www.tripos.com). Poly R was set to a random peptide conformation and then structurally optimized within Sybyl-X1.2 using the MMFF94 forcefield and partial atomic charges, conjugate gradient convergence method; termination of the optimization was achieved when the gradient difference of successive steps was <0.05 kcal/mol Å (all other parameters were at default values). The structure of the covalently bound dec-RVKR-cmk inhibitor was removed from the mouse furin crystal structure and also subjected to the same minimization process as the Poly R. Docking of dec-RVKR-cmk and Poly R into the catalytic domain of the hPC6 homology model was carried out using Surflex-Dock (within Sybyl-X1.2, Tripos International) [23]. The protomol was generated using the automated method, a threshold of 0.50 and a bloat value of 2. The Surflex-Dock GeomX mode was used, all other parameters

were at default values. The C-Score function was used to rank the docked compound poses, the top 20 ranked poses for each compound were examined visually. Poly R and dec-RVKR-cmk are flexible compounds that can adopt a multitude of conformations within the hPC6 active site. To ensure that reasonable docked poses were obtained, the docked conformations of dec-RVKR-cmk in the hPC6 homology model were compared to the conformation of the inhibitor seen in the mouse furin crystal structure. The top ranked pose of dec-RVKR-cmk in hPC6 was similar to the conformation adopted in the mouse furin crystal structure (RMSD = 1.5 Å when aligned *via* all heavy atoms), the greatest difference between the two being the conformation of the decanoyl chain. For Poly R, the docked pose occupying a majority of the hPC6 active site S1–S6 sub-pockets was selected as an illustrative example.

### 2.4. Decidualization of human endometrial stromal cells and inhibition by C-PEGylated Poly Rs

Human endometrial tissues were obtained from non-pregnant women undergoing curettage following laparoscopic sterilization or assessment of tubal patency. Ethical approval was obtained from the Human Ethics Committee at Southern Health, Melbourne, Australia and written informed consent was obtained from all participating patients prior to tissue collection. Tissues taken between days 8–24 of the menstrual cycle were collected into DMEM/F12 (Sigma, St. Louis, MO) and processed within 24 h for stromal cell isolation. Human endometrial stromal cells (HESCs) were isolated by enzymatic digestion and filtration as previously described [20,43]. Stromal cells (>97% pure) were cultured in T25 $cm^2$ flasks in DMEM/F12 medium supplemented with 10% charcoal stripped fetal calf serum (CS-FCS, Thermo Electron Corporation, Maple Plain, MN, USA), 2 mM L-glutamine (Sigma), 100 μg/ml streptomycin and 100 IU/ml penicillin (Gibco, Mulgrave, VIC, Australia) [10]. Once 70–80% confluent, the HESCs were passaged into 12-well plates ($8 \times 10^4$ cells/well) and cultured to 80% confluence. For decidualization, cells were treated with estradiol 17-β (E2, $10^{-8}$ M), medroxy-progesterone acetate (MPA, $10^{-7}$ M) and 8-bromoadenosine 3′:5′ cyclic monophosphate (cAMP, $5 \times 10^{-4}$ M) (all from Sigma) for 72 h in serum free DMEM/F12 containing 0.1% BSA. Untreated cells served as non-decidualized controls. Decidualization success was confirmed by a significant increase in the decidual marker prolactin (PRL) in the conditioned medium by ELISA (Bioclone Australia Pty Ltd., Sydney, Australia) as per the manufacturer's instructions [20]. To determine cellular uptake of PEGylated Poly Rs, HESCs were cultured without (control) or with C-30k-PEG Poly R or C-1239-PEG Poly R (1 μM) on 14 mm glass coverslips in the presence of the decidualizing stimuli [E2 ($10^{-8}$ M) + MPA ($10^{-7}$ M) + cAMP ($5 \times 10^{-4}$ M)] for 24 h. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, incubated with 2 μM nuclear stain DAPI (4′,6-diamidino-2-phenylindole, Sigma) for 10 min at room temperature and mounted with fluorosave reagent (Calbiochem Calbiochem, Kilsyth, Australia). The stained cells were analyzed using an Olympus BX60 fluorescent microscope and images taken using an Olympus DP70 camera and DP controller imaging software. To assess decidualization inhibition by Poly R derivatives, HESCs were decidualized in the absence (control) or presence of different concentrations of C-30k-PEG PolyR or C-1239-PEG PolyR (1 μM, 4 μM or 8 μM) for 72 h with the media replaced every 24 h. The time course of inhibition of decidualization was expressed as a percentage reduction in prolactin levels in the conditioned media relative to the control. Three independent experiments were performed using different cell preparations for each experiment.

### 2.5. Serum stability of Poly R and C-PEGylated Poly Rs in vitro

Poly R, C-30k-PEG Poly R or C-1239-PEG Poly R (1 μM) was mixed in 10 μl mouse serum and incubated at 37 °C for 0 or 24 h. Following incubation, the majority of the serum proteins were precipitated by adding 9 volumes of 10% (v/v) trichloroacetic acid (TCA)/acetone. The mixture was incubated for 1 h at −20 °C and centrifuged for 20 min at 15,000 rpm at 4 °C. Poly R and its derivatives remained in the supernatant and were precipitated by adding 9 volumes of 100% acetone. They were then subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gels were scanned at excitation/emission of 488/530 nm using a Typhoon 9400 (GE Healthcare, USA) to visualize the intact peptides containing the FAM tag.

### 2.6. Serum pharmacokinetics and tissue distribution of Poly R and C-PEGylated Poly Rs in mice in vivo following vaginal administration

C57BL/6J mice (6–8 weeks) were housed and handled according to the Monash University animal ethics guidelines on the care and use of laboratory animals (Animal ethics number "MMCB 2009/24"). All studies were approved by the Animal Ethics Committee at the Monash Medical Center, Melbourne, Australia. Mice were designated as Day 0 of pregnancy on the morning of finding a vaginal plug. On Day 4 of pregnancy, Poly R, C-30k-PEG Poly R or C-1239-PEG Poly R (5 μl of 10 mM in 5% acetic acid) was mixed with 10 μl of a placebo vaginal gel (CONRAD, USA), vortexed and applied into the mouse vagina using a pipette. To determine the serum concentration of administered Poly Rs in the mouse circulation, blood was collected from the mouse's tail vein at 60 min, 4 h and 8 h after administration. Serum was prepared by centrifugation of coagulated blood at 3000 rpm for 15 min. Poly Rs were recovered from 20 μl serum by sequential precipitation with 10% TCA/acetone and acetone as described in Section 2.5 and analyzed on 15% SDS-PAGE gels. To calculate the serum concentration of each peptide, standards of the corresponding peptide (Poly R, C-30k-PEG Poly R or C-1239-PEG Poly R) ranging from 12.5 to 100 nM were included on each gel, and band intensity was determined by densitometry.

To determine the tissue distribution of Poly Rs, mice were given a single dose of Poly R, C-30k-PEG Poly R or C-1239-PEG Poly R (5 μl of 10 mM in 5% acetic acid mixed with 10 μl placebo vaginal gel) through the vagina and sacrificed 1 h later. Tissues including the vagina, cervix, uterus, ovary, kidney, liver and heart were collected, fixed in Tissue-Tek® O.C.T. compound (ProSciTech, QLD, Australia) and stored at −80 °C. Cryostat sections were examined under a fluorescent microscope to detect fluorescent signals.

## 3. Results

### 3.1. Poly R is a potent PC6 inhibitor in vitro

We first confirmed that Poly R was a potent PC6 inhibitor *in vitro*, based on its ability to inhibit rhPC6 hydrolysis of a fluorogenic peptide substrate pERTKR-AMC. A progressive emission of fluorescence resulting from substrate hydrolysis was monitored over time in the absence of Poly R, validating the activity assay (Fig. 1A). Poly R inhibited the substrate hydrolysis in a dose-dependent manner (Fig. 1A). When the rate of substrate hydrolysis (AMC release per min) was calculated from the linear phase of each kinetic progression curve and used as the activity unit, Poly R inhibited rhPC6 activity by 40% at 2 μM and by >90% at 10 μM compared to the control (rhPC6 alone) (Fig. 1B). Poly R with and without a FAM tag showed no difference in inhibiting rhPC6 activity (data not shown).

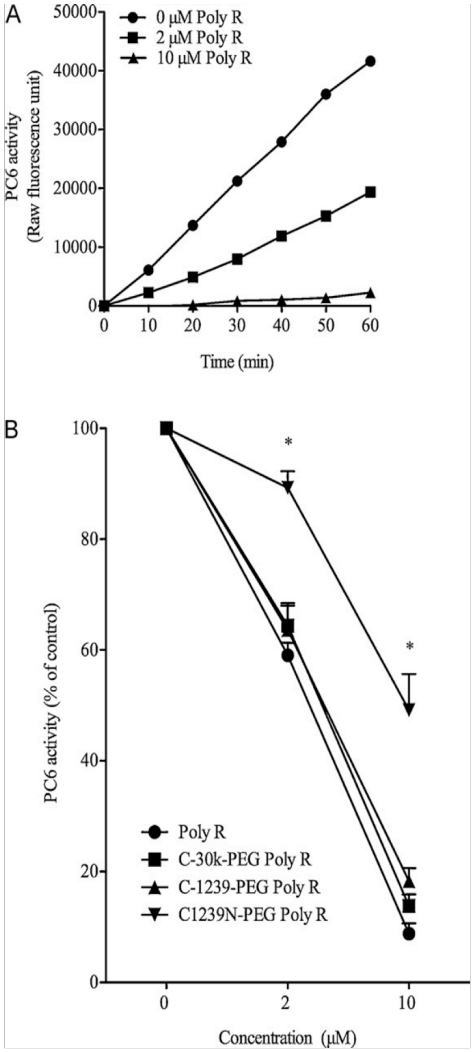


**Fig. 1.** Inhibition of PC6 activity by Poly R and its derivatives. (A) Representative progressive curve of PC6 activity in the presence of different doses of Poly R (0, 2 and 10 μM). (B) Dose-dependent inhibition of PC6 activity by Poly R and derivatives (C-30k-PEG Poly R, C-1239-PEG Poly R and C1239N-PEG Poly R) (2 μM and 10 μM). The data are expressed as percent reduction in the rate of substrate hydrolysis relative to the control (no Poly R). Each value represents mean ± SEM of three independent experiments. $^{*}P < 0.05$.

### 3.2. Strategies to modify Poly R by PEGylation

Poly R has a relatively small molecular weight (MW of 1.7 kDa). To study the effect of PEGylation on Poly R, three differently PEGylated Poly Rs were synthesized (Table 1 and Fig. 2). The C-30k-PEG Poly R had a 30 kDa PEG added to the C-terminus; its MW is 18-fold larger than the parental Poly R. The C-1239-PEG Poly R was also PEGylated at the C-terminus but with a much smaller PEG (1239 Da). The C-1239N-PEG Poly R contained a 1239 Da PEG at both the C- and N-termini. Consequently, C-1239-PEG Poly R and C-1239N-PEG Poly R are approximately 2- and 3-fold larger in MW than the parental Poly R. All the PEGs were specifically attached through a L-cysteine introduced to the Poly R (Fig. 2). All Poly Rs had a N-terminal FAM fluorescent tag (MW, 0.4 kDa) for detection purposes.

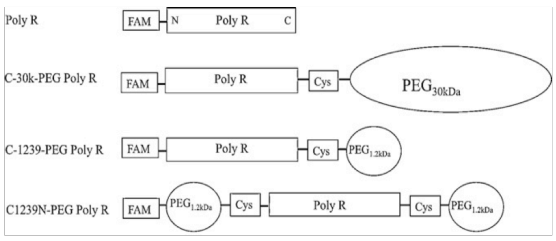


**Fig. 2.** Schematic illustration of the linear structure of Poly R and its derivatives. FAM, 5(6)-carboxyfluoresceine; N, N-terminus; C, C-terminus; Cys, cysteine.

### 3.3. PEGylation at the C-terminus does not compromise the inhibitory potency of Poly R but modification at both termini does

To determine whether various modifications alter Poly R's inhibitory activity against rhPC6, the PC6 activity assay was performed in the presence of each PEGylated Poly R (2 or 10 μM). C-30k-PEG Poly R and C-1239-PEG Poly R were comparable in their inhibitory activity to the parental Poly R (Fig. 1B). In contrast, C1239N-PEG Poly R, although much smaller than C-30k-PEG Poly R, showed a dramatically reduced potency. It inhibited rhPC6 activity by <10% at 2 μM and ~50% at 10 μM compared to 40% and >90% respectively by the parental Poly R (Fig. 1B). As C-30k-PEG Poly R and C-1239-PEG Poly R (referred to as C-PEGylated Poly Rs) showed equal potency to the parental Poly R, they were further examined in cultured cells and in mice *in vivo*.

### 3.4. In silico docking of Poly R explains how the various PEGylation may affect Poly R's inhibitory activity

The precise binding mode of Poly R to the hPC6 catalytic domain is yet to be experimentally determined. The crystal structure conformation of the covalently bound inhibitor dec-RVKR-cmk in the mouse furin catalytic domain suggested that the positively charged D-arginine residues interact with the negatively charged residues in the S1–S6 sub-pockets [21]. The docked pose where the D-arginine residues of Poly R occupy a majority of the hPC6 S1–S6 sub-pockets were therefore selected as an example Poly R–hPC6 binding mode and is depicted in Fig. 3A–C. The positively charged D-arginine side-chains make strong electrostatic interactions with the negatively charged aspartic (D) or glutamic acids (E) in the S1, S2, S3 and S6 sub-pockets. The inhibitor can also form hydrogen bonds/polar contacts with residues lining the active site of hPC6 (Fig. 3C).

The FAM moiety is an aromatic ring system, with a small MW of 0.4 kDa, capable of forming hydrogen bonds and π–π interactions with residues in the hPC6 active site (Fig. 3B and C). *In silico* docking suggested that FAM addition to the N-terminus would not interfere with Poly R interacting with the hPC6 active site (Fig. 3B and C), consistent with this attachment having no impact on the *in vitro* activity of the Poly R (data not shown). The addition of a PEG moiety regardless of the size (1239 Da or 30 kDa) at the C-terminal of Poly R has no significant effect on the inhibition activity of hPC6 (Fig. 1B). The arrow pointing to the C-terminus indicates the location of the PEG attachment to Poly R and it does not impact on any of the S1–S6 sub-pockets (Fig. 3B and C). Therefore, we assumed that Poly R remains conformationally free from the PEG and available for binding to the hPC6 active site. Although PEGylation at the C-terminal is tolerated, the addition of PEG moieties (irrespective of size) at both the N- and C-termini (C-1239N-PEG Poly R) reduced the ability of Poly R to access the hPC6 active site. This is reflected by a reduction in the inhibitory activity of C-1239N-PEG Poly R compared to the derivative modified only at the C-terminal (Fig. 1B).

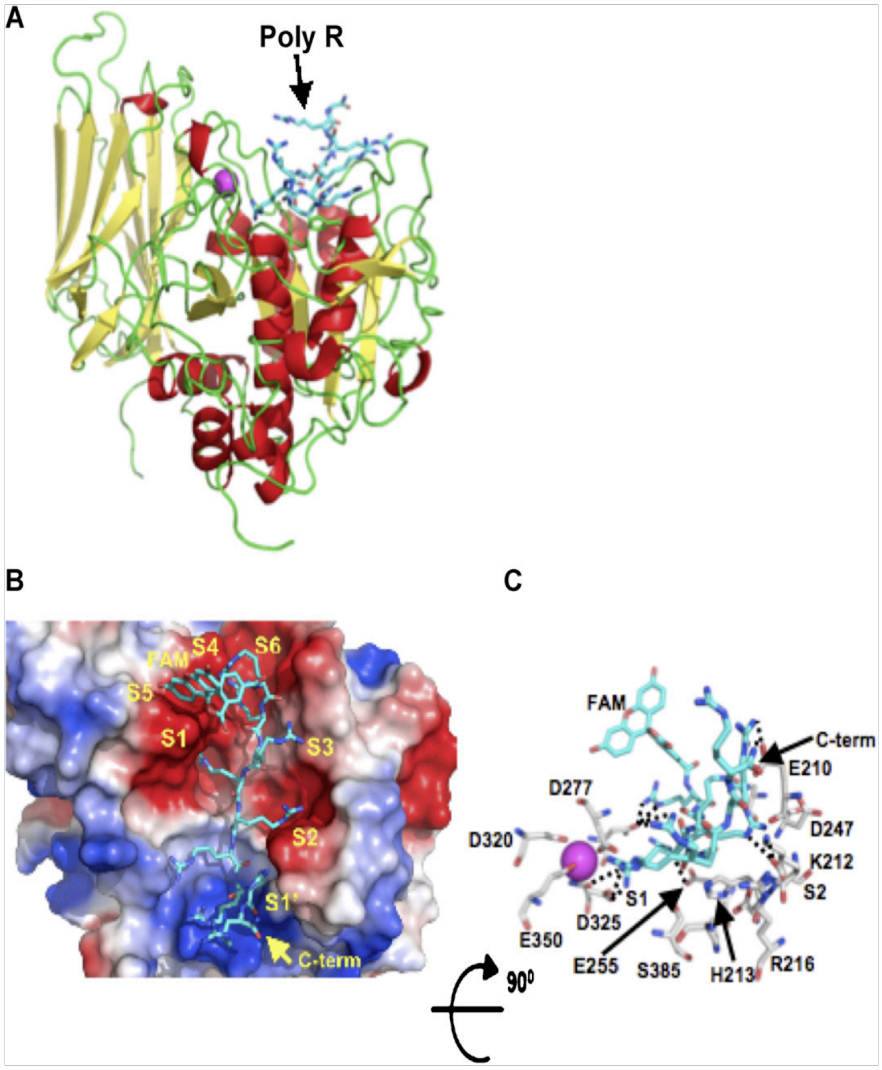


**Fig. 3.** *In silico* docking of Poly R in the active site of hPC6 (putative binding pose). (A) The catalytic domain of the hPC6 homology model with Poly R docked into the catalytic site. hPC6 is depicted as a cartoon (α-helices are colored red, β-strands yellow) and Poly R is shown in stick representation (C atoms are in cyan, N atoms blue and O atoms red). (B) Close up view looking down onto the hPC6 active site. hPC6 is depicted as a molecular surface colored by electrostatic potential (red, negative; blue, positive potential); the red patches on the surface clearly show that the active site groove is largely negatively charged. The location of the sub-pockets S1–S6 and S1′ are indicated. Poly R is shown in stick format (same color scheme as in A) and traverses the hPC6 active site groove. The PEG attachment point is indicated by the C-term label. (C) View rotated approx 90° around the horizontal axis from that shown in (B) to illustrate the depth of Poly R in the hPC6 active site. Poly R makes numerous interactions in the hPC6 active site, for example D325 and E255. Selected side-chains of the S1–S6, S1′ subpockets and catalytic triad residues are shown in C. In (A) and (C) the $Ca^{2+}$ ion closest to the active site is depicted as a magenta sphere and the coordinated water molecules as small red spheres. For clarity, only some of the electrostatic/hydrogen/polar contacts are indicated by black dashed lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### *3.5. C-PEGylated Poly Rs are readily taken up by HESCs and inhibit decidualization in a dose-dependent manner*

Decidualization of HESCs is a cellular process essential for embryo implantation. PC6 activity is absolutely required for decidualization and Poly R inhibits the process [20]. To determine whether C-PEGylation would affect cellular uptake of Poly R, HESCs were cultured without or with 1 μM C-PEGylated Poly Rs in the presence of decidualizing stimuli as previously described for Poly R [20]. Cellular uptake, represented by the strong FAM fluorescence of C-30k-PEG Poly R, was observed in the treated but not control cells (non-treated) (Fig. 4A) Similar cellular uptake was also seen for C-1239-PEG Poly R (data not shown). When the cells were treated with C-30k-PEG Poly R during decidualization, it inhibited decidualization in a dose-dependent manner, inhibiting ~20% at 1 μM, ~70% at 4 μM and >90% at 8 μM (Fig. 4B). A similar dose-dependent inhibition was also observed for C-1239-PEG Poly R (data not shown).

### *3.6. Poly R and C-PEGylated Poly Rs are equally stable in mouse serum in vitro*

Following incubation with mouse serum at 37 °C for 0 h or 24 h, intact bands of Poly R and C-1239-PEG Poly R were detected at the expected MW on SDS-PAGE gels (Fig. 5A). Bands of intact C-30k-PEG Poly R were also detected at approximately 65 kDa (Fig. 5A). The higher than expected MW for C-30k-PEG Poly R was likely due to interactions between the large PEG and SDS in the SDS-PAGE gel as previously reported [48,50]. For each peptide, the band intensity was comparable between 0 and 24 h time points ($n = 4$) (Fig. 5B), indicating that they were stable in mouse serum for at least 24 h.

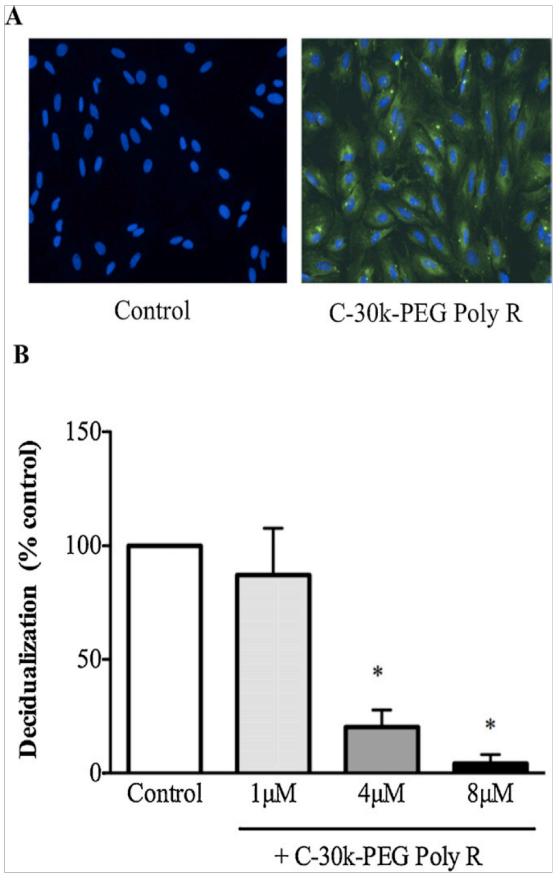


**Fig. 4.** Cellular uptake of C-30k-PEG Poly R and inhibition of decidualization of HESCs. (A) HESCs were cultured without (control) or with 1 μM C-30k-PEG Poly R for 24 h in the presence of the decidualizing stimuli. The FAM signal (green) represents cellular uptake. Nuclei are in blue and images are at 40× magnification. (B) Dose-dependent inhibition of decidualization by C-30k-PEG Poly R. The data are expressed as percentage reductions relative to control (no Poly R). Each value represents mean ± SEM of three independent experiments. *$P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 3.7. *In vivo pharmacokinetics of Poly R and C-PEGylated Poly Rs in mice following vaginal delivery*

The serum pharmacokinetics of Poly R and C-PEGylated Poly Rs in mouse *in vivo* was determined by measuring their serum concentration over 8 h following vaginal delivery (Fig. 6). All Poly Rs were detected at 1 h, but only C-30k-PEG Poly R remained detectable at 4 and 8 h (Fig. 6). The C-30k-PEG Poly R and C-1239-PEG Poly R reached a comparable maximum concentration ($C_{max}$) (19 and 22 nM respectively), which was much higher than that of Poly R (6 nM) (Table 2). The time to reach $C_{max}$ ($T_{max}$) was the same for all Poly Rs (1 h) and C-30k-PEG Poly R remained at its $C_{max}$ at 1–4 h. The area under the curve (AUC) for C-30k-PEG Poly R was 10-fold and 2-fold greater than that of Poly R and C-1239-PEG Poly R respectively (Table 2).

**Table 2**
Pharmacokinetics of Poly R, C-30k-PEG Poly R and C-1239-PEG Poly R in mouse serum following vaginal administration.

| | Poly R | C-30k-PEG Poly R | C-1239-PEG Poly R |
|---|---|---|---|
| Area under the curve (AUC) | 721 | 7443 | 2681 |
| $T_{max}$ (h) | 1 | 1–4 | 1 |
| $C_{max}$ (nM) | 6 | 19 | 22 |

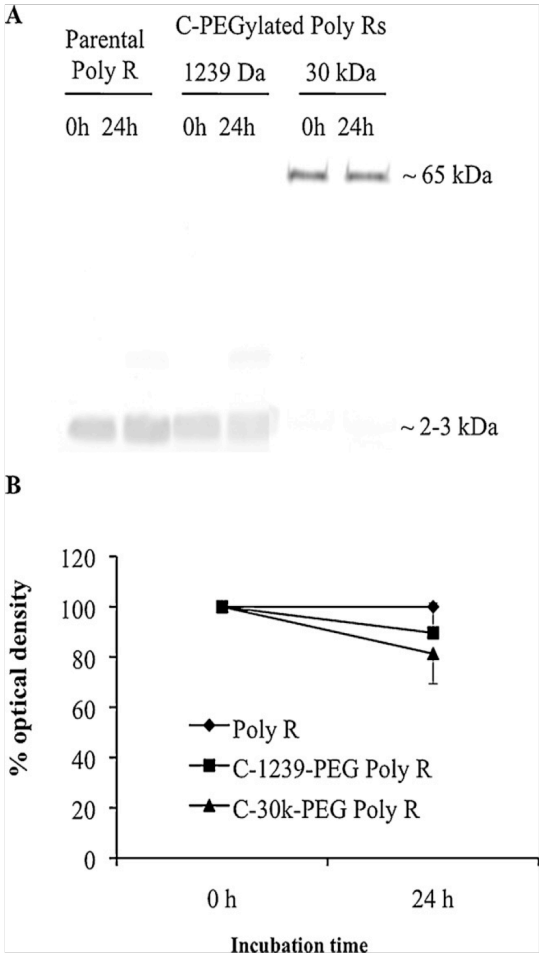


**Fig. 5.** Detection of Poly R, C-30k-PEG Poly R and C-1239-PEG Poly R in mouse serum by SDS-PAGE following incubation at 37 °C for 0 or 24 h. (A) Representative fluorescence image of SDS-PAGE. No significant degradation was observed over 24 h. (B) Relative band density of each Poly R at 0 and 24 h, each value represents mean ± SEM of four independent experiments.

### 3.8. *Tissue distribution of Poly R and C-PEGylated Poly Rs in mice in vivo following vaginal administration*

The tissue distribution of Poly R and C-PEGylated Poly Rs was examined 1 h after vaginal administration (Fig. 7). High

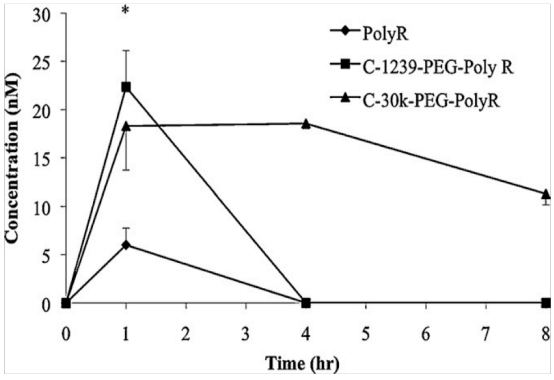


**Fig. 6.** Detection of Poly R, C-30k-PEG Poly R and C-1239-PEG Poly R in mouse circulation 8 h following vaginal administration. Each value represents mean ± SEM of at least three animals. *$P < 0.05$.

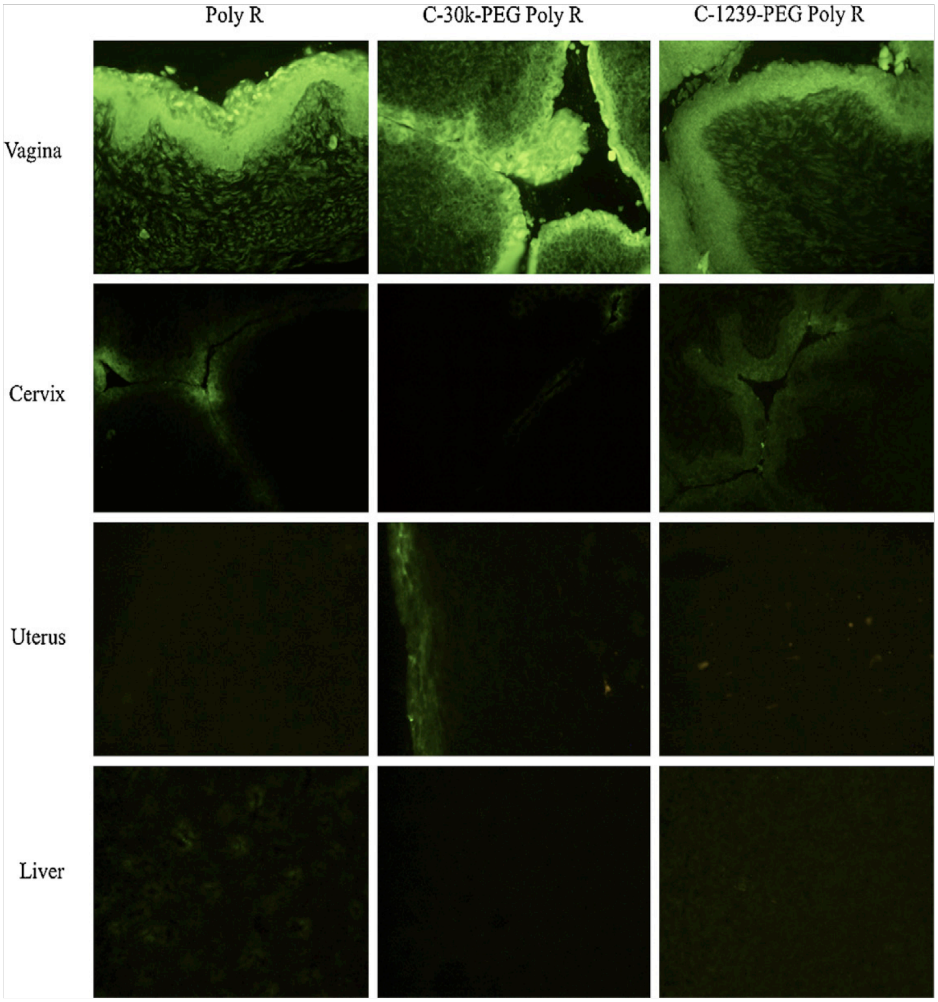


**Fig. 7.** Tissue distribution of Poly R, C-30k-PEG Poly R and C-1239-PEG Poly R in mice 1 h following vaginal administration. The vagina, cervix, uterus and liver tissues are shown.

fluorescence signal was observed in the vaginal tissues of all treated mice. For Poly R, the majority of the fluorescence signal was concentrated in the vaginal stratified squamous epithelium whereas the signal in the underlying submucosa was relatively low. The vaginal tissues of mice treated with C-30k-PEG Poly R showed a thinner layer of concentrated signal in the stratified squamous epithelium and the signal in the immediate submucosal stroma was obvious, indicating penetration or diffusion from the epithelium into the submucosa. For C-1239-PEG Poly R, the signal in both the stratified squamous epithelium and the underlying submucosa was less intense. This may be due to the higher absorption of C-1239-PEG Poly R, which was reflected by higher concentrations in the serum. In cervical tissues, clear fluorescent signal was observed for both Poly R and C-1239-PEG Poly R, but intensity was relatively low for C-30k-PEG Poly R. This may suggest that C-30k-PEG Poly R exhibited higher mucoadhesive properties and formed stronger interaction with the vaginal mucosa, resulting in less expansion to the cervix. There was no signal observed in the uteri of Poly R or C-1239-PEG Poly R treated mice. In some of the C-30k-PEG Poly R treated mice, fluorescent signal was observed at the mesometrial side of the uterus. Clear signal was observed in the liver of mice treated with Poly R or C-1239-PEG Poly R but not with C-30k-PEG Poly R, suggesting that the smaller Poly Rs were cleared more rapidly *via* hepatic metabolism. There was no significant signal detected in the kidney of all treated mice, suggesting that the Poly Rs were not predominantly excreted by renal clearance. The ovary and the heart of all the treated mice were also collected and examined, but no significant fluorescence signals were observed in these tissues (data not shown).

## 4. Discussion

This study investigated the *in vitro* bioactivity, stability and *in vivo* pharmacokinetics of Poly R and its PEGylated derivatives. We revealed that Poly R tolerated C-terminal PEGylation regardless of the PEG size. C-PEGylated Poly Rs and the parental Poly R were comparable in their inhibitory activity against rhPC6 *in vitro* and in human endometrial stromal cells. A likely binding mode of Poly R in the hPC6 active site was predicted and visualized by *in silico* docking, providing an understanding on the impact of the different PEGylations on the interaction between the peptides and the enzymatic site. Following vaginal administration, the PEGylated Poly Rs showed significantly enhanced vaginal uptake, compared to the parental Poly R, and the C-30 kDa PEGylation remarkably increased the *in vivo* serum half-life of Poly R.

Our finding that Poly R tolerates C-terminal modification without loss of inhibitory activity is important. Attachment of even a 30 kDa PEG to the C-terminal of Poly R does not affect its binding

to the active site of hPC6. PEGylated Poly R containing a moiety 18 times the size of the peptide retains full inhibitory potency against hPC6. These findings have significant implications for future design of Poly R derivatives with specific properties, such as enhanced cellular uptake and specificity, without sacrificing its inhibitory potency.

We have previously demonstrated that Poly R is readily taken up by HESCs during decidualization and inhibits decidualization in a dose-dependent manner [20]. The same results were observed for C-30k-PEG Poly R and C-1239 PEG Poly R, suggesting that C-terminal PEGylation and the increase in MW do not affect Poly R's cellular uptake or ability to inhibit decidualization of HESCs.

Although Poly R has been widely used for *in vitro* studies, there is limited data on the *in vivo* application of this peptide PC inhibitor. We report for the first time that Poly R is stable in serum for at least 24 h. The stability is likely attributed to its D-arginines. Substitution of L-amino acids with the D-isomers in several peptides that are susceptible to enzymatic cleavage is known to significantly increase their stability [34]. An example is the modification of cetrorelix, a decapeptide antagonist of luteinizing hormone releasing hormone. When five of the ten cetrorelix residues are replaced by the D-isomers, it is highly resistant to degradative enzymes and stable for up to 50 h at 37 °C [36].

Our study used the mouse as an *in vivo* model to evaluate mucosal delivery through the vagina. Vaginal absorption varies with the estrous cycle in rodents (mice and rats) and the vaginal mucosa is most permeable at diestrus [13,32]. We administered Poly R or C-PEGylated Poly Rs into the mouse vagina during early pregnancy, when the vaginal mucosa resembles that of prolonged diestrus [40]. Although the mouse is not a perfect model, it has been shown that its vaginal mucosa at diestrus is relevant for study of vaginal delivery, and results can be translated to humans [7].

The relatively low concentration and short circulating time of Poly R were likely the combined results of its low penetration across vaginal mucosa/epithelium and rapid clearance by hepatic metabolism. The significantly higher concentration of C-30k-PEG Poly R and C-1239-PEG Poly R in the circulation was likely attributed to their higher penetration efficiency. However, similar to Poly R, C-1239-PEG Poly R was rapidly cleared by hepatic metabolism. The prolonged serum half-life of C-30k-PEG Poly R in the circulation was likely due to its low hepatic clearance.

The serum pharmacokinetic profiles and vaginal tissue distribution of Poly R and PEGylated Poly Rs suggest that PEGylation significantly enhanced the vaginal absorption and the penetration of Poly R through the vaginal mucosa/epithelium. PEG is widely used as a mucoadhesive agent [22], hence it is suggested that PEGylation enhanced the mucoadhesive properties of Poly R, facilitating its the vaginal uptake. PEGylated nanoparticles penetrate the mucosal layer more efficiently than unmodified nanoparticles following intravaginal delivery [7]. To our knowledge, ours is the first report of a peptide with improved therapeutic properties for vaginal route of administration following PEGylation.

The phenomenon of preferential localization of vaginally administered drugs to the uterus has been widely reported in women [2,4–6,8]. However, our data showed limited evidence of such a transport mechanism in mice. Although a more sensitive detection technique will be required to confirm this result, similar observations have been reported [7,35]. Although signals of C-30k-PEG Poly R were detected in the uterus following vaginal administration, however the signal was relatively low, suggesting that mouse is not the best animal model to investigate the effect of vaginally administered drugs to the uterus. Further studies will investigate the effectiveness of uterine uptake of vaginally administered Poly R's in other animals.

In summary, we investigated for the first time a potent peptide PC inhibitor, Poly R, and its C-PEGylated derivatives for delivery to the uterus through the vaginal in mice. Attachment of a PEG as large as 30 kDa to the C-terminal end of Poly R does not compromise its binding to the active site of hPC6 or its inhibitory potency. These findings provide important insights into future design of Poly R derivatives. In addition, our data showed that PEGylation provides an effective strategy to modify peptides for vaginal mucosal delivery.

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# Supplementary data (unpublished)

**Note S1: Cyclic Poly R**

Cyclisation is another strategy that has been used successfully to improve pharmacokinetic and pharmacodynamic properties of peptides (Werle and Bernkop-Schnurch, 2006). It has been widely applied to modify peptides to enhance their stability against enzymatic degradation and stabilise their conformation necessary for binding (Tugyi et al., 2005, Zhao et al., 2010, Clark et al., 2005). Therefore, we modified Poly R by cyclisation and examined its inhibitory potency against PC6 activity *in vitro* and its binding mode to PC6 active site by *in silico* docking. Head-to-tail cyclisation of Poly R was achieved via a disulfide bond between the two L-cysteines introduced at both ends of the peptide (synthesized by Mimotopes Pty. Ltd., Australia) (Figure S1). In biochemical *in vitro* activity assay, cyclic Poly R showed a dramatically reduced potency, inhibited rhPC6 activity by <10% at 2 μM and ~ 50% at 10 μM compared to 40% and >90% respectively by the parental Poly R (Figure S2). Cyclic Poly R was docked into the homology model of the hPC6 catalytic domain to visualise its binding mode (Figure S3). It was shown that cyclic Poly R has decreased conformational flexibility and entropy, resulting in inability to bind into the hPC6 active site groove as deeply as the acyclic inhibitors. For example, Poly R is able to interact with D325, E255 and K212 (refer to previous data), whereas cyclic Poly R is unable to reach these residues (Figure 3S). The reduced level of interaction with hPC6 is consistent with the observed reduction in inhibitory activity of cyclic Poly R compared to the parental inhibitor (Figure 2S).

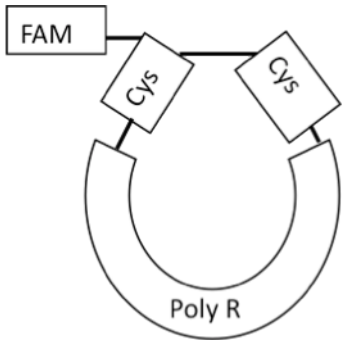


Figure S1. Schematic illustration of Cyclic Poly R

FAM, 5(6)-carboxyfluoresceine; N, N-terminus; C, C-terminus; Cys, cysteine.

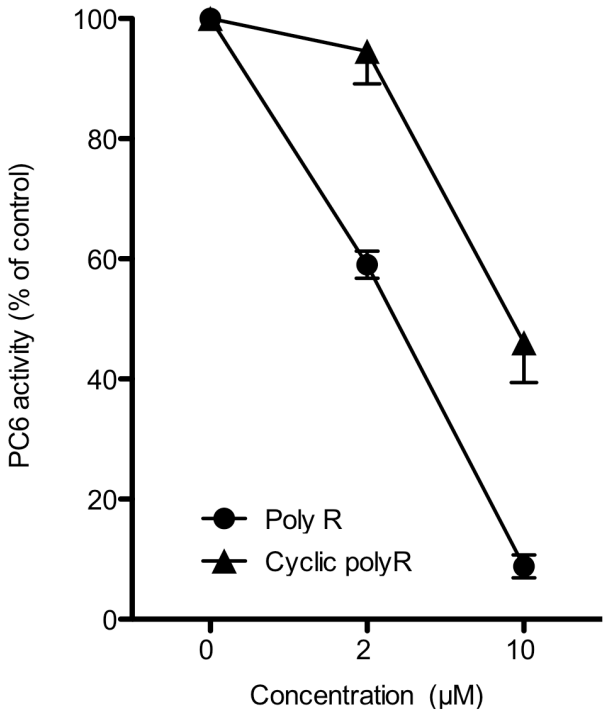


Figure S2. Inhibition of PC6 activity by Poly R and cyclic Poly R

Dose-dependent inhibition of PC6 activity by Poly R and Cyclic Poly R (2 µM and 10 µM). The data are expressed as percent reduction in the rate of substrate hydrolysis relative to the control (no Poly R). Each value represents mean ± SEM of three independent experiments.

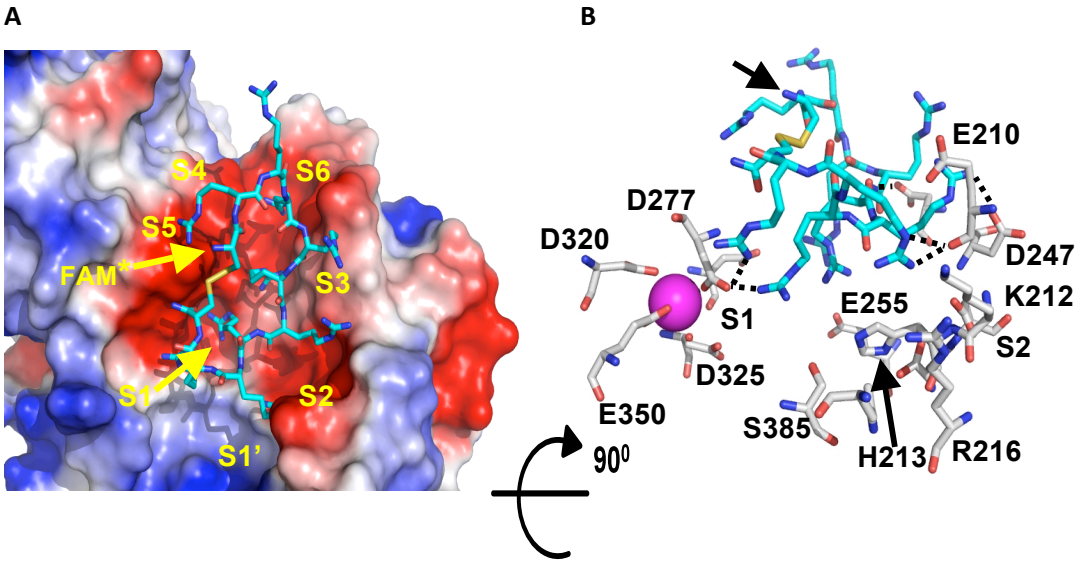


Figure S3. *In silico* docking of cyclic Poly R to the active site of hPC6

A. hPC6 is depicted as a cartoon (α-helices are coloured red, β-strands yellow) and cyclic Poly R is shown in stick representation (C atoms are in cyan, N atoms blue and O atoms red). The disulfide linker is coloured yellow. hPC6 is depicted as a molecular surface coloured by electrostatic potential (red = negative, blue = positive potential); the red patches on the surface clearly show that the active site groove is largely negatively charged. The location of the sub-pockets S1-S6 and S1' are indicated. B. View rotated approx 90$^{o}$ around the horizontal axis from that shown in A to illustrate the depth of cyclic Poly R in the hPC6 active site. Cyclic Poly R cannot bind as deeply as the acyclic Poly R in the hPC6 active site, for example it is unable to reach D325 and E255. For clarity, the FAM moiety is not shown, the attachment point is indicated by the FAM* label. In B, $Ca^{2+}$ ion closest to the active site is depicted as a magenta sphere and the coordinated water molecules as small red spheres. For clarity, only some of the electrostatic/hydrogen/polar contacts are indicated by black dashed lines.

# Chapter 3 –

# Inhibition of embryo implantation in mice following vaginal administration of C-30k-PEG Poly R

# Introduction

The World Health Organization has called for the urgent development of safe contraceptive and HIV prevention options that can be controlled and managed by women (UNAIDS, 2012). Maternal mortality associated with pregnancy and childbirth has accounted for 287,000 deaths in 2010 with 99% occurred in developing countries (WHO, 2012). Affordable and reliable contraception that can be controlled by women is critical to improve maternal and child health and will also overcome the problem of gender inequality with respect to contraceptive management. In addition, the intervention of such contraceptives will reduce poverty and chronic hunger in the world's least developed countries, where the situation is made more difficult by their high rates of population growth. Furthermore, about half of the 34 million people living with HIV are women because the infection is spread predominantly through sexual transmission. The development of contraceptives with anti-HIV agents formulated as vaginal gels is thus of great interest and significance.

The critical role of PC6 in embryo implantation and its involvement in HIV infectivity has made it a potential target for the development of non-hormonal contraceptives with the added benefit of reducing HIV. Vaginal administration of a PC6 inhibitor presents an ideal route to achieve this dual-function. An effort to develop a vaginal-deliverable PC6 inhibitor based on Poly R was described in Chapter 2. The study revealed C-30k-PEG Poly R as a potential PC6 inhibitor with unique characteristics such as enhanced vaginal absorption and penetration. Menkhorst et al (2011) reported the successful blocking of embryo implantation following vaginal administration of a PEGylated Leukemia inhibitory factor (LIF) antagonist. Therefore, this chapter aims to conduct a proof-of-concept study to evaluate the contraceptive

potential of C-30k-PEG Poly R in inhibiting PC6 to block implantation following vaginal administration in mice. PC6 is essential for decidualisation, a process critical for embryo implantation, and inhibition of uterine PC6 production inhibits decidualisation and blocks implantation in mice (Nie et al., 2005b).

## Materials and Methods

### Animals

C57BL/6J mice were housed and handled according to the Monash University animal ethics guidelines on the care and use of laboratory animals. All studies were approved by the Animal Ethics Committee at the Monash Medical Centre, Melbourne (Animal ethics number "MMCB 2009/12").

### Materials

C-30k-PEG Poly R was produced by site-specific and covalent attachment of a 30kDa PEG through an L-cysteine introduced to the C-terminus of nona-D-arginine (Poly R) (Mimotopes Pty. Ltd, Australia) (Details in Chapter 2).

### Determination of time window for C-30k-PEG Poly R delivery

Female mice (6-8 week) were mated with stud males, and the morning of finding a vaginal plug was designated as Day (D) 0 of pregnancy. At D4 2pm, D4 6pm and D5 2pm, mice were sacrificed for visual examination of implantation sites in the two uterine horns and the progress of the implantation process by haematoxylin and eosin (HE) staining of cross sections of fixed tissue.

**Vaginal application of C-30k-PEG Poly R**

C-30k-PEG Poly R (5 µl of 3mM in 5% acetic acid) was mixed with 10 µl of a placebo vaginal gel (CONRAD, USA), vortexed and applied into the mouse vagina using a 200 µl pipette. The volume of infused gel (10 µl) was optimised. The ratio of gel to inhibitor in solvent was 2 to 1 for optimal retention in vaginal cavity and 15 µl was the maximum volume that could be contained in the vaginal cavity of a mouse. A total of 5 applications at D3 10pm, D4 10am, 4pm, 10pm and D5 10am of pregnancy were given to each mouse as this time window of treatment was found to be most optimal (see results 3.3). Control mice treated with the gel, which was mixed with PEG in place of C-30k-PEG Poly R were included in each experiment. Treated mice were killed at D5 2pm of pregnancy and their implantation sites were examined and weighted. The size of implantation sites of treated mice were compared to that of control mice, and the effect was determined by ratio (treated/control). The ratio is < 1 if implantation sites were smaller (partially inhibited), ≥ 1 if uninhibited and 0 if is completely inhibited.

**Desmin immunohistochemistry**

Paraffin-embedded, formalin-fixed implantation sites were dewaxed in histosol and rehydrated through ethanol to water. Endogenous hydrogen peroxidase activity was quenched using 3% $H_2O_2$ in methanol for 10 min at RT followed by washing with high salt tris-buffered saline (TBS) (300 mM NaCl, 5mM TrisCl in distilled water pH7.6). Sections were blocked in non-immune serum (15% normal goat serum in 0.1% Tween-20 high salt TBS) for 30 min at RT followed by incubation in primary antibody (Desmin, 2.9 µg/ml, M0760, DAKO or negative mouse IgG 2.9 µg/ml DAKO) in blocking buffer (10% normal goat serum in 0.1% Tween-20 high salt TBS) for 30 min at RT. Sections were washed 3 times with 0.6% Tween-20 in high salt TBS before the incubation with the Envision$^+$ system labelled polymer-HRP anti-mouse (K4001,

DAKO) for 30 min at RT. The sections were washed twice with 0.6% Tween-20 in high salt TBS and twice with high salt TBS, the substrate 3'3-diaminobenzidine (K3466, DAKO) was then applied to visualize the signal. Negative controls were included in each run.

# Results

**Determination of the optimal time window for C-30k-PEG Poly R delivery**

To determine the optimal time window for vaginal administration of C-30k-PEG Poly R, the progress of mouse embryo implantation in control mice was examined by analysing implantation sites collected at D4 2pm, D4 6 and D5 2pm. Before D4 12pm, the embryos are located in the uterine lumen and can be flushed out (White et al., 2007). Around 2pm on D4, embryos were spaced out along the uterine horns and the implantation sites were just visible as faint bands as a result of increased vascular permeability due to the initiation of decidualisation (Herington and Bany, 2009) (Figure 1A). Before this time point, implantation sites were not visible by eye (data not shown). At D4 6pm and D5 10am, the implantation sites became more obvious as they swelled in accordance with the expansion of the decidual zone (Figure 1B and C). Therefore, the size/weight of the implantation sites directly reflects the extent of decidualisation. Following sectioning through fixed implantation sites, Figure 2A shows a cross section of the implantation site at D4 2pm; the embryo is located in the uterine lumen. At D4 6pm, the embryo has started to invade into the uterine tissue (Figure 2B). At D5 2pm, the embryo is completely embedded within the uterine tissue (Figure 2C).

Our strategy was to inhibit implantation by multiple administration of C-30k-PEG Poly R before (D3 10pm and D4 10am) and during the early stages (D4 4pm, 10pm and D5 10am) of

decidualisation to stop the decidualisation from initiation and/or progression (Figure 1). Administration later than this window was not tested, as our aim was to stop implantation rather than induce abortion after the embryo has embedded in the uterus tissue.

**Contraceptive efficacy of C-30k-PEG Poly R**

At Day 5 2pm of pregnancy, control (n = 7) and treated mice (n = 17) were sacrificed and their uteri examined for implantation sites. Visible implantation sites were excised and weighed to determine the size (in accordance to the extent of decidualisation). The size would be reduced if C-30k-PEG Poly R affects the decidualisation process. The ratio of implantation site weight (treated/mean control) was calculated for each treated mouse. The data were plotted and analysed by the Wilcoxon Signed Rank Test (Figure 3). The mean ratio = 0.7465 with a $p$ = 0.0017, suggesting a highly significant inhibition.

Of the 17 mice treated with C-30k-PEG Poly R, 24% displayed a complete inhibition of implantation, 47% showed a partial inhibition, while in 29% there was no effect (Figure 4 and Table 1). Implantation sites of those mice showing a complete inhibition were visible only as bands (no swelling); these were confirmed by histology to contain blastocysts but not many decidual cells (details in section below). Representative uterine horns with control, partially and completely inhibited implantation sites are shown in Figure 5. The number of implantation sites in the groups with partial and uninhibited implantation was not different.

**Histological examination and immunohistology**

The C-30k-PEG Poly R delivered through the vagina was expected to affect decidualisation of uterine stromal cells. In control mice, the embryo is completely embedded within the uterine tissue, and the uterus is highly decidualised, showing a large decidual zone

identified by immunostaining with desmin (Figure 6A). Mice showing a partial inhibition of implantation had smaller decidual areas, indicated by less immunostaining of desmin within the uterus (Figure 6B). In addition, within the uterine tissue, embryo invasion towards the antimesometrial side was much less advanced in the treated than control mice (Figure 6A-B).

In the implantation sites of mice showing a complete inhibition, the decidual reaction was clearly reduced (Figure 6C). Importantly, although the embryo had attached to the uterine luminal epithelium, it remained within the lumen and did not invade into the stromal endometrial (Figure 6C).

## Discussion

In this chapter, *in vivo* experiments using mice as the animal model showed that vaginal administration of a PC6 inhibitor, C-30k-PEG Poly R, had some potential as a contraceptive. In some mice, C-30k-PEG Poly R stopped implantation by inhibiting the decidualisation process during early pregnancy. This study proved the concept that the inhibition of PC6 in the uterus, which has been shown to be critical for decidualisation in mice and women (Heng et al., 2010, Okada et al., 2005, Wong et al., 2002), has potential for the development of non-hormonal contraceptives for women.

Numbers of difficulties were encountered when using mice as a model for this study. Firstly, the vaginal cavity of a mouse is very small, which severely limits the amount of inhibitor that can be administered. In addition, it is difficult to prevent the leakage of the inhibitor from the vaginal cavity or to prevent the mice from cleaning their vagina following administration. To minimise these problems, C-30k-PEG Poly R at high concentration was used to maximise its

delivery in a small volume, and mice were monitored for at least 10 minutes following the administration to ensure it was not immediately removed by licking. However, it was difficult to be sure that all of the inhibitor applied was retained in the vaginal cavity for absorption. It should be noted that detection of plugging of mice following mating does not ensure pregnancy. To make sure that each mated mouse was truly pregnant prior to vaginal administration (particularly important for the complete inhibition group), they were examined at the end of the study for the presence of multiple corpora lutea (CL) in their ovaries to confirm that normal ovulation had occurred. Any mice showing no implantation sites and without multiple CL were excluded from the experiment. This made the accumulation of a sufficient number difficult.

Vaginally administered C-30k-PEG Poly R impaired implantation in the majority of mice (71% when the complete and partial inhibitions are combined). However, the treatment caused a complete inhibition of implantation only in 24% of mice. This result is most likely attributed to the limited localisation of C-30k-PEG Poly R to the uterus, specifically the stromal cells following vaginal administration (discussed in Chapter 2). The pharmacokinetic properties of C-30k-PEG Poly R described in Chapter 2 showed relatively low signals of the inhibitor reaching the uterine stromal cells, even though it could be effectively absorbed from the vaginal cavity (Ho et al., 2012b). This study suggests that the "first uterine pass effect" (vaginally administered drugs preferentially localise to the uterus) that has been widely demonstrated in women (see Chapter 1), is lacking in mice. Within this context, inhibition of implantation in 71% of mice (24% complete and 47% partial) was encouraging, strongly suggesting that the inhibition could be much higher if efficacy of delivery can be improved in a more appropriate animal model.

In light of these findings, our group subsequently tried the rabbit as an alternative model and achieved a much higher percentage (60%) of complete inhibition of embryo implantation (Aljofan et al., 2012), further proving the concept that PC6 could be targeted for contraception. The efficiency of C-30k-PEG Poly R in inhibiting vaginally acquired HIV infection is yet to be determined. Furthermore, future studies are required to establish the efficiency of C-30k-PEG Poly R in non-human primates before progressing to human clinical trials.

**Figure 1**

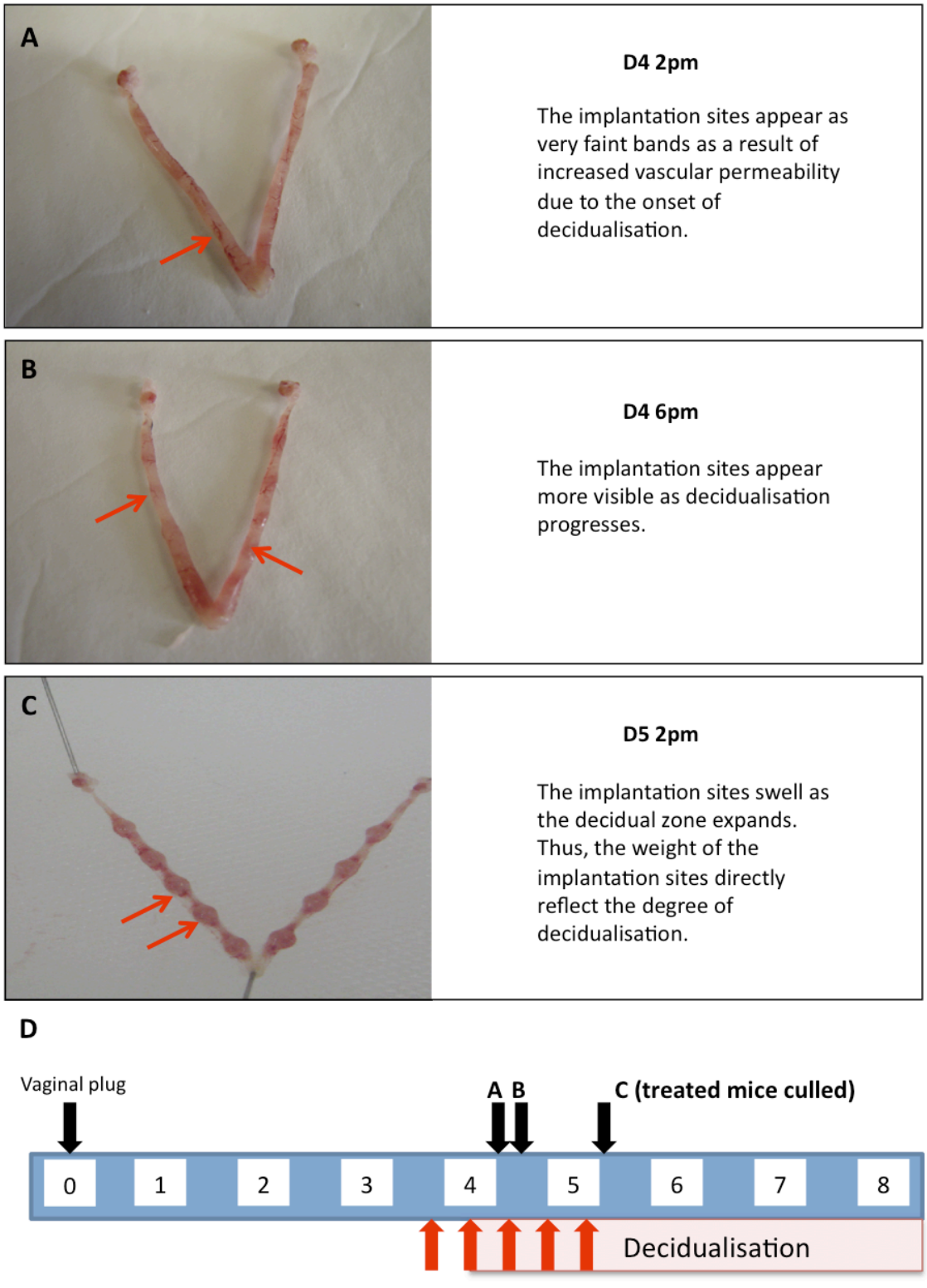
A
D4 2pm
The implantation sites appear as very faint bands as a result of increased vascular permeability due to the onset of decidualisation.
B
D4 6pm
The implantation sites appear more visible as decidualisation progresses.
C
D5 2pm
The implantation sites swell as the decidual zone expands. Thus, the weight of the implantation sites directly reflect the degree of decidualisation.
D
Vaginal plug
A B
C (treated mice culled)
0
1
2
3
4
5
6
7
8
Decidualisation

Figure 1. Mouse uterine horns from control animals showing the appearance of implantation sites between D4 and D5 of pregnancy, and timing of experimental events from D0 to D8

(A) D4 2pm; (B) D4 6pm; (C) D5 2pm, some of the implantation sites are pointed out with arrows and (D) timing of event from D0 to D8. Red arrows represent the time of C-30k-PEG Poly R treatment. Treated mice were culled at D5 2pm. Decidualisation phase is referenced from (Herington and Bany, 2009).

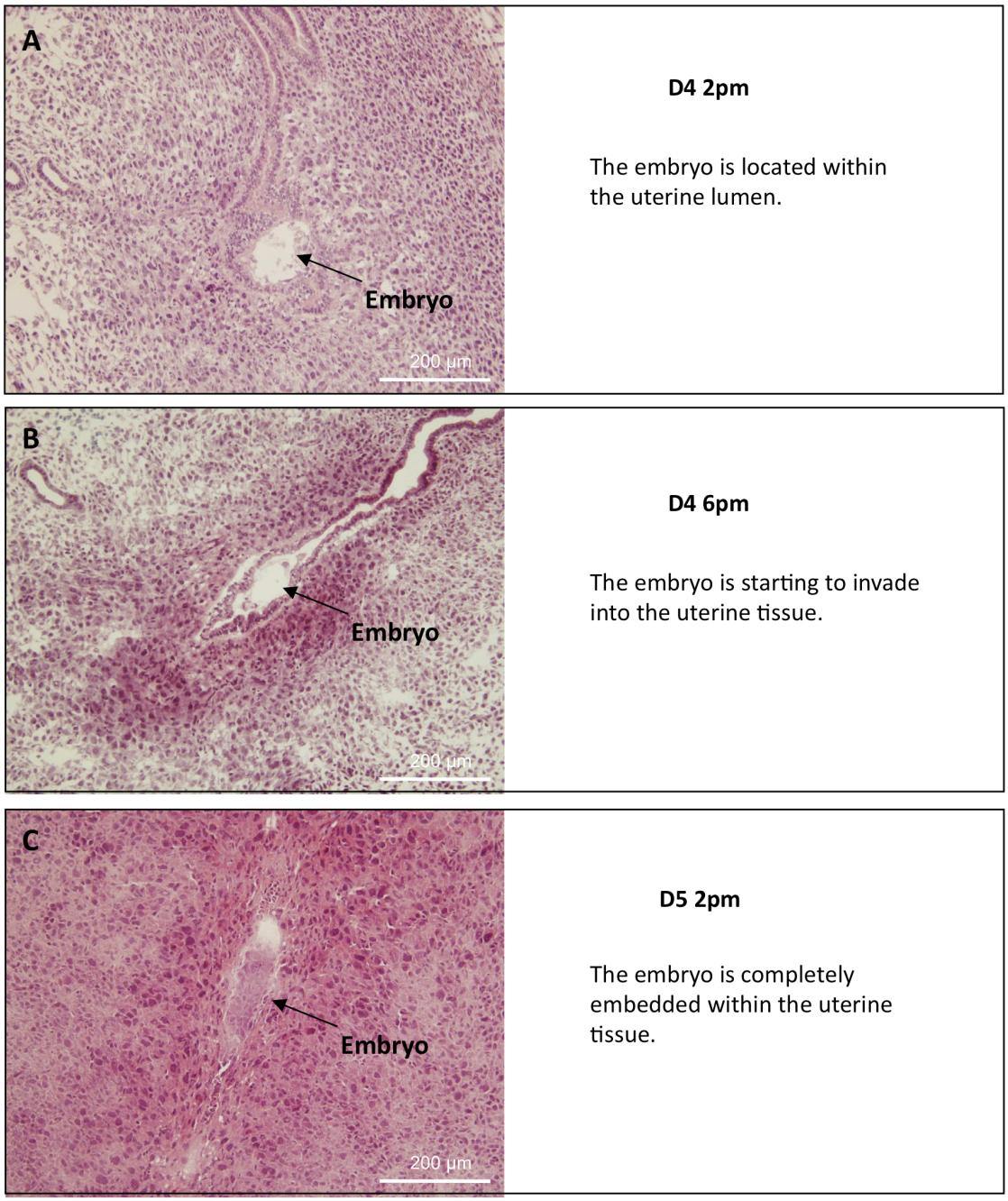


Figure 2. The progress of normal mouse embryo implantation from D4 to D5 afternoon

HE staining of implantation site cross sections. (A) D4 2pm; (B) D4 6pm and (C) D5 2pm.

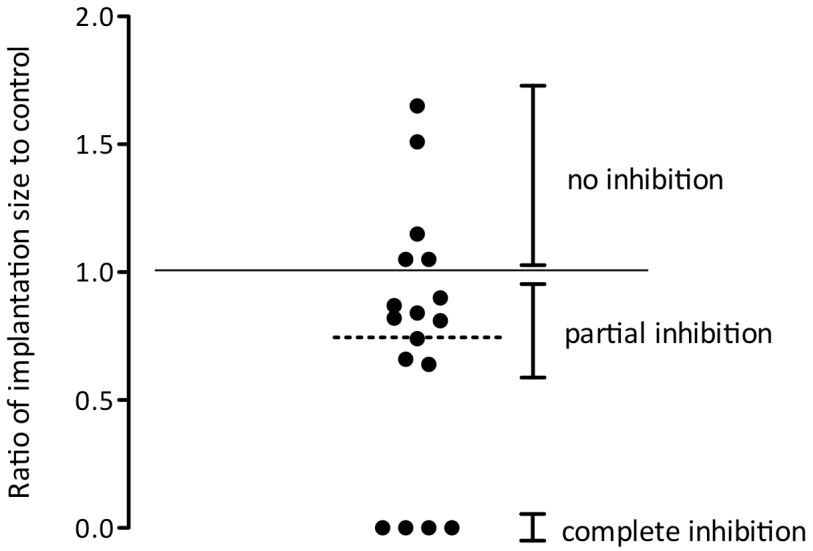


Figure 3. Contraceptive efficacy of C-30k-PEG Poly R in mice following vaginal delivery

The ratio (treated/control) of implantation site weight was plotted for each mouse (n=17) following the treatment with C-30k-PEG Poly R. The dash line represents the mean = 0.7465, *p* = 0.0017.

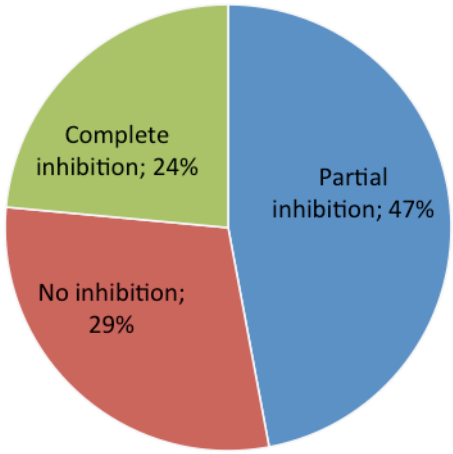


Figure 4. Percentage of mice showing a complete, partial and no inhibition of implantation in mice following vaginal delivery of C-30k-PEG Poly R. Same raw data as in Figure 3.

Table 1. Contraceptive efficacy of C-30k-PEG Poly R in mice following vaginal delivery

| Phenotype | Number of mice /Total | Percentage of mice |
|---|---|---|
| Complete inhibition | 4/17 | 24% |
| Partial inhibition | 8/17 | 47% |
| No inhibition | 5/17 | 29% |

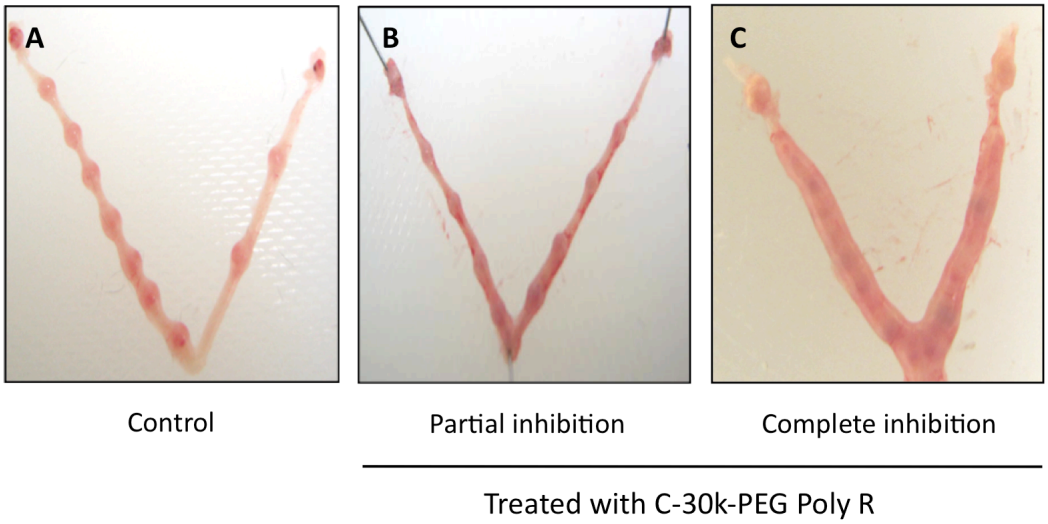


Figure 5. Representative images of mouse uterine horns at D5 2pm of pregnancy following vaginal delivery of C-30k-PEG Poly R

(A) Control; (B) and (C) treated mice with partial and complete inhibition of implantation respectively.

**Figure 6**

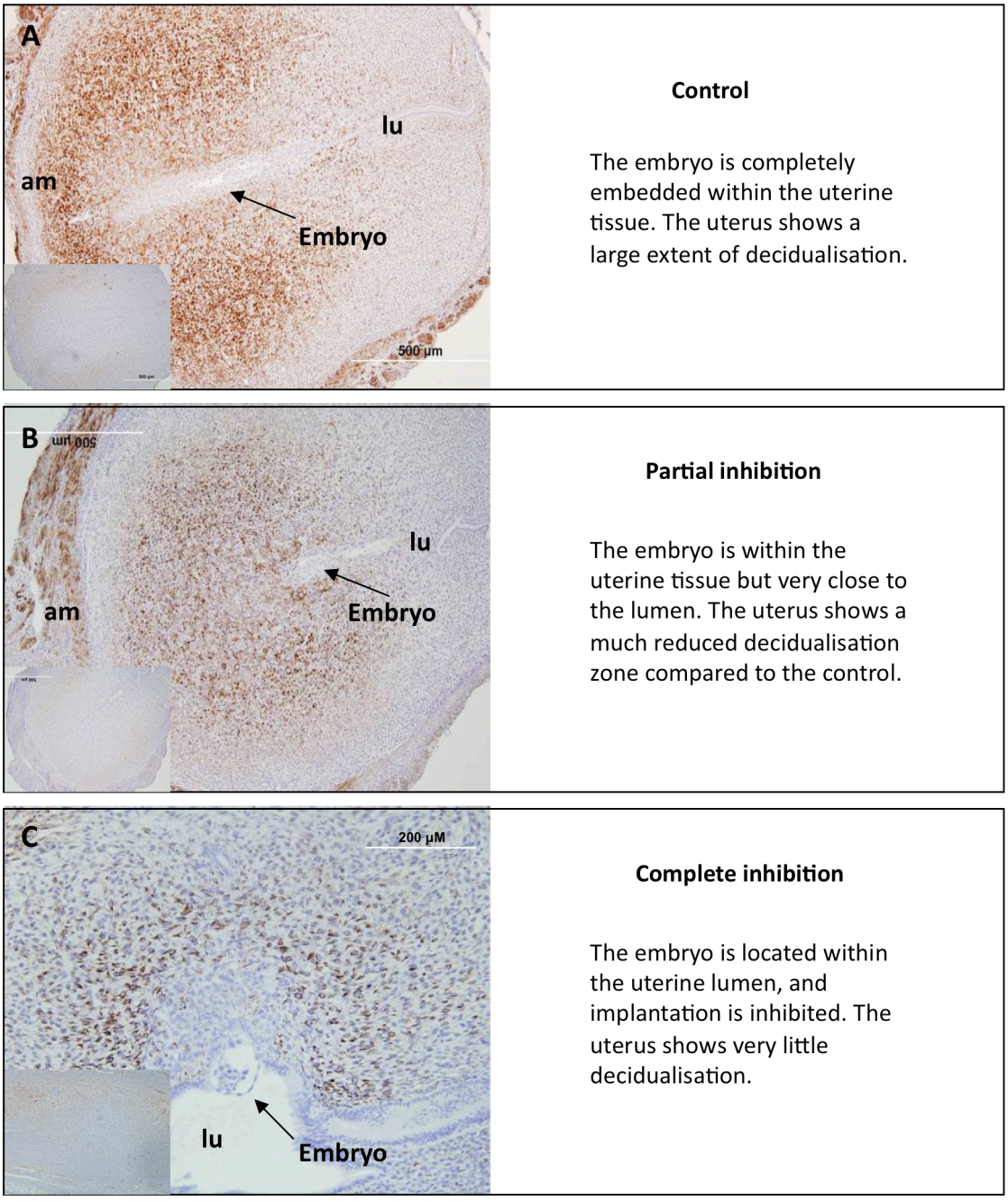
A
lu
am
Embryo
500 µm
Control
The embryo is completely embedded within the uterine tissue. The uterus shows a large extent of decidualisation.
B
lu
am
Embryo
Partial inhibition
The embryo is within the uterine tissue but very close to the lumen. The uterus shows a much reduced decidualisation zone compared to the control.
C
200 µM
lu
Embryo
Complete inhibition
The embryo is located within the uterine lumen, and implantation is inhibited. The uterus shows very little decidualisation.

Figure 6. Histological examination of implantation sites at D5 2pm following vaginal administration of C-30k-PEG Poly R

Representative sections were stained for the decidual marker desmin. am, antimesometrial side; lu, uterine lumen. Representative microphotographs of (A) control (cross section); (B) partial (cross section) and (C) complete inhibition (longitudinal section) are shown.

# Chapter 4 –

# A high-throughput *in vitro* model of human embryo attachment

# Declaration for Thesis Chapter 4

## Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

| **Nature of Contribution** | **Extent of contribution (%)** |
|---|---|
| Performed all experiment, conducted optimisation, interpretation of all data, statistical analysis and manuscript writing. | 85 |

The following co-authors contributed to the work. Co-authors who are students at Monash University have the extent of their contribution indicated in percentage terms:

| **Name** | **Nature of contribution** | **Extent of contribution for <u>student</u> co-authors only (%)** |
|---|---|---|
| Harmeet Signh | Conception of project | Not applicable |
| Mohamad Aljofan | Conception of project | Not applicable |
| Guiying Nie | Conception of project and supervision | Not applicable |

Candidate's Signature

| | **Date** |
|---|---|

## Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

| | |
|---|---|
| **Location (s)** | **Prince Henry's Institute of Medical Research, Monash Medical Centre Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia** |

| | | |
|---|---|---|
| **Signature 1** | [redacted] | **Date** 20/05/2013 |
| **Signature 2** | | 2 January 2013 |
| **Signature 3** | | 21/05/2013 |

ORIGINAL ARTICLES: REPRODUCTIVE BIOLOGY

# A high-throughput in vitro model of human embryo attachment

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**Objective:** To develop a simple, high-throughput and widely applicable in vitro human implantation model that assesses trophoblast spheroid attachment to human uterine epithelial cells.
**Design:** Experimental study to establish and validate the model.
**Setting:** Cell culture.
**Cell(s):** Lines BeWo, RL95-2, and AN3-CA.
**Intervention(s):** Labeling trophoblast spheroids with green fluorescence, selecting spheroids of size similar to implanting blastocysts by use of cell strainers, and assessing spheroid attachment by use of an automated microplate reader.
**Main Outcome Measure(s):** Establishment of a simple, reliable, and high-throughput in vitro model to study human embryo implantation.
**Result(s):** The assay enabled rapid fluorometric assessment of spheroid attachment to human endometrial epithelial cells (RL95-2 and AN3-CA) under different experimental conditions. The high-throughput assay was confirmed by conventional counting method to be highly reproducible and accurate.
**Conclusion(s):** The described methodology provides for the first time a high-throughput assay for the study of human embryo implantation and a promising tool for screening potential inhibitors or enhancers of embryo attachment. (Fertil Steril® 2012;97:974–8. ©2012 by American Society for Reproductive Medicine.)
**Key Words:** Embryo attachment, endometrial epithelial cells, implantation, trophoblast spheroids

Embryo implantation is critical in establishing pregnancy. The implantation process is complex and highly coordinated, starting when a blastocyst interacts with the uterus. Adhesive interaction between the trophectoderm and endometrial epithelium is followed by blastocyst penetration between the epithelial cells and invasion through the underlying basal lamina (1). There are continuing efforts to understand the cellular basis of interactions between the trophectoderm and endometrial epithelium at the initiation of implantation in the human, but such experimental studies are severely limited by ethics and practical difficulties. It is impossible to perform in vivo studies in humans, and access to human embryos is very restricted (2). Consequently, experimental investigations rely on model experiments that use human trophoblast and endometrial cell lines.

Attachment models involving coculture of a monolayer of human endometrial epithelial cells (to mimic the uterine epithelium) and trophoblast spheroids (to mimic blastocysts) have been widely used (3–9). However, these are technically demanding and time consuming because spheroids are selected microscopically and their attachment is quantified by manual counting. These assays are highly variable and not useful for high-throughput studies. To study the interaction between human endometrial epithelial cells and blastocysts, the field requires an effective assay that is simple and highly reproducible.

Our study establishes a simple, high-throughput attachment assay that can be performed in most laboratories. New methodologies for spheroid selection and quantification of attachment also are developed. We validated the assay by testing human endometrial epithelial cells of different receptivity (receptive: RL95-2; nonreceptive: AN3-CA) and by applying a known inhibitor of spheroid attachment to RL95-2 (a calcium chelator) and an enhancer to AN3-CA (a DNA methylation inhibitor) (6, 7). Our results demonstrate that this high-throughput assay is efficient and reproducible to assess attachment under different experimental conditions. This assay will also permit high-throughput screening of drugs affecting embryo attachment.

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H.H. and H.S. contributed equally to this article.
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## MATERIALS AND METHODS

### Cell Culture

Endometrial epithelial carcinoma cells RL95-2 (ATCC: CRL-1671) and AN3-CA (ATCC: HTB-111) and choriocarcinoma BeWo cells (ATCC: CCL-98) were obtained from the American Type Culture Collection. Both RL95-2 and BeWo cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 and AN3-CA cells

in minimal essential medium (MEM) (GIBCO). All media were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin (all from GIBCO). The RL95-2 medium was additionally supplemented with 5 μg/mL of insulin (Actrapid; Novo Nordisk). The cells were cultured at 37°C in a humidified 5% $CO_2$ incubator and were subcultured every 3 days by trypsinization (trypsin-ethylenediaminetetraacetic acid [EDTA] solution; GIBCO).

## Spheroid Attachment Assay

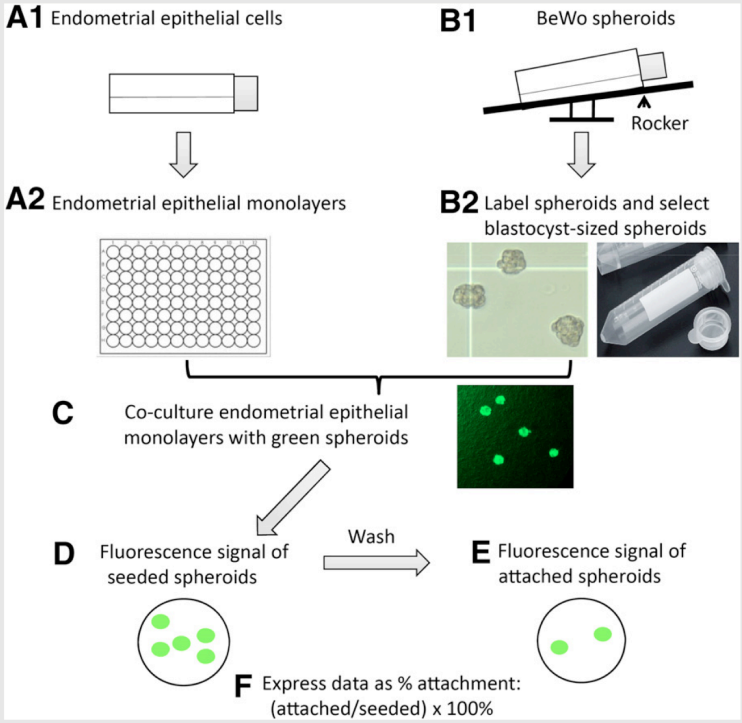
A schematic illustration of the spheroid attachment assay is shown in Figure 1, involving the preparation of endometrial epithelial monolayers (step A) and BeWo spheroids (step B), their coculture (step C), and the assessment of attachment (steps D–F). Each step is described here.

**Preparation of endometrial epithelial cell monolayers.** Human endometrial epithelial cells were grown in T-75 Nunc tissue culture flasks, trypsinized, and seeded onto black 96-well plates with clear bottoms (Nunc; Thermo Scientific Pty. Ltd.) at a density of $3 \times 10^5$ (RL95-2) or $1 \times 10^4$ (AN3-CA) per well. Confluent monolayers were formed after 24 hours of incubation (see Fig. 1, step A).

**Preparation of trophoblast spheroids.** BeWo cells were grown in suspension in 15% fetal calf serum (FCS) DMEM/Ham's F-12 at a density of $2 \times 10^5$ cells/mL in polyvinylpyrrolidone (PVP) (Sigma-Aldrich) coated T75 Nunc tissue culture flasks on a fast-rocking shaker (84 counts/minute; Ratek Instruments). Spheroids formed spontaneously by cell aggregation after 17 hours of culture (see Fig. 1, step B).

**Fluorescence labeling and selection of spheroids.** At the end of spheroid preparation, Calcein-AM fluorescent dye (0.5 μg/mL of spheroid suspension medium; BD Biosciences) was added to the spheroid suspension and was incubated for 30 minutes on the fast-rocking shaker. To select spheroids of size similar to human blastocysts, the spheroid suspension was first passed through a cell strainer (BD Biosciences), sieve size 100 μm, to eliminate large cell aggregations. The flow through was then passed through a cell strainer of 70 μm sieve size to capture spheroids of size between 70 and 100 μm (see Fig. 1, step B).

**FIGURE 1**




A schematic illustration of the spheroid attachment assay. (A) The endometrial epithelial cells were grown in flasks, trypsinized, and seeded onto 96-well plates to form monolayers (A1, A2). (B) Meanwhile, BeWo spheroids were prepared by rocking cell suspension overnight (B1). Spheroids were labeled with the fluorescence dye Calcein-AM and selected using cell strainers (B2). (C) Approximately 40 spheroids were seeded onto endometrial epithelial cell monolayers in each well and cocultured for 1 hour. (D) The fluorescence signal of seeded spheroids was recorded using a plate reader. (E) The wells were washed with phosphate-buffered saline, and the fluorescence signal of the attached spheroids was then recorded. (F) The attachment was expressed as percentage of the ratio of fluorescence signals (Attached spheroids/Seeded spheroids).

*Ho. In vitro model of human embryo attachment. Fertil Steril 2012.*

**Assessment of spheroid attachment.** Fluorescence-labeled spheroids (70–100 μm, ~40/well) were seeded on the top of RL95-2 monolayers in 96-well plates and were cocultured for 1 hour in an atmosphere of 5% $CO_2$ at 37°C (see Fig. 1, step C). The plate was scanned by an automated plate reader (Ex/Em = 495 nm/515 nm; PerkinElmer) using a scanning mode (10 × 10 horizontal and vertical points) to record the signals of all seeded spheroids in each well (see Fig. 1, step D). The media were removed, and all wells were washed with 200 μL phosphate-buffered saline (PBS) to remove unattached spheroids and were replenished with culture media. The plate was scanned again to record the signals of the attached spheroids (see Fig. 1, step E). The percentage attachment was calculated as the percentage of attached/seeded fluorescence signals [(Attached spheroids/Seeded spheroids) × 100%]. To cross validate the fluorescence reading, percent spheroid attachment is confirmed by manual counting of spheroid before and after wash.

**Validation of spheroid attachment assay.** For the highly adhesive cell line RL95-2, $Ca^{2+}$ influx is essential for spheroid attachment; blocking $Ca^{2+}$ influx greatly reduces spheroid attachment (9). The RL95-2 monolayer was pretreated with a $Ca^{2+}$ chelator, EDTA (1 mM, pH7.4; Sigma-Aldrich), for 5 minutes before the addition of spheroids. For the nonadhesive cell line AN3-CA, inhibition of DNA methylation by 5′-aza-2′-deoxycytidine (AZA) increases AN3-CA receptivity to BeWo spheroids (7). The AN3-CA monolayers were incubated with 20 μM AZA (Sigma-Aldrich) starting just before full confluency for 48 hours. Medium containing EDTA and AZA were removed, BeWo spheroids were added, and the spheroid attachment assay was performed as already described.

## Statistical Analysis

Data are obtained from at least three independent experiments with three replicate wells per treatment within each experiment. Data are expressed as mean ± standard error. Statistical significance was evaluated by the *t*-test using Prism software. $P<.05$ and $P<.01$ were considered statistically significant (*) and highly statistically significant (**), respectively.

# RESULTS

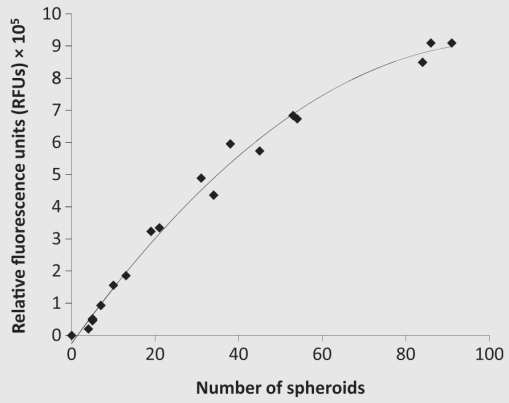
## Selection of Equal-Sized Spheroids by Use of Cell Strainers

To simplify spheroid selection, filtration of spheroid suspensions sequentially through cell strainers of 100 μm and 70 μm sieve sizes provided a quick and easy selection of similar-sized spheroids. Larger and smaller cell aggregates that could compromise the accuracy of the assay were eliminated (see Fig. 1, B2). Approximately 75% of spheroids >100 μm or <70 μm were discarded; approximately 25% of spheroids were harvested from each preparation.

## Optimization of Spheroid Quantification

To quantify the seeded and the attached spheroids robustly using a plate reader, BeWo spheroids were labeled with Calcein-AM, a cell-permeable fluorescent dye that stains viable cells (10). The fluorescence labeling had no effect on the attachment properties of BeWo spheroids, and the fluorescence signal was retained after PBS washing (data not shown). To ensure that all spheroids scattered throughout the wells were detected, the plate reader was set to scan the entire area of the well, taking readings from 10 × 10 horizontal and vertical points. The fluorescence signal increased with the increasing number of spheroids in a linear pattern between 0 and 60 spheroids, with a lower limit of detecting less than five spheroids (Fig. 2). The linear relationship no longer held when the spheroid number was >60 (see Fig. 2), when the fluorescence signal reached saturation. Based on this and a criterion of signal to background ratio of >2.0, we selected approximately 40 spheroids as the optimal seeding number for the attachment assay.

**FIGURE 2**



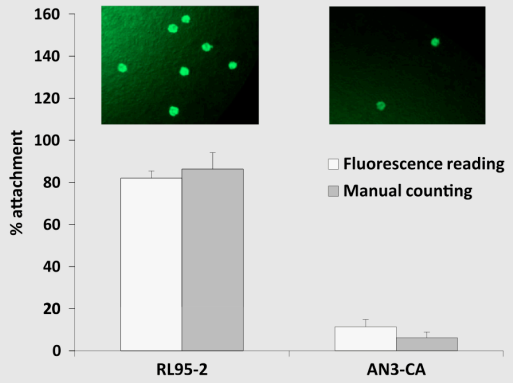

Correlation between fluorescence signal and the number of spheroids. Calcein-labeled spheroids were serially diluted and seeded onto monolayers of RL95-2. The fluorescence signal was plotted against the number of seeded spheroids in each well (determined by manual counting).

*Ho. In vitro model of human embryo attachment. Fertil Steril 2012.*

## Quantitative Assessment of Spheroid Attachment

The percentage of spheroids attached was calculated based on fluorescence signals of the attached to seeded spheroids in individual wells. This ensured high accuracy by taking into account any variability in the number of spheroids seeded. To confirm the accuracy of the fluorescence labeling/reading method, the number of the seeded and attached spheroids was also manually counted under a microscope. We compared spheroid attachment to RL95-2 and AN3-CA, which have been extensively used as highly receptive and nonreceptive uterine epithelial cell lines, respectively (7, 8). The RL95-2 cells exhibited a statistically significantly higher attachment compared with AN3-CA (Fig. 3), consistent with previous reports (7). In addition, the data from the fluorescence-reading method was highly consistent with that from manual counting (Fig. 3).

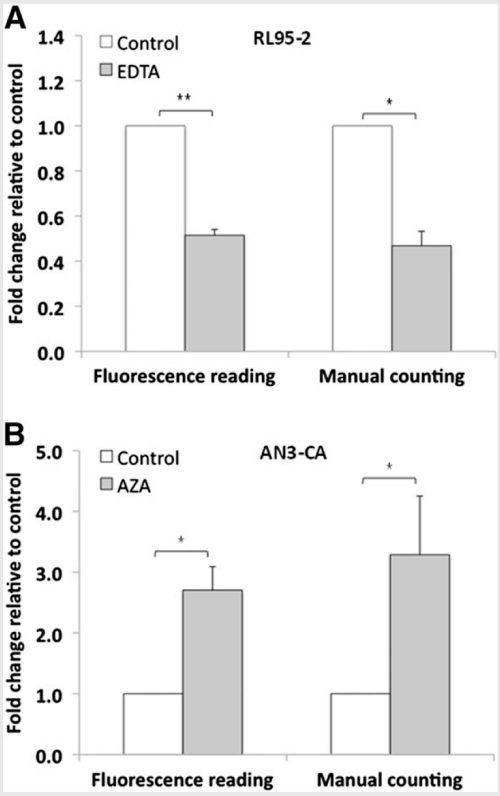
**FIGURE 3**




Assessment of spheroid attachment to RL95-2 (receptive) and AN3-CA (nonreceptive) cell monolayers by both fluorescence reading (*open bar*) and manual counting methods (*gray bar*). Representative micrographs of BeWo spheroids attached to RL-95 monolayer and AN3-CA respectively are shown on the top. The experiment was repeated three times in triplicate wells.

*Ho. In vitro model of human embryo attachment. Fertil Steril 2012.*

**FIGURE 4**




Assessment of spheroid attachment reduction or enhancement after treatment of endometrial epithelial cell monolayers. The experiments were repeated three times in triplicate wells. (*$P<.05$; **$P<.001$.) (A) RL95-2 wells pretreated without or with 1 mM ethylenediaminetetraacetic acid (EDTA) for 5 minutes. Control = open bar; EDTA = gray bar. (B) AN3-CA cells treated without or with 20 $\mu$M AZA for 48 hours. Control = open bar; AZA = gray bar.

*Ho. In vitro model of human embryo attachment. Fertil Steril 2012.*

## Validation of the Fluorescence-Labeling Method

To further validate the assay, EDTA, a known inhibitor of spheroid attachment to RL95-2 (9) and AZA, a methylation inhibitor that enhances spheroid attachment to AN3-CA (7), were tested. Pretreatment of RL95-2 monolayers with EDTA significantly decreased spheroid attachment by approximately 50% (Fig. 4A). The RL95-2 monolayers remained intact after the EDTA treatment. For the nonadhesive AN3-CA, treatment with the methylation inhibitor AZA increased the spheroid attachment by approximately threefold (see Fig. 4B). In both validations, no statistically significant differences were found between the fluorescence reading and manual counting methods. Indeed, the error bars of the fluorescence reading data were smaller than that of manual counting (0.12 vs. 0.44 and 0.39 vs. 0.97, respectively), demonstrating the greater reproducibility of this high-throughput assay.

## DISCUSSION

We have developed a new, simple, high-throughput attachment assay for the study of trophoblast spheroid attachment to endometrial epithelial cells. Spheroid selection was substantially simplified from that in previous methods. Assessment of spheroid attachment was achieved by fluorescently labeling spheroids and quantifying them using a plate reader. The assay was validated and proved to be highly sensitive and reproducible.

BeWo multicellular spheroids were initially established in culture by Grummer et al. (11, 12). They demonstrated that such spheroids show similar morphologic characteristics and hormonal secretion profiles to human cytotrophoblast. Likewise, Abaidoo et al. (13) showed marked similarities between BeWo cell morphology and that reported for human trophoblast, demonstrating that BeWo spheroids are useful for in vitro studies of human implantation. In addition, BeWo has been well characterized to express several trophoblast markers, including cytokeratin 7, human chorionic gonadotrophic hormone, human leucocyte antigen-G, and cluster of differentiation antigen 9 (2). Although BeWo is the choice of trophoblast cell line for attachment studies in the present assay, it does not fully represent the trophectoderm: however, there is currently no readily available cell line that better represents the blastocyst.

The conventional method of spheroid selection uses a microdenuding pipette under a microscope (6, 7, 14). Here, we report a quick and simple method of selection for spheroids of size similar to that of implanting human blastocysts, by sequential filtration through 100 and 70 $\mu$m cell strainers. This method is simple, accurate, and considerably minimizes spheroid manipulation and handling time. In addition, the use of similarly sized spheroids avoids inconsistency and increases reproducibility.

To enable robust quantification of spheroid attachment using a microplate reader, BeWo spheroids were labeled with Calcein-AM, a cell-permeant fluorescent dye that is readily taken up by live cells (15). The fluorescence signal increased in proportion to the number of spheroids, demonstrating that the fluorescence signal effectively represented the spheroid quantity in each well. In addition, the Calcein-AM is of very low toxicity, highly stable, and bleach resistant (16), making it an ideal fluorescent dye to use in this high-throughput assay.

The variable receptive properties of different epithelial cell lines have been well-documented using trophoblast cells and monolayers (3–7, 9). The RL95-2 cells express a number of known blastocyst attachment-mediating molecules, including CD9, ezrin, osteopontin, and various integrins ($\alpha 3$, $\alpha 6$, $\beta 1$, $\beta 4$) (2, 17). In contrast, AN3-CA cells lack these marker molecules (8). RL95-2 closely mimics human receptive uterine epithelium, as they are highly adhesive to trophoblast (2, 4, 8, 18), whereas AN3-CA more closely represents a non-receptive cell layer (4, 7). To validate the high-throughput assay, we compared BeWo spheroid attachment to RL95-2 and AN3-CA, revealing high and low percentage of attachment to RL95-2 and AN3-CA, respectively. Further confirmation was gained by manual counting.

We also demonstrated that this high-throughput assay was able to detect spheroid attachment under different experimental conditions. $Ca^{2+}$ signaling has been postulated to play an important role in the attachment of embryos to the endometrium (19, 20). Attachment of trophoblast spheroids to RL95-2 monolayers was significantly reduced when $Ca^{2+}$ influx was blocked with $Ca^{2+}$ channel blockers (9) or extracellular $Ca^{2+}$ chelators (e.g., EDTA) (6). Our study has confirmed that the attachment of spheroids statistically significantly decreased when the RL95-2 monolayer was pretreated with EDTA, further validating the assay.

Similar assays have been applied to examine other features of spheroid-endometrium interactions. Inhibition of DNA methylation by AZA increased the receptivity of AN3-CA to BeWo spheroids due to increased expression of E-cadherin (7). Likewise, the present fluorescence assay detected increased spheroid attachment to AN3-CA monolayers after treatment with AZA.

Although the fluorescence-reading method was highly comparable with that of manual counting, it was more reproducible. All these results demonstrate that this high-throughput spheroid attachment assay can effectively replace the conventional spheroid attachment assay with high sensitivity and accuracy. One limitation of this study is the use of BeWo, RL95-2, and AN3-CA cell lines, which may not fully represent the in vivo situation, but this model can be easily applied using other trophoblast or endometrial epithelial cell types of interest for specific studies. To further improve/standardize the assay, a centrifugation step can be incorporated to remove the loosely bound spheroids as reported (6).

In summary, we have established a reliable, high-throughput spheroid attachment assay using fluorescence-labeled spheroids and 96-well plates, which can be standardized and easily performed in most laboratories. This assay can be used to study effects of attachment-associated factors and to robustly screen potential inhibitors or enhancers of embryo attachment.

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# Chapter 5 –

# Small molecule proprotein convertase inhibitors for inhibition of embryo implantation

# Declaration for Thesis Chapter 5

## Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

| **Nature of Contribution** | **Extent of contribution (%)** |
|---|---|
| Performed experiments, conducted optimisation, interpretation of data, statistical analysis and manuscript writing. | 70% |

The following co-authors contributed to the work. Co-authors who are students at Monash University have the extent of their contribution indicated in percentage terms:

| **Name** | **Nature of contribution** | **Extent of contribution for <u>student</u> co-authors only (%)** |
|---|---|---|
| Harmeet Signh | Conducted experiments and data analysis | Not applicable |
| Sophea Heng | Conducted experiments | Not applicable |
| Tracy Nero | Conducted structural biology analysis and contributed to manuscript drafting | Not applicable |
| Michael Parker | Contributed to manuscript revision | Not applicable |
| Guan-Sheng Jiao | Contributed research material | Not applicable |
| Alan T. Johnson | Contributed research material | Not applicable |
| Guiying Nie | Conception of project and supervision | Not applicable |

Candidate's Signature

| | **Date** |
|---|---|

## Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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| **Location (s)** | **Prince Henry's Institute of Medical Research, Monash Medical Centre Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia** |

| | | |
|---|---|---|
| **Signature 1** | [redacted] | **Date** 20/05/2013 |
| **Signature 2** | | 28/05/2013 |
| **Signature 3** | | 6/5/2013 |
| **Signature 4** | | 6/5/13 |
| **Signature 5** | | 5/2/13 |
| **Signature 6** | | 5/2/13 |
| **Signature 7** | | 21/05/2013 |

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# Small molecule proprotein convertase inhibitors for inhibition of embryo implantation

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[§]G.-S. J is co-corresponding author regarding the chemistry of the inhibitors

[redacted]

## Abstract

Uterine proprotein convertase (PC) 6 plays a critical role in embryo implantation and is pivotal for pregnancy establishment. Inhibition of PC6 may provide a novel approach for the development of non-hormonal and female-controlled contraceptives. We investigated a class of five synthetic non-peptidic small molecule compounds that were previously reported as potent inhibitors of furin, another PC member. We examined (i) the potency of these compounds in inhibiting PC6 activity *in vitro;* (ii) their binding modes in the PC6 active site *in silico;* (iii) their efficacy in inhibiting PC6-dependent cellular processes essential for embryo implantation using human cell-based models. All five compounds showed potent inhibition of PC6 activity *in vitro,* and *in silico* docking demonstrated that these inhibitors could adopt a similar binding mode in the PC6 active site. However, when these compounds were tested for their inhibition of decidualisation of primary human endometrial stromal cells, a PC6-dependent cellular process critical for embryo implantation and successful pregnancy, only one (compound 1o) showed potent inhibition. The lack of activity in the cell-based assay may reflect the inability of the compounds to penetrate the cell membrane. Because compound's lipophilicity is linked to cell penetration, a measurement of lipophilicity (log*P*) was calculated for each compound. Compound 1o is unique as it appears the most lipophilic among the five compounds. Compound 1o also inhibited another crucial PC6-dependent process, the attachment of human trophoblast spheroids to endometrial epithelial cells (a model for human embryo attachment). We thus identified compound 1o as a potent small molecule PC6 inhibitor with pharmaceutical potential to inhibit embryo implantation. Our findings also highlight that human cell-based functional models are vital to complement the biochemical and *in silico* analyses in the selection of promising drug candidates. Further investigations for

compound 1o are warranted in animal models to test its utility as an implantation-inhibiting contraceptive drug.

## Introduction

The proprotein convertases (PCs) are a family of nine serine proteases implicated in the processing of a multitude of precursor proteins (Couture et al., 2011, Seidah and Prat, 2012). The first seven members [PC1/3, PC2, furin, PACE4, PC4, PC5/6 (to be referred as PC6 in this report) and PC7] activate a large number of polypeptide hormones, growth factors, adhesion molecules, various viral surface proteins and pro-toxins of bacteria by cleavage at basic residues (Seidah and Prat, 2012). The eighth and ninth members (SKI-1 and PCSK9) do not require a basic residue for cleavage and they play major roles in regulation of lipid homeostasis (Seidah and Prat, 2012, Seidah, 2011). Accumulated evidence over the last decade has confirmed PCs as potential therapeutic targets for several important pathologies including osteoarthritis, cancer, cardiovascular disease and viral infections (Couture et al., 2011). Therefore, development of PC inhibitors is clearly an important research and development field.

Our interest in PC inhibitors originated from studies aiming at inhibiting PC6 in the female reproductive tract to inhibit embryo implantation. Uterine PC6 is pivotal in embryo implantation and is essential for the establishment of pregnancy (Freyer et al., 2007). To enable implantation, the uterus must acquire epithelial receptivity and undergo a process known as decidualisation to differentiate stromal fibroblasts into phenotypically and functionally distinct decidual cells (Salamonsen et al., 2009). We have previously shown that

PC6 is critical for both uterine epithelial receptivity and stromal cell decidualisation (Heng et al., 2011a, Heng et al., 2011b, Heng et al., 2010, Ho et al., 2012a). Knockdown of PC6 in a human endometrial epithelial cell line HEC1A significantly reduced its receptivity for blastocyst adhesion (Heng et al., 2011a). Decidualisation of primary human endometrial stromal cells (HESCs) was inhibited when PC6 activity was blocked (Heng et al., 2010, Aljofan et al., 2012). It has also been demonstrated in mice that when uterine PC6 production was blocked, decidualisation was inhibited and implantation was prevented (Nie et al., 2005b). In addition, PCs including PC6 also play an important role in HIV infection (Decroly et al., 1996, Miranda et al., 1996, Vollenweider et al., 1996). Therefore, inhibition of PC6 is an attractive approach to develop novel, non-hormonal and female-controlled contraceptives that could also protect women from HIV infection.

The majority of PC inhibitors reported in the literature to date have been proteins or peptides (Jiao et al., 2006). Nona-D-arginine (Poly R) is one of the most potent peptide based PC inhibitors known to date. Poly R inhibits PC6 *in vitro* with a Ki in the nanomolar range and has been shown to inhibit HIV in cell culture (Fugere et al., 2007, Kibler et al., 2004). We have previously demonstrated that Poly R inhibits decidualisation of HESC in culture and have evaluated the therapeutic potential of a PEGylated Poly R [covalently attached with polyethylene glycol (PEG) polymers] in inhibition of implantation in rabbits (Heng et al., 2010, Aljofan et al., 2012). However, the physiochemical properties of Poly R could limit their usefulness in therapeutic applications in women. Therefore, we continue to search for potent PC6 inhibitors with the desired characteristics such as serum stability and cell permeability.

In this study, we evaluated five synthetic small molecule compounds derived from 2,5-dideoxystreptamine chemical scaffold previously reported by Jiao *et al.*, 2006 (Jiao et al., 2006).

Four of these compounds (1e, 1f, 1g, 1n) were previously shown to be potent inhibitors of both human furin and PC6 *in vitro* (Jiao et al., 2006). Compound 1o was shown to be a relatively poor inhibitor of furin but no data on PC6 was reported (Jiao et al., 2006). Here, the inhibitory potency of all five compounds against human PC6 (hPC6) was determined *in vitro*. *In silico* docking studies were performed to visualise the potential binding mode of these inhibitors in the active site of hPC6 and to gain an understanding of how this may relate to their inhibitory activity. The therapeutic potential of these small molecule inhibitors was then examined in *in vitro* human cell-based models to investigate their ability to inhibit two important PC6-mediated cellular processes essential for embryo implantation: (1) decidualisation of primary HESCs and (2) attachment of human trophoblast spheroids (surrogate for embryos) to endometrial epithelial cells.

## Materials and Methods

### Small molecule PC inhibitors

Small molecule compounds 1e, 1f, 1g, 1n and 1o (Table 1), derived from 2,5-dideoxystreptamine, were synthesized as previously reported by Jiao, *et al.*,(Jiao et al., 2006).

### Cell culture

Ishikawa (Clone 3-H-12), a human endometrial adenocarcinoma cell line of epithelial origin, was kindly provided by Dr. Masato Nishida (National Kasumigaura Hospital, Ibarak, Japan) (Nishida, 2002). JAR cells, a human choriocarcinoma cell line, were purchased from ATCC (American Type Culture Collection). Ishikawa and JAR cells were cultured in phenol-red MEM

without glutamine and RPMI 1640 Medium, GlutaMAX™, respectively, supplemented with 10% charcoal stripped fetal bovine serum (CS-FBS, Thermo Electron Corporation, Maple Plain, MN) and 100 μg/ml streptomycin and 100 IU/ml penicillin (Gibco®, Mulgrave, VIC, Australia). Ishikawa cells were used within 10 passages according to the provider's recommendation to avoid changes in cell characteristics such as down-regulation of estrogen receptor and progesterone receptor expression.

**Inhibition of PC6 activity by small molecule compounds**

An *in vitro* PC6 activity assay as described previously (Heng et al., 2010, Ho et al., 2012a) was used to evaluate PC6 inhibition by small molecule compounds. In brief, 2 units of active recombinant human PC6 (rhPC6) (PhenoSwitch BioScience Inc., Quebec, Canada) were incubated with small molecule compounds (10 μM), in Dulbecco's modified Eagle's medium/Ham's F12 culture medium (DMEM/F12, Sigma, St. Louis, MO) in the presence of 100 μM fluorogenic substrate pERTKR-AMC (Bachem, Torrance, CA) at 37$^{o}$C. The real-time kinetic progression of substrate hydrolysis [release of fluorescent 7-amino-4-methylcoumarin (AMC)] was monitored every 5 min at excitation/emission of 355/460 nm (Wallac, Victor 2 spectrophotometer; PerkinElmer, Boston, MA) for 1 h. Inhibition of PC6 activity was expressed as a reduction in the rate of substrate hydrolysis relative to the control (PC6 activity in the absence of inhibitory peptides). At least two independent experiments were performed for each compound.

**In silico docking of small molecule compounds into the catalytic site of hPC6**

As no crystal structure of PC6 from any species is available, a homology model of hPC6 was used for the *in silico* docking studies. The construction of the hPC6 homology model has been previously reported (Ho et al., 2012b). The hPC6 active site is a canyon-like groove lined with clusters of negatively charged groups that are classified into sub-pockets that are defined as S1-S6 and S1′ (Fugere and Day, 2005). The important residues in each sub pockets are S1 – D277, D325; S2 – D173, E210; S3 – L246, W273; S4 – E255, D283; S5 – D276, D283; S6 – D249, D252; S1′ – K212, R216, H381 (Fugere and Day, 2005). The hPC6 catalytic triad consists of D172, H213 and S385 (Fugere and Day, 2005). The five compounds were constructed using standard bond lengths and bond angles within SYBYL-X 2.0 (Certara L.P., http://www.tripos.com) and then structurally optimized using the MMFF94s force field and partial atomic charges, conjugate gradient convergence method; termination of the optimization was achieved when the gradient difference of successive steps was < 0.05 kcals/mol Å (all other parameters were at default values). Docking of the compounds into the catalytic domain of the hPC6 homology model was carried out using Surflex v2.6. The protomol was generated using the automated method, a threshold of 0.50 and a bloat value of 2. The GeomX mode was used, all other parameters were at default values. The C-Score function was used to rank the docked compound poses, the top twenty ranked poses for each compound were examined visually. Docked poses of compounds 1g and 1o have been chosen to illustrate how these compounds can bind into the hPC6 active site.

**Decidualisation of HESCs and inhibition by small molecule compounds**

Human endometrial tissues were obtained from non-pregnant women undergoing curettage following laparoscopic sterilization or assessment of tubal patency. Ethical approval was

granted by the Human Ethics Committee of Southern Health, Melbourne, Australia and written informed consent was obtained from all tissue donor patients. Tissues collected between Day 8-24 were processed within 24 h. Human endometrial stromal cells (HESCs) were isolated by enzymatic digestion and filtration as previously described (Ho et al., 2012b, Heng et al., 2010, Singh et al., 2011). HESCs (> 97%) were cultured in T25 $cm^2$ flasks in DMEM/F12 medium supplemented with 10% CS-FBS, 2 mM L-glutamine (Sigma), 100 µg/ml streptomycin and 100 IU/ml penicillin (Dimitriadis et al., 2002). Once 70-80% confluent, the HESCs were passaged into 12-well plates ($8 \times 10^8$ cells/well) and cultured to 80% confluence. For decidualisation, cells were treated with estradiol 17-β (E2, $10^{-8}$ M), medroxy-progesterone acetate (MPA, $10^{-7}$ M) and 8-bromoadenosine 3′:5′ cyclic monophosphate (camp, $5 \times 10^{-4}$ M) (all from Sigma) for 72 h in serum free DMEM/F12 containing 0.1% BSA. Decidualisation success was confirmed by a significant increase in the decidual marker prolactin (PRL) in the conditioned medium by ELISA (Bioclone Australia Pty Ltd., Sydney, Australia) as per the manufacturer's instructions (Heng et al., 2010). To access decidualisation inhibition by the small molecule compounds, HESCs were decidualised in the absence (control) or presence of 10 µM of each compound for 72 h with the media replaced every 24 h. Compound 1o was also tested for dose-dependent inhibition at 1 and 5 µM. The time course of inhibition of decidualisation was expressed as a percentage reduction in prolactin levels in the conditioned media relative to the control. Three or four independent experiments were performed using different cell preparations for each experiment. $P < 0.05$ was considered statistically significant.

**Lipophilicity calculation**

The log*P* value is a measure of the lipophilicity of a compound; the larger the log*P* value, the more lipophilic the compound is. Lipophilicity (or hydrophobicity) is linked to the compound's ability to penetrate the cell membrane; if a compound is too hydrophilic then it will not be able to cross the cell membrane and if it is too lipophilic, it may remain in the membrane and not pass through into the cell. The log*P* of each compound in Table 1 was calculated using ACDlabs Structure Designer Suite log*P* software (http://www.acdlabs.com).

***In vitro* human trophoblast spheroid attachment assay**

The *in vitro* efficacy of compound 1o to inhibit embryo attachment was determined using a human trophoblast spheroid attachment model involving the co-culture of trophoblast JAR spheroids and monolayers of Ishikawa endometrial epithelial cells (Ho et al., 2012c).

To generate JAR spheroids, JAR cells were grown in suspension in culture media (10ml) at a density of $2.5 \times 10^5$ cells/ml in T75 Nunc tissue culture flask with rocking at a speed of 50 rpm (ERPM4, Ratek Instruments, Victoria, Australia) for 20 – 22 h. Selection of JAR spheroids of size similar to human blastocyst were done as described previously (Ho et al., 2012c). The spheroid suspension was passed first through a cell strainer (BD Bioscience, NSW, Australia) with sieve size 100 µm, to eliminate large cell aggregates, then through a cell strainer of 70 µm sieve size to capture spheroids of size between 70 and 100 µm. Ishikawa cells ($1.5 \times 10^4$ cells/well) were cultured in 96-well plates with or without compound 1o (5 µM or 10 µM) for 3 days to form a cell monolayer, media was then replaced with 50-100 spheroids/well in 100 µl media, and the Ishikawa monolayer and spheroids were co-cultured for 1 h in an atmosphere of 5% $CO_2$ at $37^oC$. Loosely attached spheroids were removed by washing twice with phosphate-buffered saline (PBS), first with 200 µl and second with 100 µl. The percentage of attachment

(attached/seeded spheroids) was calculated and the data was presented in relative to control. Data presented are from three independent experiments, each with four duplicate wells.

# Results

**The five small molecule compounds are potent PC6 inhibitors *in vitro***

Of the five compounds, four of them (1e, 1f, 1g and 1n) were previously shown to be potent inhibitors of both human furin and PC6 *in vitro* (Jiao et al., 2006) but compound 1o had not been screened against PC6. We confirmed that all five compounds inhibit *in vitro* rhPC6 hydrolysis of the fluorogenic peptide substrate pERTKR-AMC (Ho et al., 2012b). At 10 μM, compounds 1e, 1f, 1g and 1n inhibited rhPC6 ≥ 90%, whereas compound 1o had a slightly lower inhibitory potency of 85% (Figure 1).

***In silico* docking reveals a similar binding mode for all five compounds in the hPC6 active site**

Putative binding modes of compounds 1g and 1o are shown in Figure 2. The hPC6 active site is a long groove able to accommodate hexa-peptide length compounds. The small molecule inhibitors, compounds (1e, 1f, 1g, 1n and 1o), need to be able to "hook" themselves into the hPC6 active site via strong interactions with the negatively charged residues that line this site. The five compounds contain these "hooks" in the form of four guanidino moieties. The four guanidino substituents on the 2,5-dideoxystreptamine ring can adopt a variety of conformations within the hPC6 active site; however the 2,5-dideoxystreptamine ring is physically restricted to the triangular region connecting sub-pockets S1, S2 and S1′ (Figure 2).

The binding modes depicted in Figure 2 for compounds 1g and 1o block access to the catalytic site of hPC6. The electrostatically positive guanidino moieties of the compounds are able to interact with the negatively charged residues lining the sub-pockets of the hPC6 active site (these are the red regions in Figure 2). The compounds can also make numerous hydrogen bonds, polar contacts and π-π stacking interactions with hPC6 active site residues. The G, $R_1$, $R_2$ and $R_3$ substituents (Table 1) of the di-aryl 2,5-dideoxystreptamine compounds 1e, 1f and 1g can occupy one or more of the sub-pockets S1, S2 and S4 and also the region near the catalytic triad (D172, H213, S385, Figure 2A). In contrast, the G, $R_1$, $R_2$ and $R_3$ substituents (Table 1) of the tri-aryl 2,5-dideoxystreptamine compounds 1n and 1o are able to occupy the S1, S2 and S3 sub-pockets, in addition to the region near the catalytic triad (Figure 2B). Compound 1n can also occupy the S4 sub-pocket; however, the physical sizes of the 3 naphthyl rings prevent compound 1o from doing so. The binding modes for the five compounds (1e, 1f, 1g, 1n and 1o) in the hPC6 active site were consistent with the binding mode of compound 1n in human furin described previously by Jiao *et al* (Jiao et al., 2006).

**Only compound 1o inhibits decidualisation of HESCs**

Decidualisation of HESCs is a cellular process essential for embryo implantation and successful pregnancy. PC6 is critical for decidualisation and blocking of PC6 activity inhibits the process (Heng et al., 2010, Okada et al., 2005). To determine whether the five compounds would also inhibit PC6-dependent decidualisation, HESCs were cultured without (control) or with 10 μM of each compound in the presence of decidualising stimuli (Heng et al., 2010). Of the five compounds, only compound 1o significantly inhibited decidualisation, whereas the other four compounds had no effect (Figure 3A). Further experiments showed that compound 1o

inhibited decidualisation in a dose-dependent manner, inhibiting ~60% at 1 µM, ~80% at 5 µM and ~ 85% at 10 µM (Figure 3B).

**Compound 1o is the most lipophilic of the five compounds**

Lipophilicity is a major determining factor in a compound's pharmaceutical properties such as penetration across cellular membranes. We therefore calculated the lipophilicity (log*P*) of the five compounds (Table 1). Based on their predicted log*P* values, the relative order of lipophilicity is: compound 1o (moderately lipophilic) > compound 1f > compound 1e > compound 1n > compound 1g (most hydrophilic). All five compounds are potent *in vitro* inhibitors of rhPC6, but only the most lipophilic of the compounds (compound 1o) inhibited PC6-dependent decidualisation of HESCs, suggesting that the other four compounds failed to inhibit decidualisation because of their inability to get into the cell.

**Compound 1o reduced receptivity of Ishikawa endometrial epithelial cells to JAR spheroids**

We next focused on compound 1o and investigated its effect on the attachment of trophoblast spheroids to endometrial epithelium, employing an *in vitro* implantation model (Ho et al., 2012c). This is another PC6-dependent cellular process essential for implantation (Heng et al., 2011a, Heng et al., 2011b). Human choriocarcinoma JAR cells in suspension were rocked overnight to form spheroids (surrogates for embryos). Endometrial epithelial Ishikawa cell monolayers were formed by culturing them, in the absence or presence of compound 1o for 3 days. JAR spheroids were then co-cultured with Ishikawa monolayers and the number of attached spheroids was calculated. Compound 1o significantly reduced the receptivity of

Ishikawa to JAR spheroids in a dose-dependent manner, with an inhibition of ~50% at 10 µM and ~30% at 5 µM (Figure 4).

## Discussion

PC6 plays a crucial role in embryo implantation and HIV infection; it is therefore highly desirable to develop inhibitors of PC6 for potential non-hormonal female contraceptives that could also protect women from HIV. In the ongoing search for PC6 inhibitors with appropriate physiochemical characteristics for therapeutic applications, we investigated five synthetic small molecule compounds that had been previously reported as inhibitors of furin, another PC member (Jiao et al., 2006). Our studies revealed that all five compounds (1e, 1f, 1g, 1n and 1o) were potent inhibitors against rhPC6 *in vitro* and they were able to adopt similar binding modes in the hPC6 active site. However, the functional studies by *in vitro* cell-based model demonstrated that only compound 1o was able to inhibit decidualisation of HESCs. Prediction of lipophilicity, a physiochemical property related to a compound's ability to cross cellular membranes, revealed that compound 1o was distinct in lipophilicity, being the most lipophilic. Compound 1o was further demonstrated to be potent in inhibiting the receptivity of human endometrial epithelial cells for trophoblast spheroid attachment in an *in vitro* human cell-based model.

It is well established that PC6 is the only PC member that is up-regulated during decidualisation, and knockdown of PC6 production by morpholino antisense oligonucleotides in mice *in vivo* resulted in inhibition of decidualisation and pregnancy failure (Freyer et al., 2007, Nie et al., 2005b). Although compound 1o can inhibit furin and possibly other PC

members (Jiao et al., 2006), the inhibitory effect of the compound on decidualisation of HESCs was PC6 specific as only PC6 is involved in decidualisation (Heng et al., 2010). The lack of activity displayed by the other four compounds is likely to be attributed to their poor lipophilicity. Lipophilicity is a key factor that determines how well a molecule can pass through cell membranes (Kenakin, 2012). The data presented here suggests that compound 1o has the ideal lipophilicity to cross the cell membrane and reach its site(s) of action, although the exact cell localization of the compound is yet to be determined.

The drug efficiency of compound 1o in the inhibition of PC6 was further evidenced by its ability to significantly reduce the receptivity of endometrial epithelial cells. It is established that PC6 is up-regulated in the human endometrium specifically at the time of epithelial receptivity (Heng et al., 2011a). The critical role of PC6 in receptivity has been demonstrated by a significant reduction in the attachment of mouse blastocysts to endometrial epithelial cells after specific knockdown of PC6 by small interfering RNA (Heng et al., 2011a). Furthermore, PC6 regulation of receptivity has been validated in the human endometrium *in vivo* in fertile and infertile women (Heng et al., 2011a).

In conclusion, our studies have discovered that compound 1o is a potent PC6 inhibitor with potential pharmaceutical properties to inhibit embryo implantation. In both pharmaceutical and academic research, there have been increasing emphases and demand on cell-based assays to reduce the costly failure of drug development in late stages. Here, we highlight the importance of human cell-based functional assays to investigate drug efficiency. These assays provide invaluable information and demonstrate that physicochemical properties of drugs such as lipophilicity must be investigated in addition to biochemical assays; otherwise highly potent drugs selected based on biochemical characteristics may not be necessarily useful.

While further studies in animal models are yet to be performed, our data showed for the first time of the potential of a non-peptide small molecule PC inhibitor for the development of contraceptives.

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## Figure Legends

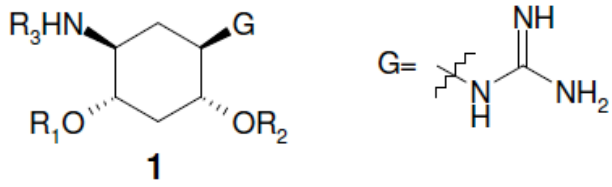
**Figure 1:** Inhibition of PC6 activity by small molecule compounds. The data are expressed as percent reduction of PC6 activity based on the rate of substrate hydrolysis relative to the control. Each value represents mean ± SEM of at least two independent experiments.

**Figure 2:** Putative binding modes of compounds 1g and 1o in the active site of hPC6. hPC6 is depicted as a molecular surface coloured by electrostatic potential (red – regions of negative potential, blue – regions of positive potential), selected residues of the S1-S6, S1′ sub-pockets and catalytic triad are shown as sticks. The $Ca^{2+}$ cation close to the active site is shown as a magenta sphere (labelled as Ca) and the coordinated waters as small red spheres (labelled as H2O). The location of sub-pockets S1-S6 and S1′ are indicated. Electrostatic/hydrogen bonds/polar contacts are indicated by black dashed lines. (A) Binding mode of compound 1g (green coloured sticks). The four guanidino moieties are located in S1, S2 and S4 sub-pockets and near the catalytic triad (D172, H213, S385). (B) Binding mode of compound 1o (yellow coloured sticks). The four guanidino moieties are located in the S1, S2 and S3 sub-pockets and near the catalytic triad (D172, H213, S385).

**Figure 3:** Inhibition of decidualisation of HESCs by small molecule compounds. (A) Inhibition of decidualisation by the five compounds at 10 μM. (B) Dose-dependent inhibition of decidualisation by compound 1o. The data are expressed as percentage reductions relative to control (no inhibitors). Each value represents mean ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$

**Figure 4:** Inhibition of the receptivity of endometrial epithelial cell line Ishikawa to JAR spheroids by compound 1o in a dose-dependent fashion. The data are expressed as the attachment ratio relative to control. Each bar indicates the mean ± SEM of three independent experiments. **P* < 0.05; ***P* < 0.01

Table 1: The chemical structure, molecular weight and log*P* values for the 2,5-dideoxystreptamine-derived small molecules.



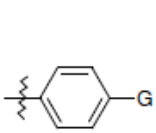
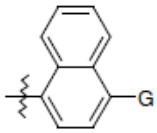
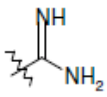
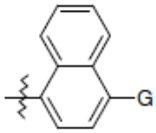
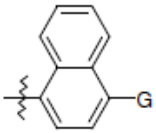
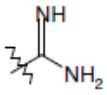
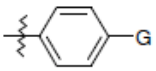
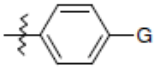
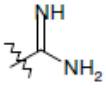
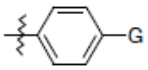
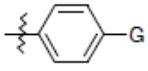
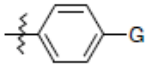
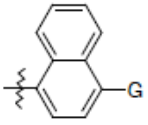
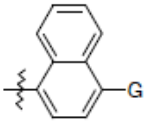
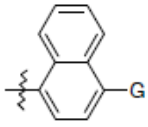
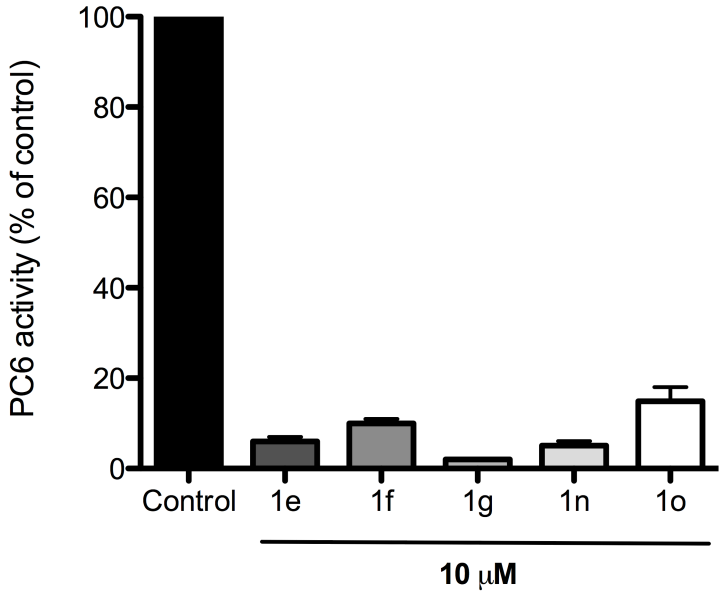

| Compound | $R^1$ | $R^2$ | $R^3$ | Molecular Weight | Log*P* |
|---|---|---|---|---|---|
| 1e |  |  |  | 547 | 0.49 |
| 1f |  |  |  | 597 | 1.67 |
| 1g |  |  |  | 497 | -0.70 |
| 1n |  |  |  | 588 | 0.23 |
| 1o |  |  |  | 738 | 3.79 |

**Figure 1**


PC6 activity (% of control)
100
80
60
40
20
0
Control
1e
1f
1g
1n
1o
10 μM

## Figure 2

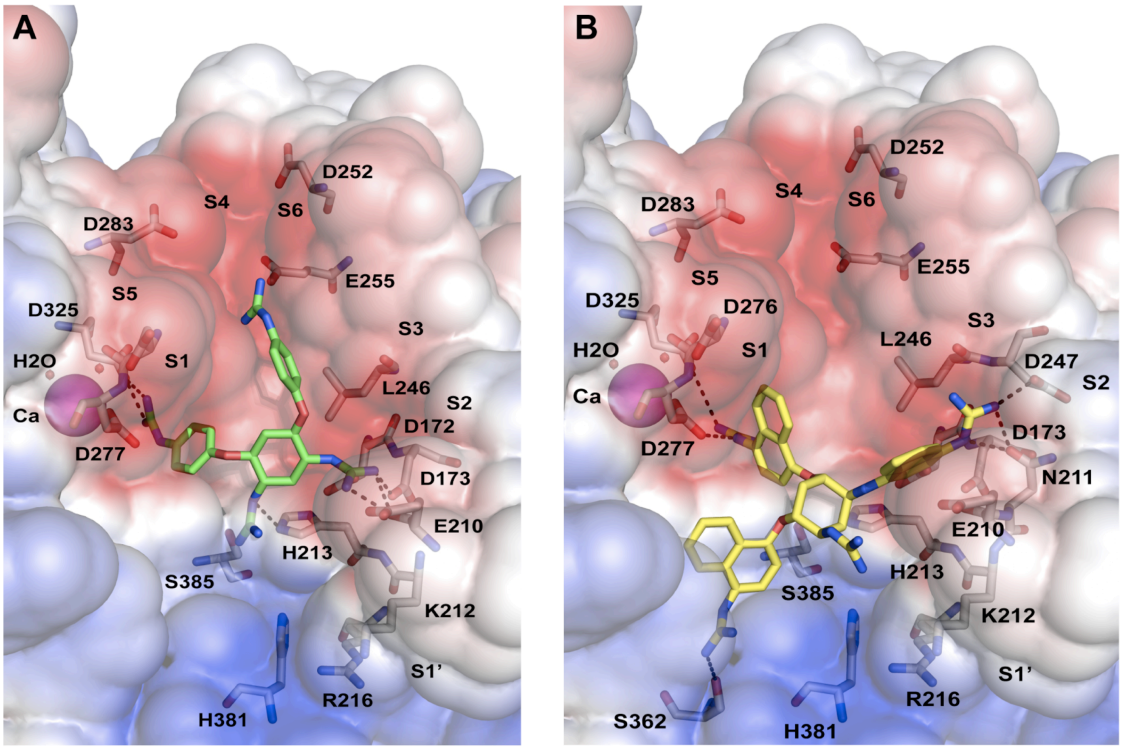

A
D252
S4
S6
D283
E255
S5
D325
S3
H2O
S1
L246
S2
Ca
D172
D277
D173
E210
H213
S385
K212
S1'
R216
H381
B
D252
S4
S6
D283
E255
S5
D325
D276
S3
H2O
S1
L246
D247
S2
Ca
D173
D277
N211
E210
H213
S385
K212
S1'
R216
S362
H381

**Figure 3**

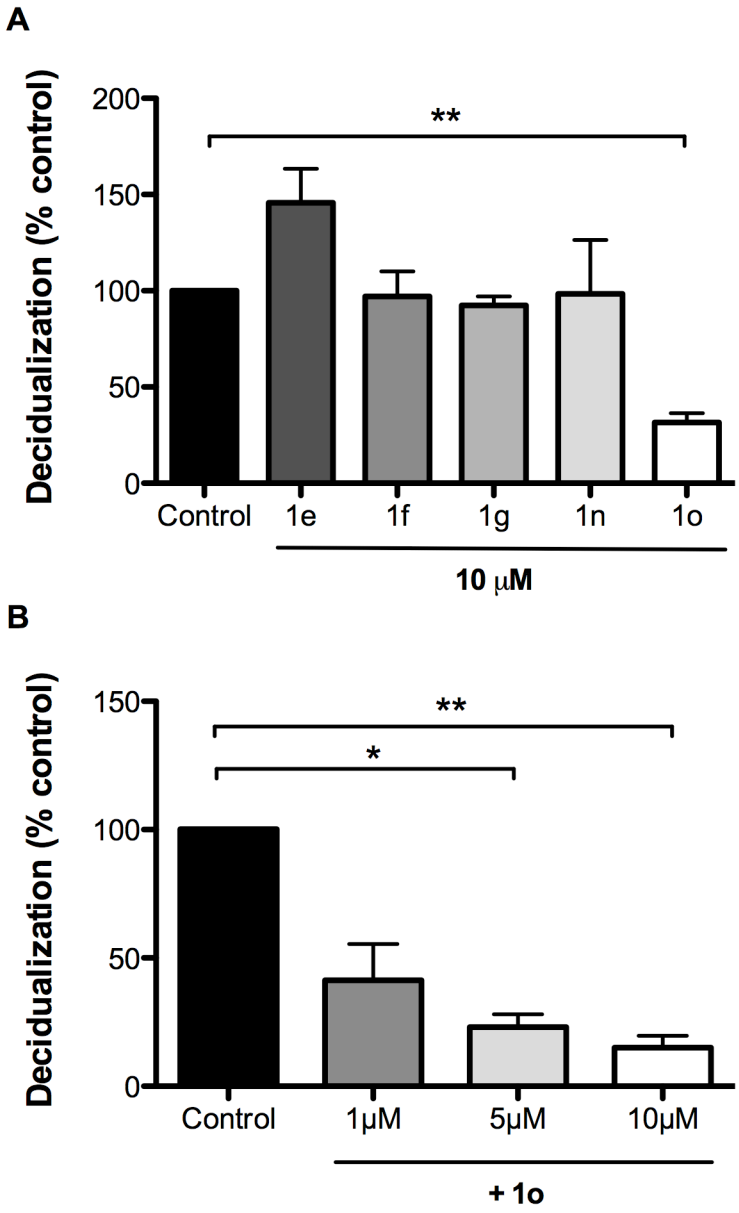
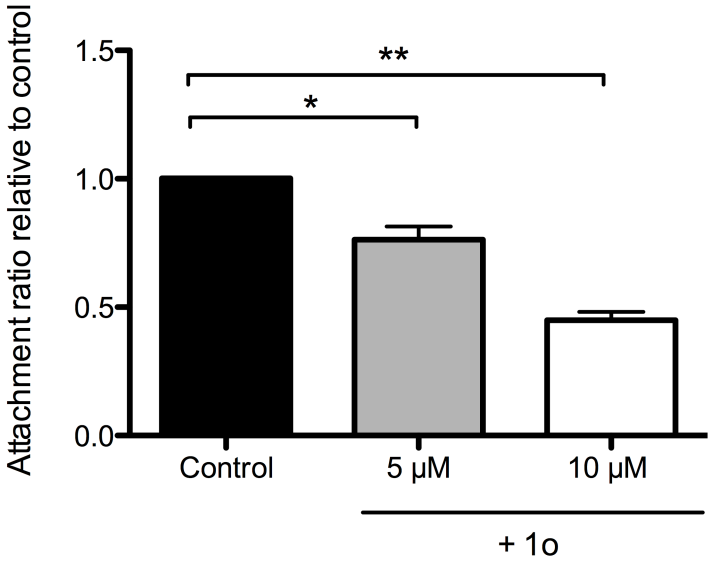

A
Decidualization (% control)
200
150
100
50
0
**
Control
1e
1f
1g
1n
1o
10 μM
B
Decidualization (% control)
150
100
50
0
**
*
Control
1μM
5μM
10μM
+ 1o

**Figure 4**


Attachment ratio relative to control
1.5
1.0
0.5
0.0
**
*
Control
5 µM
10 µM
+ 1o

# Supplementary data (unpublished)

## Note S1: Comparison of C-30k-PEG Poly R and compound 1o in inhibition of decidualisation of primary HESCs

Inhibition of decidualisation of primary HESCs by C-30k-PEG Poly R (the most promising peptide-based PC6 inhibitor with enhanced vaginal absorption, based on the findings of this thesis) and compound 1o was compared (Figure S1). These two inhibitors were comparable and both inhibited decidualisation in a dose-dependent manner.

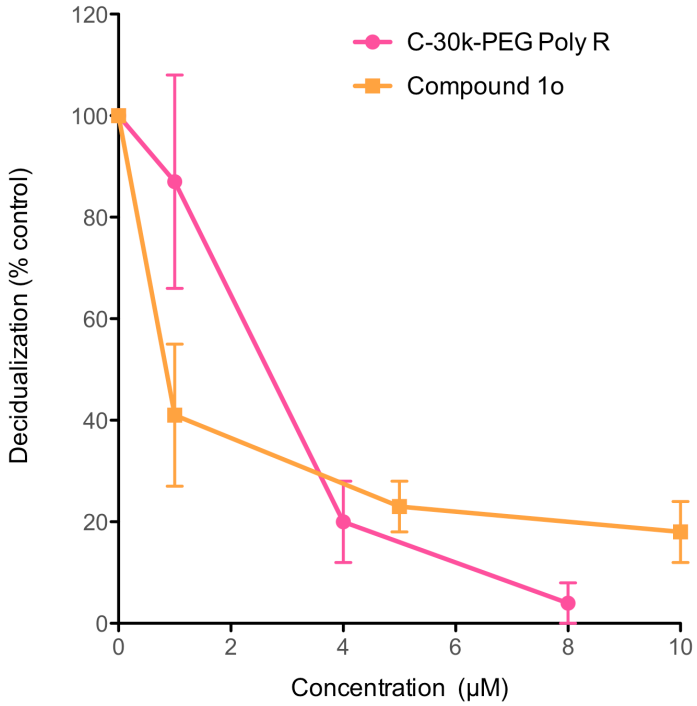



Figure S1. Comparison of C-30k-PEG Poly R and compound 1o in inhibition of decidualisation

Each value represents mean ± SEM of three independent experiments. Source of Data: C-30k-PEG Poly R (Ho et al., 2012a) and compound 1o (Chapter 5).

## Note S2: Comparison of C-30k-PEG Poly R and compound 1o in inhibition of endometrial epithelial cell receptivity to trophoblast spheroids

C-30k-PEG Poly R and compound 1o were also compared for their inhibition of trophoblast JAR spheroids to endometrial epithelial Ishikawa cell monolayers. Results are shown as the attachment ratio relative to the vehicle control (Figure S2). In this particular model, C-30k-PEG Poly R was significantly less potent than compound 1o, and doubling the concentration did not improve the inhibition. In contrast, compound 1o inhibited spheroid attachment (or epithelial receptivity) in a clear dose-dependent manner (0.76 at 5 μM *vs* 0.45 at 10 μM, $p < 0.01$), and was significantly more inhibitory than C-30k-PEG Poly R.

Early studies identified compound 1o as the most lipophilic among the five compounds with a log*P* value of 3.79. Log*P* is a measurement of lipophilicity that links to cell penetration. The exact log*P* for C-30k-PEG Poly R cannot be calculated because PEGs do not have a single defined structure. However, it is known that Poly R is extremely hydrophilic (log*P* = -12.03) and addition of PEG, a polymer, increases the lipophilicity of Poly R. It is thus predicted that compound 1o is relatively more lipophilic than C-30k-PEG Poly R. This may explain (at least partially) why compound 1o was more potent in inhibiting receptivity because spheroid attachment involves the plasma membrane of the endometrial epithelial cells.

**Conclusions:**

Based on the above comparisons, it can be concluded that the most promising peptide-based PC6 inhibitor (C-30k-PEG Poly R) and the small molecule inhibitor (compound 1o) can equally inhibit decidualisation in a functional human cell-based assay. However, compound 1o is superior to C-30k-PEG Poly R in the inhibition of epithelial receptivity.

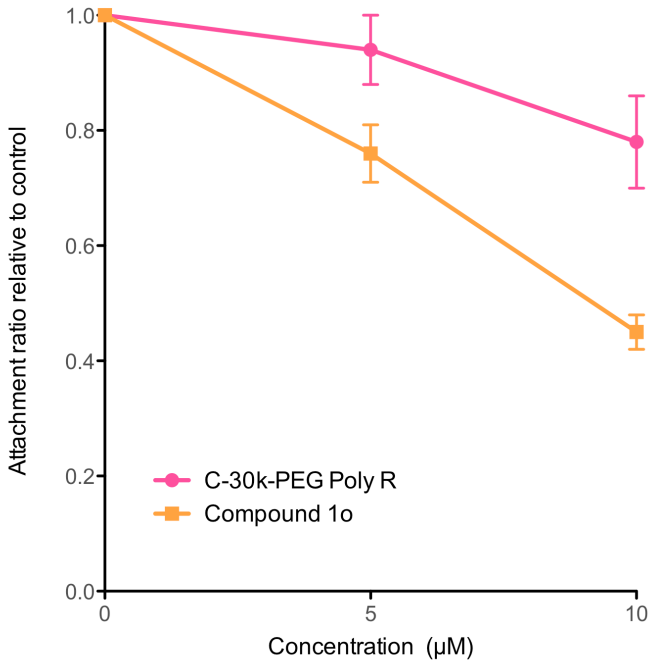



Figure S2. Comparison of C-30k-PEG Poly R and compound 1o in inhibition of Ishikawa cell receptivity to trophoblast JAR spheroids

The data presented are mean ± SEM of three independent experiments.

# Chapter 6 –

# General discussion, conclusion remarks and future directions

# General discussion

At the commencement of this thesis, it was well established that uterine PC6 was essential for decidualisation, a cellular process critical for embryo implantation and pregnancy establishment in both mice and women (Nie et al., 2005b, Okada et al., 2005). It was also confirmed that PC6 was the only PC member associated with embryo implantation (Freyer et al., 2007, Tang et al., 2005). In addition, PCs including PC6 also play a critical role in HIV infectivity (Decroly et al., 1996), and inhibition of PC activity inhibits HIV infection in cells (Anderson et al., 1993, Kibler et al., 2004). Therefore, the research studies described in this thesis aimed to prove the hypothesis that inhibition of PC6 could provide a novel approach to the development of non-hormonal and women-controlled contraceptives that could also protect women from HIV infections. One of the keys to prove this concept is to develop potent and appropriate PC6 inhibitors and test them in cell and animal models. The PC6 inhibitors for this purpose should be vaginally deliverable, because the vagina is the initial entry site of sexually transmitted HIV infection and vaginal application of anti-HIV drugs is essential to stop the initial HIV transmission. Therefore, vaginal delivery of inhibitors to block PC6 activity in the female reproductive tract is the ideal route of administration to achieve the dual protection from HIV infection and pregnancy.

The concept of targeting PCs as a strategy for therapeutic purposes is not completely new. There have been significant proofs of concept in inhibiting PCs for treatments of viral infections, cancer and cardiovascular diseases (Couture et al., 2011). However, there had been no studies investigating the therapeutic applications of inhibiting PC6, until the discoveries of its critical role in embryo implantation by our group (Nie et al., 2005b, Nie et al., 2003).

In this thesis, the contraceptive potential of two types of PC6 inhibitors were established: (i) peptide-based; (ii) non-peptidic small molecule compounds. In the beginning of the research, the studies were focused on modifying a peptidic PC inhibitor, Poly R, which was published as one of the most potent PC6 inhibitors and that could also inhibit HIV replication in cell culture (Fugere et al., 2007, Kibler et al., 2004). However, none of the PC inhibitors are highly specific for a particular PC member because of highly conserved catalytic sites of PCs (50-70%) (Henrich et al., 2005). It is a challenge to develop an inhibitor that is highly specific to only one PC member. Therefore, there are concerns about targeting these enzymes for a therapeutic approach, particularly regarding potential side effects since some PCs, particularly furin are expressed ubiquitously. One of the strategies to overcome this concern is to deliver drugs specifically to the target organs.

It has been demonstrated in women that vaginally administered drugs are preferentially localised to the uterus, a phenomenon called “first uterine pass effect” (Bulletti et al., 1997, De Ziegler et al., 1997). As such, it could be envisaged that, in addition to inhibiting sexually transmitted HIV in the vagina, vaginally administered PC6 inhibitors could also reach the uterus to prevent embryo implantation. In an effort to develop a PC6 inhibitor that shows appropriate pharmacokinetics for optimal vaginal absorption, two strategies were used to modify Poly R: (i) PEGylation and (ii) cyclisation. Both PEGylation and cyclisation are strategies known to improve pharmacokinetics of peptides or proteins (Werle and Bernkop-Schnurch, 2006). PEGylation at the C-terminus of Poly R, regardless of the PEG size, did not compromise its inhibitory potency. In contrast, modifications at N-terminus by PEGylation or cyclisation dramatically reduced its inhibitory activity. These findings provide important insights to assist future design of Poly R derivatives. Furthermore, the studies revealed that C-terminal PEGylation of Poly Rs enhanced vaginal absorption and penetration across the vaginal mucosa/epithelium. This was the first report of a peptide with improved therapeutic

properties for vaginal route of administration following PEGylation. Thus, the strategy of PEGylation should be considered for future design of drugs for vaginal administration.

The critical role of PC6 in decidualisation and embryo implantation was well evidenced in mice (Nie et al., 2005b, Nie et al., 2003). As mice are known as a good model to study decidualisation, *in vivo* studies were conducted using mice as an animal model to prove the concept that vaginal delivery of PC6 inhibitors could block uterine PC6 activity and inhibit embryo implantation. However, it was unknown whether the phenomenon of "first uterine pass effect" existed in mice. Pharmacokinetic studies of Poly R and C-PEGylated Poly Rs in mice following vaginal administration revealed that the majority of absorbed Poly Rs were detected in the blood circulation. Poly R and PEGylated Poly Rs with low molecular weight were rapidly cleared by hepatic metabolism and barely detected in the uterus. In contrast, C-30k-PEG Poly R with a larger molecular weight (32 kDa) remained in the blood circulation for a prolonged time and some signal was detected in the uterus.

Although the study showed limited evidence of a vaginal-uterus transport mechanism in mice, in the absence of a better *in* vivo model, an *in vivo* study was still conducted to test the efficiency and utility of vaginally administered C-30k-PEG Poly R as a contraceptive in mice, because low C-30k-PEG Poly R signals were detected in the uterus. It was noted that C-30k-PEG Poly R showed a prolonged time in the blood circulation, although this would not target the uterus specifically, it was speculated that the prolonged circulation might help its localisation to the uterus at a higher concentration. Should embryo implantation be inhibited, the data would serve as a proof-of-concept. Indeed, the study with a relatively large number of mice showed that vaginally administered C-30k-PEG Poly R inhibited implantation in 71% of mice (24% complete and 47% partial inhibition). Within the context of limited C-30k-PEG Poly R localised to the uterus, this data strongly suggests that inhibition could be higher if efficacy of

delivery could be improved in a more appropriate animal model. In addition, PC6 is abundant in the epithelium in women, critical for both embryo attachment and stromal decidualisation, whereas it is present only in decidua in mice, suggesting that vaginal administration of PC6 inhibitors in women could well be more efficacious for implantation inhibition than in mice.

A later study published (Menkhorst et al. 2011) demonstrated that vaginal administration of a PEGylated LIF antagonist (PEGLA) (a large, PEGylated protein) effectively blocked embryo implantation in mice. The tissue distribution of PEGLA was sensitively detected by radioactive isotope in the study. It was revealed that vaginally administered PEGLA localised to the ovary and oviduct more rapidly and at higher concentration compared to other organs including the uterus (Menkhorst et al., 2011), and it could be that inhibition of LIF in oviduct enhanced the effect on implantation inhibition. In addition, Okada et al. demonstrated that a potent luteinising hormone-releasing hormone analogue (leuprolide) was effective in ovaries following vaginal administration in rats (Okada et al., 1982, Okada et al., 1983, Okada et al., 1984). These two reports suggest that mice/rats are animal models more suitable for vaginal drug delivery studies, where the target organ is the ovary and/or oviduct. This is in contrast to human studies, which showed a preferential localisation of drugs to the uterus following vaginal administration by the “first uterine pass effect” (Bulletti et al., 2001, De Ziegler et al., 1997). My study thus concluded that an alternative animal model is required for future research of vaginal administration of PC6 inhibitors as contraceptives. Indeed, a subsequent study by Aljofan et al. (2012) from our group suggested that the rabbit might be a more appropriate animal model. Transcervical delivery of surgical delivery of PEG Poly R to assure intrauterine placement was not performed because the aim was to develop a vaginal deliverable formulation to inhibit uterine PC6 protein.

During this thesis research, it was shown that PC6 also plays a critical role in the establishment of endometrial epithelial receptivity in women (Heng et al., 2011a, Heng et al., 2011b). Therefore, it is undoubtedly logical to demonstrate that potential PC6 inhibitors will also inhibit endometrial receptivity via the epithelium. An *in vitro* model for human embryo attachment was essential to evaluate the effects of PC6 inhibitors on the endometrial epithelial receptivity. Although attachment models involving co-culture of a monolayer of human endometrial epithelial cells (to mimic the uterine epithelium) and trophoblast spheroids (to mimic blastocysts) had been published (John et al., 1993, Li et al., 2002, Wang et al., 2011), the methodologies are technically demanding, time consuming and variable. In an effort to establish an effective, reliable and high-throughput model to test the PC6 inhibitors, significant optimisations were introduced to the conventional methodology. Substantial efforts led to the establishment of a simple, high-throughput and widely applicable *in vitro* human embryo attachment model for the assessment of trophoblast spheroid attachment to human endometrial epithelial cells. Since the availability of a good model is imperative to advance research in embryo implantation, this model has attracted much attention from researchers in the field. In addition to email enquiries for the details of this model, a researcher from overseas obtained funding to visit me for 2 weeks to learn this model. Therefore, my establishment and publication of this *in vitro* model for human embryo attachment has made significant contributions to the field.

It is important to note that caution is needed when using any *in vitro* human embryo attachment model because none of the human trophoblast or endometrial epithelial cell lines exactly represents the blastocyst or the endometrium. Each cell line has different characteristics; thus the selection of appropriate cell line for functional studies of implantation is vitally important (Hannan et al., 2010). The endometrial epithelial cell line, Ishikawa, and trophoblast cell line, JAR, were used in the attachment model to assess the inhibitory effect of

PC6 inhibitors for this thesis. Ishikawa cells possess apical adhesiveness for JAR spheroids (Heneweer et al., 2005). The cell line is widely regarded as a good model for the study of normal endometrial function as it expresses many enzymes, functional steroid receptors, and structural proteins found in the normal endometrium (Castelbaum et al., 1997, Lessey et al., 1996). Other trophoblast and endometrial epithelial cell lines and rarely available human blastocyst and primary endometrial epithelial cells could be used in limited studies to validate the cell line data.

Another main aim of this thesis was to identify/develop new, potent and non-peptidic PC6 inhibitors that could have advantageous pharmacological properties. We focused on a class of five small synthetic molecule compounds derived from 2,5-dideoxystreptamine that were previously reported as potent inhibitors of furin, another PC member (Jiao et al., 2006), and made available to us by PanThera Biopharma LLC through collaboration. Although the number of small molecule compounds that target PC family members is increasing, these classes of inhibitors remain simple observations and only one (dicoumarol derivative) has been tested in a cellular assay (Komiyama et al., 2009). Therefore, it is yet unknown whether these compound inhibitors will have therapeutic potential or even research-based applications.

The five small 2,5-dideoxystreptamine molecule compounds were investigated here as non-peptidic PC6 inhibitors. All five compounds showed potent inhibition of PC6 activity *in vitro* and could adopt a similar binding mode to the PC6 active site as analysed by *in silico* docking studies. Surprisingly, when tested functionally for their inhibition of decidualisation of primary HESCs, only compound 1o was able to inhibit the process. The lack of inhibitory activity of the other inhibitors in the cell-based assay may reflect the inability of the compounds to penetrate the cell membrane. Lipophilicity is a key-determining factor of a compound's ability to penetrate cell lipid membranes (discussed further below). Compound 1o

is unique, as it is the most lipophilic among the five compounds. Compound 1o also inhibited attachment of human trophoblast spheroids to endometrial epithelial cells, suggesting that it is a potent small molecule PC6 inhibitor with pharmaceutical potential to inhibit embryo implantation. This finding highlights that human cell-based functional models are vital to complement the biochemical and *in silico* analyses in the selection of promising drug candidates. Therefore, our human cell-based models that can reproduce PC6-dependent cellular processes critical for embryo implantation should be used in future as the basis to evaluate and validate potential PC6 inhibitors as contraceptive drugs.

The data from studies of the small molecule compounds indicates that lipophilicity may contribute significantly to the overall quality of drugs. Drugs that are not lipophilic tend not to enter the membrane, while those that are extremely lipophilic easily dissolve in the lipid region of the membrane, but will not diffuse through. Log*P* is a measurement of a compound's overall lipophilicity; a negative value represents hydrophilic. It has been found that a log*P* in the range of 2 to 3 results in the maximum rate of transport by diffusion through a lipid membrane (Byers and Sarver, 2009). PC6 has 2 isoforms and only one isoform, PC6A was detected in the uterus tissues (unpublished data). PC6A is soluble, associated with the regulated secretory pathway, and recent data showed that PC6A could also localise to and retain activity at the cell surface through association with HSPGs (Bergeron et al., 2000, Nour et al., 2005) (see Figure 2 in Chapter 1). PC6 localised in different cellular compartments or at the cell surface may contribute differently to the process of decidualisation or in establishment of endometrial epithelial receptivity. However, this speculation is yet to be experimentally confirmed. Compound 1o (log*P* = 3.79) was able to inhibit decidualisation as well as endometrial epithelial receptivity in cellular assays, suggesting that it may have the ideal lipophilicity to inhibit both intracellular and cell surface PC6. On the other hand, Poly Rs that were less potent than compound 1o in inhibiting endometrial epithelial receptivity could have

different cellular localisation profiles to inhibit PC6. Although Poly R (nono-D-arginine) is extremely hydrophilic (log*P* = -12.03), its ability to readily enter into stromal cells and inhibits decidualisation (Heng et al., 2010) may suggest it has the properties of cell penetrating peptides (CPPs). CPPs are rich in basic amino acids such as arginine, and are able to translocate across membranes or through endocytotic pathways to acquire access to the cell interior (Madani et al., 2011).

## Conclusion remarks

In conclusion, this thesis has established vital information regarding the contraceptive potential of two types of PC6 inhibitors, (i) peptide based and (ii) non-peptidic small molecule compounds. The Poly R studies demonstrating the benefits of PEGylation have provided important insights into future design of drugs for vaginal delivery. However, the subsequent functional studies in mice revealed that, the mouse is not the ideal animal model for testing vaginal administration of PC6 inhibitors as contraceptives. This important information will inform future research; stressing mice are not an appropriate animal model to investigate vaginally administered drugs where the target organ is the uterus. To assist my research and that of others, and to fill a knowledge gap in the field, a simple and reliable *in vitro* model for human embryo attachment was established and proven to be efficient and reproducible. This model has attracted attention from researchers in the field, strongly indicating its significant contribution. From the study of a class of small molecule compounds, we discovered that compound 1o is a potent PC6 inhibitor with potential pharmaceutical properties to inhibit embryo implantation. Furthermore, this study has highlighted the importance of functional human cell-based models to determine the suitability and quality of promising drug candidates. The data of this thesis have provided important insight into proving that inhibiting PC6 in

female reproductive tract may provide a novel approach to the development of non-hormonal and women-controlled contraceptives.

## Future directions

Although both C-30k-PEG Poly R and compound 1o can equally inhibit decidualisation of stromal cells, compound 1o was significantly more potent than C-30k-PEG Poly R in inhibiting epithelial receptivity. These suggest that the small molecule inhibitor may have advantageous therapeutic effects than the peptide-based inhibitor. The cellular localisation of compound 1o and C-30k-PEG Poly R should be investigated in future studies. This will provide an insight into their sites of action, and could explain their differences. Future *in vivo* studies also need to evaluate whether compound 1o is superior to C-30k-PEG Poly R as a vaginally deliverable PC6 inhibitor for inhibition of embryo implantation. Identification of an appropriate animal model or development of an *ex-vivo* model to examine the vaginal absorption and localisation of vaginally administered compound 1o will advance the evaluation of its pharmacokinetic properties for vaginal administration. Further investigations in an appropriate animal model, ideally primate, will provide substantial proof of inhibition of epithelial receptivity and decidualisation *in vivo* by compound 1o, and its efficacy as an implantation-inhibiting contraceptive drug can be determined.

Investigation of the ability of C-30k-PEG Poly R and compound 1o in inhibiting HIV infection is yet to be conducted. This will further prove the hypothesis that inhibiting PC6 in the female reproductive tract can provide the dual protection (contraception and HIV infection). Following confirmation of their potency against HIV replication in cell culture, these inhibitors could be further tested in an animal model, such as the humanised BLT (bone marrow, liver, thymus) mice that have been established for the study of HIV infection, as these

mice are susceptible to intravaginal HIV infection and had been used to test anti-HIV drugs administered through the vagina (Denton et al., 2008).

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