ONLINE SUPPORTING INFORMATION

Fragment-based discovery of potent and selective DDR1/2 inhibitors.

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2. Extended selectivity data

Supplementary Table S1. Extended selectivity data on selected compounds. All data are in μM .

Kinase	Compounds				
	4	5	8	9	dasatanib
DDR2	0.0033	0.0081	0.0058	0.0061	0.002
DDR1	54% at 0.0015	0.006	44% at 0.0015	59% at 0.005	40% at 0.0015
c-Kit	0.019	0.31	49% at 3	0.16	90% at 0.003
Abl	53% at 0.1	60% at 1	39% at 1	70% at 1	<0.001 ^b
b-raf	51% at 0.1	49% at 1	44% at 0.1	30% at 1	
p38	39% at 0.1	38% at 0.1	58% at 0.1	27% at 0.1	0.1 ^b
PDGFRb	73% at 1	NA ^a	NA ^a	56% at 1	0.012
EphA3	63% at 1	73% at 1	73% at 1	31% at 1	
Lck	50% at 1	38% at 1	40% at 1	NA ^a	0.0004 ^b
EphB4	48% at 1	45% at 1	74% at 1	44% at 1	
PDGFRa	37% at 1	NA ^a	NA ^a	32% at 1	
Yes	NA ^a	NA ^a	NA ^a	34% at 1	0.0005 ^b
MuSK	NA ^a	NA ^a	NA ^a	NA ^a	
Syk	NA ^a	NA ^a	NA ^a	NA ^a	
c-src	NA ^a	NA ^a	NA ^a	NA ^a	0.0005 ^b , 99% at 0.003
Aurora A	NA ^a			NA ^a	
chk1	NA ^a			NA ^a	
GSK3b	NA ^a			NA ^a	
InsR	NA ^a			NA ^a	
Erk2	NA ^a				
MAPKAP2	NA ^a				
Met	NA ^a			NA ^a	
PKA	NA ^a			NA ^a	
VEGFR2	NA ^a			NA ^a	2.8

^a Not Active, i.e., less than 30% activity at 1 μM

^b Data taken from Das *et al.*, *J. Med. Chem.* **2006**, 49, 6819-6832.

Supplementary Table S2. Key enzyme activity data for compounds used in the cell assay (i.e., Figure 3 of main text)^a. All data are in nM.

	4	Dasatanib	Nilotinib	Saracatanib
DDR2	3.3	2	5.6	300
DDR1	54% at 1.5	40% at 1.5	ND ^b	ND ^b
c-Kit	19	90% at 3	ND ^b	540
c-src	<3000	99% at 3	810	3.8

^a Note that further selectivity data is available in the literature for (i) dasatanib (Karaman *et al.*, *Nat. Biotech.* **2008**, 26, 127-132); (ii) nilotinib (Manley *et al.*, *Biochim Biophys. Acta.* **2010**, 1804, 445-453; and (iii) saracatinib (Green *et al.*, *Mol. Oncology.* **2009**, 3, 248-261).

^b No inhibition value was determined in this case.

3. Kinase inhibitory assays

In vitro Binding Assay for DDR1 and DDR2

DDR1 and DDR2 assays were performed using a time resolved fluorescence (TRF) binding assay (Life Technologies). Semi-log serial dilutions of compounds were used to produce a 10-point dose-response curve and were incubated with 0.5 nM DDR1 or 0.25 nM DDR2 (Life Technologies) for 1 hour at room temperature in low volume black 384 well assay plates (Corning). Each well contained 5 nM or 10 nM Kinase Tracer 178 respectively and 2 nM Europium labelled anti-GST antibody (Life Technologies) in assay buffer (50 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM EGTA and 0.01% BRIJ35) in a total volume of 20 µL. The ratio of fluorescence emission at 665 nm/ 615 nm after excitation at 340 nm was obtained using the PHERAstar® plate reader (BMG Labtech). IC₅₀ values were determined by fitting the data to a four parameter logistic fit using Graphpad Prism 6.0. In Table 1 of the main text, all DDR1 and DDR2 binding assays are average values from at least two determinations which were in good agreement with each other. The exception is for the initial fragment **1** and the inactive enantiomer **6** for which the DDR2 binding assay was performed once.

In vitro Activity Assay for c-Src and c-Kit

c-Src and c-Kit (Merck Millipore) assays were performed using time resolved fluorescence (TRF) activity assays. For c-Src, each well contained 1 nM Src enzyme, 30 nM biotinylated HER2 peptide (GGMEDYYFEFMGGKKK) (Enzo Life Sciences), 1 µM ATP in a total volume of 12 µL assay buffer (50 mM HEPES pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂, 1 mM EGTA, 0.01% BRIJ35). For c-Kit, each well contained 50 nM biotinylated HER2 peptide, 200 µM ATP in a total volume of 30 µL assay buffer (20 mM HEPES pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂, 0.01% BRIJ35). Detection mix containing 0.5 M EDTA and HTRF pair; a europium-labelled anti-phosphotyrosine antibody (Perkin Elmer) and streptavidin-labelled XL665 (Cisbio) was then added and the ratio of fluorescence emission at 665 nm/ 615 nm after excitation at 340 nm was obtained using the PHERAstar® plate reader (BMG Labtech). IC₅₀ values were determined by fitting the data to a four parameter logistic fit using Graphpad Prism

Kinase Panel

Selectivity of DDR inhibitors was determined using the SelectScreen kinase profiling service at Life Technologies.

4. Cell-based assays

Cell Culture

NCI-H2286 and HCC-366 cell lines were obtained from ATCC and DSMZ, respectively and cultured in RPMI-1640 medium + 10% (v/v) FBS. Cells were maintained at 37°C in an atmosphere of 5% CO₂

The HEK293 cell line was obtained from ATCC and cultured in EMEM + 10% FBS + 1% NEAA + 2 mM L-Glutamine. All cells were maintained at 37°C in an atmosphere of 5% CO₂. The complete open reading frame of DDR2 was cloned into the pcDNA3.1(+) (Life Technologies) mammalian expression vector for transfection into HEK293 cells using lipofectamine 2000 and the manufacturers (Life Technologies) standard protocols. An I638F point mutation was also introduced into the construct above using the primer sequence: 5'GGACCCAAACATCTTCCATCTATTAGCTG 3' and utilizing the Quick-change site directed mutagenesis kit from Agilent Technologies. HEK293 cells stably expressing wt DDR2 or the DDR2 I638F construct were selected by adding geneticin (Life Technologies) to the medium at 0.5 mg/ml.

Determination of DDR2 cellular activity

DDR2 Immunoprecipitation and Western blotting

Cells were treated with 10 µg/ml collagen (Sigma) for 30 minutes prior to treatment with a range of compounds. Following a 16 hour incubation, cells were harvested and lysed in ice-cold TG lysis buffer. Protein concentrations were determined using the BCA protein assay (Pierce) and lysates adjusted to equal protein concentrations. 10 µg biotinylated DDR2 antibody (R&D Systems) was added to lysates and incubated overnight at 4°C. Pre-washed Streptavidin magnetic beads (ThermoScientific) were added to the lysate/biotinylated antibody mixture. Following a 1 hour incubation at RT the beads were collected and washed in TBS-T. Beads were boiled in protein sample buffer for 5 minutes and collected. The resulting supernatant was analysed by western blot analysis. Samples were resolved by SDS-PAGE on NuPage Novex Tris-Glycine gel systems, transferred to nitrocellulose membranes and incubated with primary antibodies specific for DDR2 (R&D Systems) and phospho-tyrosine proteins (Millipore). Blots were incubated with infrared-dye-labeled secondary antibodies and detected on the Odyssey infrared imaging system (LI-COR Biosciences).

Mesoscale Discovery Assay

Cells were treated with 10 µg/ml collagen (Sigma) for 30 minutes prior to addition of compound **4**. Following a 16 hour incubation, cells were harvested and lysed in MSD lysis buffer (Mesoscale Discovery). Protein concentrations were determined using the BCA protein assay (Pierce) and lysates adjusted to equal protein concentrations. 96-well streptavidin high-bind MSD plates (Mesoscale Discovery) were coated with 25 µl per well of 1 µg/ml biotinylated anti-DDR2 antibody (R&D Systems). Plates were incubated for 1 hour at room temperature, prior to blocking for 1 hour with 150 µl 3% BSA. 25 µl cell lysate per well was added to the anti-DDR2-coated MSD plates in duplicate wells and incubated overnight at 4°C. Plates were washed with TBS-T prior to the addition of 25 µl detection antibody specific to phospho-tyrosine proteins (4G10, Millipore). Following a 2 hour incubation at room temperature, plates were washed with TBS-T. 150 µl of read buffer (Mesoscale Discovery) was added, and the plates were analyzed on a SECTOR Imager 2400 reader (Mesoscale Discovery). IC₅₀ values were calculated using a sigmoidal dose–response equation (Prism GraphPad software).

Figure S1. Mesoscale Discovery (MSD) assay for quantification of DDR2 activity in HEK293 cells stably transfected with wild type DDR2 and treated with compound **4**.

Cellular DDR2 expression and activity

Cellular DDR2 expression and activity levels were determined following DDR2 immunoprecipitation, as described, DDR2 activity was stimulated by a 16 hour treatment with 10 µg/ml collagen (Sigma).

Figure S2. Cellular DDR2 expression and activity levels. The mutant-DDR2 cell lines NCI-H2286 and HCC-366 lack detectable DDR2 expression or activity. Total DDR2 and activated DDR2 (pY) were observed in HEK-293 cells stably transfected with wild-type DDR2.

Cell proliferation assays

Cell proliferation assays were carried out using Alamar Blue (Invitrogen) as described previously (Squires et al., Biological characterization of AT7519, a small-molecule inhibitor of cyclin-dependent kinases, in human tumor cell lines. *Mol. Cancer Ther.* **2009**,8, 324-32). Briefly, cells were seeded into 96-well plates at 5×10^3 per well 1 day before addition of either a compound in 0.1% DMSO or DMSO alone (control). Cells were incubated with compound for 72 hours before viability was assessed using Alamar blue. Raw values were expressed as a % of DMSO control. IC_{50} values were calculated using a sigmoidal dose-response equation (Prism GraphPad software).

Cell proliferation assays were also carried out on a number of comparator cell lines in which DDR2 is not mutated. This included compounds **3** and **4** in A549 (lung), A2780 (ovarian) and A375 (melanoma) cell lines; the compounds had no significant effect on proliferation at 10µM, the highest concentration tested.

5. *In vivo* Pharmacokinetic data, methods and analysis

Method

Compounds **4**, **8** and **9** were administered either intravenously at a dose of 1 mg/kg, formulated in 10% DMSO/20% PEG200/70% saline, or via oral gavage at a dose of 5 mg/kg, formulated in 10% DMSO/20% PEG200/70% water, to groups of nine male balb/c mice. Blood samples were taken via either saphenous vein bleeding or cardiac puncture at various time points over 24 hours and centrifuged to obtain plasma (2000 g at 4°C, 10 min). All animal experimentation was performed under the control of the Animal (Scientific Procedures) Act (1986).

Compounds **4**, **8** and **9** were extracted from plasma using protein precipitation. Samples (50 µL) were precipitated with internal standard in acetonitrile (150 µL). Calibration standards and quality controls were prepared in blank matrix and extracted under the same conditions. All samples were centrifuged at 3700 rpm at 4°C for 20 min. Supernatant was analysed and quantified by reverse-phase liquid chromatography-mass spectrometry (LC-MS). Non-compartmental pharmacokinetic analyses were performed using Phoenix® (Certara USA, Inc) software.

Pharmacokinetic Summary

Supplementary Table S3. *In vivo* pharmacokinetic parameters* for compounds **4**, **8** and **9** following intravenous dosing at 1 mg/kg

Compound		Clearance (mL/min/kg)	V _{ss} (L/kg)	Half Life (h)	AUC _{last} (h.ng/mL)	AUC _{0-∞} (h.ng/mL)
4	Mean	7.65	0.42	0.8	2166 ± 31	2178
8	Mean	2.99	0.31	1.4	5338 ± 460	5576
9	Mean	16.6	1.9	1.8	1002 ± 100	1004

* Non-compartmental PK fitting to sparse sampling data allows the calculation of standard errors on AUC

Supplementary Table S4. *In vivo* pharmacokinetic parameters* for compounds **4**, **8** and **9** following oral dosing at 5 mg/kg

Compound		T _{max} (h)	C _{max} (ng/mL)	Half Life (h)	AUC _{last} (h.ng/mL)	AUC _{0-∞} (h.ng/mL)	F %
4	Mean	0.5	3257 ± 1246	1.8	6453 ±1200	6755	62
8	Mean	0.25	4180 ± 584.2	1.3	15270 ± 1400	15270	55
9	Mean	0.25	1022 ± 200.1	1.5	4864 ± 450	4868	97

* Non-compartmental PK fitting to sparse sampling data allows the calculation of standard errors on AUC and C_{max}

6. *In vitro* pharmacokinetic data, methods and analysis

Methods

Microsomal stability

Compounds **4**, **8** and **9** and a positive control, verapamil, were incubated in duplicate at 1 μ M in male CD-1 mouse liver microsomes at 37°C for 40 min at a microsomal protein concentration of 0.25 mg/mL, in the presence of NADPH (n=2). Negative control incubations, in the absence of NADPH, were performed. Incubations were sampled (50 μ L) at 0, 5, 10, 20 and 40 min, except for the negative controls which were sampled at 0 and 40 min. All samples were centrifuged at 3700 rpm at 4°C for 20 min. Supernatant was analysed and quantified by reverse-phase liquid chromatography-mass spectrometry (LC-MS).

Plasma protein binding

Plasma protein binding of compounds **4**, **8** and **9** (1 μ M) was determined by equilibrium dialysis between male mouse balb/c plasma against phosphate buffered saline using the Rapid Equilibrium Dialysis (RED) device, at 37°C in a shaking incubator at 400 rpm for 6 hours (n=2). Positive reference controls (warfarin, carbamazepine, verapamil) were included. All samples were centrifuged at 3700 rpm at 4°C for 20 min. Supernatant was analysed and quantified by reverse-phase liquid chromatography-mass spectrometry (LC-MS).

Caco 2

Permeability of compounds **4**, **8** and **9** was assessed using the CacoReady™ system. All test and control compounds (propranolol, antipyrine, vinblastine) were incubated at a final concentration of 10 μ M in duplicate to either the apical (180 μ L) of the monolayer to measure apical to basolateral transport (A>B) across the cell barrier or to the basolateral side (750 μ L) to measure the basolateral to apical transport (B>A). For A>B spiking, test and control compounds were diluted from 10 mM DMSO stocks to 10 μ M in Hanks Balanced Salt Solution (HBSS) buffer with 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mM $\text{MgCl}_2 \cdot 5\text{H}_2\text{O}$ and 0.5 mg/mL lucifer yellow made to volume with sterile water (lucifer yellow was used to determine the integrity of the Caco-2 monolayer). For B>A compound spiking, test and control compounds were prepared as for the A>B solutions without the addition of lucifer yellow. Test and control compounds were incubated for one hour at 37°C in a highly humidified atmosphere of 95% air and 5% CO_2 . All samples were centrifuged at 3700 rpm at 4°C for 20 min. Supernatant was analysed and quantified by reverse-phase liquid chromatography-mass spectrometry (LC-MS).

CYP Inhibition

The potency of compounds **8** and **9** against CYP450s 1A2, 2C9, 2C19, 3A4 and 2D6 at 10 μ M were determined using Pan Vera Vivid Cyp450 screening kits (Life Technologies, Paisley, UK). CYPs were supplied in the form of baculosomes containing the CYP450 and NADPH reductase. Test compounds were initially dissolved in methanol and further diluted 20 fold in buffer. The final methanol concentration in the assay was 0.5%. Fluorescence was monitored continuously for 20 min at 30 s intervals on a Molecular Devices Spectramax Gemini reader (Molecular Devices Corporation, Union City, CA, USA), using excitation and emission wavelengths of 390 nm and 460 nm for 1A2, 2C19 and 3A4; 390 nm and 485 nm for 2D6 and 485 nm and 530 nm for 2C9. Initial rates were determined from progress curves.

Kinetic Solubility

Solubility of compounds **4**, **8** and **9** was determined by quantitative ^1H NMR spectroscopy using a Bruker 500MHz Avance III spectrometer equipped with a TXI cryoprobe. Samples were prepared by diluting 20 mM DMSO- d_6 stocks in buffer (40 mM phosphate buffer, 100 mM NaCl, 15% D_2O , pH=7.0) to give a final nominal concentration of 1mM. The raw data files were processed using MNova 10 with qNMR plug in. A sample of hydroxybenzoic acid (2.5 mM) was used as the quantitation reference.

In Vitro Pharmacokinetic Summary

Supplementary Table S5. *In vitro* pharmacokinetic parameters for compounds **4**, **8** and **9**

Compound	Metabolic Clearance In Mouse Liver Microsomes		Mouse Plasma Protein Binding	Caco 2 Permeability and Efflux		CYP450 Inhibition	Kinetic Solubility at 1mM
	Half-Life (min)	Intrinsic Clearance (μL/min/mg protein)	% Bound	Papp A>B (x10 ⁻⁶ cm/sec)	Efflux Ratio	% Inhibition at 10μM	Measured Concentration (μM)
4	48	58	99.2	2.9	26	ND ^a	10
8	59	47	99.7	37	1	1A2: 18 2C9: 82 2C19: 70 3A4: 65 2D6: 29	20
9	18	150	99.3	4.5	4	1A2: 22 2C9: 84 2C19: 79 3A4: 89 2D6: 26	20

^a Not determined due to insufficient solubility. Solubility limit of assay is 2mM in methanol or acetonitrile

7. DDR1 crystallography

Expression, purification and crystallisation of DDR1

Residues 595-913 of DDR1 (IMAGE clone 2964574) were cloned into pFastbac HTb and expressed in sf21 insect cells at 27 °C. Cell pellets were stored at -80 °C. Pellets were resuspended in lysis buffer (25 mM HEPES pH 8.0, 300 mM NaCl, 10% glycerol, 1.5 mM β -mercaptoethanol), lysed by sonication on ice and clarified by centrifugation at 14000 rpm. The supernatant was tumbled with NiNTA resin at 4 °C for 1 hour, the resin washed with lysis buffer and protein eluted in lysis buffer containing 250 mM imidazole. TEV protease was added to eluted protein and dialysed overnight at 4 °C in 20 mM HEPES pH 8.0, 1 mM DTT, 10% glycerol. TEV cleaved protein was further purified by gel filtration (Sephacryl S200, 25 mM HEPES pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM DTT), concentrated to 10 mg/ml and stored at -80 °C. If necessary an ion exchange chromatography step (HiTrap QFF) was used prior to gel filtration.

Sunitinib or 3-bromo-1H-pyrrolo[2,3-b]pyridine were added to a final concentration of 1mM prior to crystallisation. Protein crystals were obtained by the vapour diffusion method following the addition of an equal volume of 15-25% (W/V) PEG3350, 0.2 M NH_4I and incubation at 4 °C. The initial structure of DDR1 was solved by molecular replacement using FGFR1 as a search model. Following manual rebuilding in Coot and refinement using Refmac5, the final model contained residues 604-631, 648-721, 736-912, 154 water molecules, 12 iodide ions and ligand.

Protein crystals were obtained as described above. The crystals were back-soaked with compounds **1**, **4**, **9** or dasatanib in 0.1 M MES pH 6.4, 20% PEG3350, 15% glycerol, 0.2 M NH_4I . Typical soaking concentrations were 25mM for 24-72 hours.

Supplementary Table S6. X-ray diffraction data reduction and structure refinement.

Compound PDB ID	1 5BVK	Dasatinib 5BVW	4 5BVN	9 5BVO
Data collection				
Space Group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell				
a, b, c (Å)	62.03, 71.50, 76.68	61.64, 72.78, 75.79	61.04, 74.50, 78.50	61.92, 75.73, 78.36
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
X-ray source	ESRF ID23.1	ESRF ID29	FRE+	Diamond I04-1
Wavelength (Å)	1.0726	0.976	1.5419	0.9200
Resolution (Å)	52.27-2.27(2.31- 2.27)	33.61-1.94(2.02- 1.94)	78.50-1.92(2.29- 2.21)	78.36-1.85(2.06- 1.98)
Total reflections	46475	68029	66004	162974
Unique reflections	14992	25196	16924	24693
Completeness (%)	99.4 (99.7)	98.2 (98.9)	96.8 (95.8)	99.4 (99.4)
R _{merge} (%)	7.6 (37.9)	5.6 (46.8)	12.5 (104.6)	8.1 (184)
<I/σ(I)>	6.0 (1.3)	8.6 (1.6)	3.4 (1.2)	11.7 (1.2)
Multiplicity	3.1	2.7	3.9	6.6
Wilson B factor (Å)	35.6	35.5	26.8	32.0
Refinement Statistics				
Resolution (Å)	52.35-2.29	33.61-1.94	78.50-2.21	48.58-1.98
R factor (%)	17.7	18.5	33.5	18.8
Free R factor (%)	23.0	23.4	37.0	24.3
Free R reflections (%)	5	5	5	5
No. free R reflections	827	1266	922	1312
Molecules in AU	1	1	1	1
Ions built	6 Iodide	8 Iodide	6 Iodide	7 Iodide
No. non-H atoms	2268	2253	2270	2265
No. water molecules	113	287	67	243
Model quality				
R.m.s.d. bond lengths (Å)	0.011	0.013	0.012	0.011
R.m.s.d. bond angles (°)	1.5	1.1	1.4	1.4
Mean B factors (Å ²)				
Protein	38.2	43.1	25.2	44.3
Ligand	42.9	38.4	13.9	31.4
water	38.6	56.1	17.8	48.9

8. Experimental Details for the Synthetic Procedures and Characterization Data of Compounds 2 - 9

Analytical and Preparative LC-MS systems

Analytical LC-MS system and method description

In the following examples, compounds were characterised by mass spectroscopy using the systems and operating conditions set out below. Where atoms with different isotopes are present and a single mass quoted, the mass quoted for the compound is the monoisotopic mass (i.e. ³⁵Cl; ⁷⁹Br etc.).

Waters Platform LC-MS system:

HPLC System: Waters 2795

Mass Spec Detector: Micromass Platform LC

PDA Detector: Waters 2996 PDA

Platform MS conditions:

Capillary voltage: 3.6 kV (3.40 kV on ES negative)

Cone voltage: 30 V

Source Temperature: 120 °C

Scan Range: 125-800 amu

Ionisation Mode: ElectroSpray Positive or ElectroSpray Negative or ElectroSpray Positive & Negative

Waters Fractionlynx LC-MS system:

HPLC System: 2767 autosampler – 2525 binary gradient pump

Mass Spec Detector: Waters ZQ

PDA Detector: Waters 2996 PDA

Fractionlynx MS conditions:

Capillary voltage: 3.5 kV (3.25 kV on ES negative)

Cone voltage: 40 V (25 V on ES negative)

Source Temperature: 120 °C

Scan Range: 125-800 amu

Ionisation Mode: ElectroSpray Positive or ElectroSpray Negative or ElectroSpray Positive & Negative

Agilent 1200SL-6140 LC-MS system - RAPID:

HPLC System: Agilent 1200 series SL

Mass Spec Detector: Agilent 6140 single quadrupole

Second Detector: Agilent 1200 MWD SL

Agilent MS conditions:

Capillary voltage: 4000V on ES pos (3500V on ES Neg)

Fragmentor/Gain: 100

Gain: 1

Drying gas flow: 7.0 L/min

Gas Temperature: 345 °C

Nebuliser Pressure: 35 psig

Scan Range: 125-800 amu

Ionisation Mode: ElectroSpray Positive-Negative switching

Preparation LC-MS system and method description

Waters Fractionlynx system:

Hardware:

2767 Dual Loop Autosampler/Fraction Collector

2525 preparative pump

CFO (column fluidic organiser) for column selection

RMA (Waters reagent manager) as make up pump

Waters ZQ Mass Spectrometer

Waters 2996 Photo Diode Array detector
Waters ZQ Mass Spectrometer
Waters MS running conditions:
Capillary voltage: 3.5 kV (3.2 kV on ES Negative)
Cone voltage: 25 V
Source Temperature: 120 °C
Scan Range: 125-800 amu
Ionisation Mode: ElectroSpray Positive or
ElectroSpray Negative

Agilent 1100 LC-MS preparative system:

Hardware:

Autosampler: 1100 series "prepALS"
Pump: 1100 series "PrepPump" for preparative flow gradient and 1100 series
"QuatPump" for pumping modifier in prep flow
UV detector: 1100 series "MWD" Multi Wavelength Detector
MS detector: 1100 series "LC-MSD VL"
Fraction Collector: 2 x "Prep-FC"
Make Up pump: "Waters RMA"
Agilent Active Splitter
Agilent MS running conditions:
Capillary voltage: 4000 V (3500 V on ES Negative)
Fragmentor/Gain: 150/1
Drying gas flow: 12.0 L/min
Gas Temperature: 350 °C
Nebuliser Pressure: 50 psig
Scan Range: 125-800 amu
Ionisation Mode: ElectroSpray Positive or
ElectroSpray Negative
Columns:

Typically 5 micron particle sized columns were used where available. Waters XBridge™ Prep OBD™
C18 Phenyl and Diacel® Chiralpak® AD-H

Eluents: the mobile phase eluent was chosen in conjunction with column manufacturers'
recommended stationary phase limitations in order to optimise a column's separation performance.

Methods:

Achiral preparative chromatography

The compound examples described have undergone HPLC purification, where indicated, using
methods developed following recommendations as described in Snyder L. R., Dolan J. W., High-
Performance Gradient Elution The Practical Application of the Linear-Solvent-Strength Model, Wiley,
Hoboken, 2007.

Chiral Preparative Chromatography

Preparative separations using Chiral Stationary Phases (CSPs) are the natural technique to apply to
the resolution of enantiomeric mixtures. Equally, this technique can be applied to the separation of
diastereomers and achiral molecules. Methods are well known in the literature for optimising
preparative chiral separations on CSPs and then using them to purify compounds. Such methods are
described in Beesley T. E., Scott R.P.W.; Chiral Chromatography; Wiley, Chichester, 1998.

Compounds Synthesis and Characterization

Imidazo[1,2-a]pyridine-3-carbonyl chloride

To imidazo[1,2-a]pyridine-3-carboxylic acid (1.0 eq) in DCM (0.2 M) at 0 °C was added dropwise oxalyl chloride (3.0 eq, 2.0 M solution in DCM) and then 1 drop of DMF. The mixture was slowly warmed to room temperature and stirred for 16 h. LCMS analysis of the mixture showed >95% of the desired product (appeared as methyl ester on LCMS upon reaction with MeOH). The reaction was concentrated *in vacuo* and azeotroped with toluene (x3). The acid chloride was obtained as a pale yellow amorphous solid and was used without further purification (quant. yield).

General procedure A

1-(3-Amino-benzyl)-3-phenyl-urea

Phenylisocyanate (4.9 mL, 45 mmol) was added to a solution of 3-aminomethyl-phenylamine (5.0 g, 41 mmol) in DCM (80 mL) at 0°C. The reaction mixture was allowed to warm up to room temperature over 16 hours. The white solid that formed was collected by filtration, washed with Et₂O and dried *in vacuo*. The product was used without further purifications (quant. yield). MS: [M+H]⁺ = 242.

General procedure B

Compound 2

Imidazo[1,2-a]pyridine-3-carboxylic acid {3-[(3-phenyl-ureido)-methyl]-phenyl}-amide

1-(3-amino-benzyl)-3-phenyl-urea (590 mg, 2.47 mmol) was added to a solution of imidazo[1,2-a]pyridine-3-carboxylic acid (200 mg, 1.23 mmol), EDC (260 mg, 1.36 mmol) and HOAt (185 mg, 1.36 mmol) in DMF (75 mL) and the reaction mixture was stirred under N₂ at room temperature for 16 hours. The solvent was removed *in vacuo* and the residue partitioned between water and EtOAc. The aqueous layer was basified with 2N NaOH and extracted with EtOAc. The organic phase was collected, dried over MgSO₄, filtered and concentrated *in vacuo*. Part of the crude material was purified by preparative HPLC to give the desired product as a white solid. MS: [M+H]⁺ = 386. ¹H NMR (400 MHz, DMSO-d₆): 10.21 (1H, s), 9.48 (1H, d), 8.58 (2H, d), 7.81-7.63 (3H, m), 7.57-7.47 (1H, m), 7.47-7.13 (6H, m), 7.06 (1H, d), 6.90 (1H, t), 6.63 (1H, t), 4.33 (2H, d).

5-Aminomethyl-2-methyl-phenylamine

Lithium aluminium hydride (1M in THF, 13.3 mL, 13.3mmol) was added, dropwise, to a solution of 3-amino-4-methyl-benzamide (1.0 g, 6.7 mmol) in THF (25 mL). The reaction was heated to reflux for 2 hours. The reaction was cooled to room temperature and water (2 mL) and 15% sodium hydroxide (0.5 mL) were added cautiously. Stirring was continued for 30 minutes. The solid that formed was

filtered off and washed thoroughly with Et₂O. The filtrate was evaporated to dryness to give the title compound as a brown solid (80% yield). MS: [M+H]⁺ = 137.

1-(3-Amino-4-methyl-benzyl)-3-phenyl-urea

The title compound was prepared starting from 5-aminomethyl-2-methyl-phenylamine and phenyl isocyanate following methods similar to those described in general procedure A (66% yield). MS: [M+H]⁺ = 256.

Compound 3

Imidazo[1,2-a]pyridine-3-carboxylic acid {2-methyl-5-[(3-phenyl-ureido)-methyl]-phenyl}-amide

The title compound was prepared starting from 1-(3-amino-4-methyl-benzyl)-3-phenyl-urea and imidazo[1,2-a]pyridine-3-carboxylic acid following methods similar to those described in general procedure B (15% yield). MS: [M+H]⁺ = 400. ¹H NMR (400 MHz, DMSO-d₆): 9.94 (1H, s), 9.45 (1H, d), 8.56 (2H, d), 7.77 (1H, d), 7.55-7.46 (1H, m), 7.40 (2H, d), 7.34-7.09 (6H, m), 6.89 (1H, t), 6.64 (1H, t), 4.30 (2H, d), 2.24 (3H, s).

(3-Amino-4-methyl-benzyl)-carbamic acid tert-butyl ester

5-aminomethyl-2-methyl-phenylamine (11.7 g, 86.1 mmol) was dissolved in DCM (430 mL), filtered and di-tert-butyl dicarbonate (16.9 g, 77.5 mmol) in DCM (5 mL) was added over 20 min. The reaction was stirred for 2 hours at room temperature and then the solvent was removed *in vacuo*. The residue was triturated with Et₂O and petrol, the solid was collected by filtration and dried in a vacuum oven to give the titled compound as a beige solid (44% yield). ¹H NMR (400 MHz, DMSO-d₆): 7.18 (1H, d), 6.83 (1H, d), 6.47 (1H, s), 6.34 (1H, d), 4.82-4.68 (2H, m), 3.95 (2H, d), 2.01 (3H, s), 1.39 (9H, s).

{3-[(Imidazo[1,2-a]pyridine-3-carbonyl)-amino]-4-methyl-benzyl}-carbamic acid tert-butyl ester

To a solution of (3-amino-4-methyl-benzyl)-carbamic acid tert-butyl ester (6.5 g, 27.7 mmol) and imidazo[1,2-a]pyridine-3-carboxylic acid (5.4 g, 33.2 mmol) in DMF (150 mL) were added DIPEA (9.7 mL, 55.4 mmol) and HBTU (14.1 g, 36.0 mmol) and the reaction was stirred at room temperature for 16 hours. Water was added to the reaction mixture and the precipitate collected by filtration and dried *in vacuo*. The crude brown solid was triturated in EtOAc and petrol and the desired product was collected by filtration (84% yield). MS: $[M+H]^+ = 381$.

Imidazo[1,2-a]pyridine-3-carboxylic acid (5-aminomethyl-2-methyl-phenyl)-amide hydrochloride

To {3-[(imidazo[1,2-a]pyridine-3-carbonyl)-amino]-4-methyl-benzyl}-carbamic acid tert-butyl ester (10.5 g, 27.7 mmol) was added 4M HCl in dioxane (20.0 mL, 80.0 mmol). The reaction was stirred for 2 hours at room temperature. The solvent was removed *in vacuo* and the residue dried in a vacuum oven to give the desired product as an off white solid (36% yield). MS: $[M+H]^+ = 281$.

Compound 4

N-[5-{{{[3-fluorophenyl]carbamoyl}amino}methyl}-2-methylphenyl]imidazo[1,2-a]pyridine-3-carboxamide hydrochloride.

3-fluorophenyl-isocyanate (0.8 mL, 6.8 mmol) was slowly added to a solution of imidazo[1,2-a]pyridine-3-carboxylic acid (5-aminomethyl-2-methyl-phenyl)-amide (1.8 g, 5.7 mmol) and triethylamine (1.8 mL, 12.6 mmol) in DCM (100mL). The reaction was stirred at room temperature for 16 hours. The reaction mixture was concentrated *in vacuo* and the crude material was purified by chromatography on silica gel (gradient 0-15% methanol in DCM). 1M HCl in Et₂O (10mL) was added

to the clean combined fractions and the solvent was removed *in vacuo*. The white solid was then washed with diethyl ether (100mL x2), DCM (100mL) and water (100mL x2). The solid was dried in a vacuum oven to yield the title compound as a white solid (7% yield). MS: $[M+H]^+ = 418$. ^1H NMR (400 MHz, DMSO- d_6): 9.91 (1H, s), 9.45 (1H, d), 8.87 (1H, s), 8.55 (1H, s), 7.76 (1H, d), 7.55-7.41 (2H, m), 7.34-7.09 (5H, m), 7.05 (1H, d), 6.82-6.64 (2H, m), 4.30 (2H, d), 2.24 (3H, s).

1-(3-Amino-4-methyl-phenyl)-ethanone

Tin (II) chloride (15.9 g, 83.7 mmol) was added to a solution of 1-(4-methyl-3-nitro-phenyl)-ethanone (5.0 g, 27.9 mmol) in ethanol (200 mL). The reaction was heated to reflux for 1 hour. The reaction was cooled to room temperature and the pH of the solution was adjusted to 8 by adding a saturated solution of NaHCO_3 . The solid that formed was filtered off and discarded. The filtrate was concentrated *in vacuo* and then partitioned between EtOAc and water. The organic layer was dried over MgSO_4 , filtered and evaporated to dryness to give the title compound as a yellow oil (92% yield). MS: $[M+H]^+ = 150$.

1-(3-Amino-4-methyl-phenyl)-ethanone oxime

Triethylamine (1.4 mL, 10.1 mmol) was added to a solution of 1-(3-amino-4-methyl-phenyl)-ethanone (1.0 g, 6.7 mmol) and hydroxylamine hydrochloride (700 mg, 10.1 mmol) in EtOH (10 mL). The reaction was heated to 120 °C for 20 min under microwave irradiation. The reaction was cooled to room temperature, the solvent was removed *in vacuo* and the residue was partitioned between EtOAc and water. The organic layer was dried over MgSO_4 , filtered and concentrated *in vacuo* to give the title compound as a yellow solid (quant. yield). MS: $[M+H]^+ = 165$.

5-(1-Amino-ethyl)-2-methyl-phenylamine

Conc. HCl (13 drops) was added to a suspension of 1-(3-amino-4-methyl-phenyl)-ethanone oxime (1.1 g, 6.8 mmol) and 10% Pd/C (203 mg) in MeOH (34 mL). The reaction was stirred under H_2 (1 atm) for 3 hours, the catalyst was removed by filtration and the filtrate was concentrated *in vacuo* to give the title compound as a yellow solid (quant. yield). MS: $[M+H]^+ = 150$.

1-[1-(3-Amino-4-methyl-phenyl)-ethyl]-3-phenyl-urea

Phenylisocyanate (123 μ L, 1.1 mmol) was added dropwise to a solution of 5-(1-amino-ethyl)-2-methyl-phenylamine hydrochloride (500 mg, 2.3 mmol) and triethylamine (631 μ L, 4.5 mmol) in DCM (40 mL). The reaction was stirred at room temperature, under N_2 , for 30 min. The volatiles were removed *in vacuo* to give the title compound as a yellow gum which was used in the next step without any further purification. MS: $[M+H]^+ = 270$.

Compound 5 and 6

Imidazo[1,2-a]pyridine-3-carboxylic acid {2-methyl-5-[(S)-1-(3-phenyl-ureido)-ethyl]-phenyl}-amide and Imidazo[1,2-a]pyridine-3-carboxylic acid {2-methyl-5-[(R)-1-(3-phenyl-ureido)-ethyl]-phenyl}-amide

Imidazo[1,2-a]pyridine-3-carboxylic acid (484 mg, 2.7 mmol) in DCM (10 mL) was added to a solution of 1-[1-(3-amino-4-methyl-phenyl)-ethyl]-3-phenyl-urea (600 mg, 2.2 mmol) in DCM (100 mL). The reaction was stirred at room temperature, under N_2 , for 18 hours. The volatiles were removed *in vacuo* and part of the crude material was purified by preparative HPLC. The two enantiomers were then separated by chiral HPLC to give the desired products as white solids.

Fast running enantiomer; MS: $[M+H]^+ = 414$. ^1H NMR (400 MHz, DMSO- d_6): 9.93 (1H, s), 9.46 (1H, d), 8.55 (1H, s), 8.37 (1H, s), 7.77 (1H, d), 7.56-7.45 (1H, m), 7.41-7.11 (8H, m), 6.88 (1H, t), 6.61 (1H, d), 4.88-4.78 (1H, m), 2.24 (3H, s), 1.40 (3H, d).

Slow running enantiomer; MS: $[M+H]^+ = 414$. ^1H NMR (400 MHz, DMSO- d_6): 9.93 (1H, s), 9.46 (1H, d), 8.55 (1H, s), 8.38 (1H, s), 7.77 (1H, d), 7.56-7.45 (1H, m), 7.41-7.29 (3H, m), 7.29-7.11 (5H, m), 6.88 (1H, t), 6.62 (1H, d), 4.88-4.78 (1H, m), 2.24 (3H, s), 1.40 (3H, d).

1-[1-(3-Amino-4-methyl-phenyl)-ethyl]-3-(3-fluoro-phenyl)-urea

3-fluoro-phenylisocyanate (52 μ L, 0.5 mmol) was added dropwise to a solution of 5-((S)-1-amino-ethyl)-2-methyl-phenylamine hydrochloride (200 mg, 0.9 mmol) and triethylamine (252 μ L, 1.8 mmol) in DCM (10 mL). The reaction was stirred at room temperature for 16 hours. The solid that formed was removed by filtration. The filtrate was concentrated *in vacuo* to give the desired product as a yellow gum which was used in the next step without any further purification. MS: $[M+H]^+ = 288$.

Compound 7

Imidazo[1,2-a]pyridine-3-carboxylic acid 5-((S)-1-[3-(3-fluoro-phenyl)-ureido]-ethyl)-2-methyl-phenyl)-amide

The title compound was prepared starting from 1-[1-(3-amino-4-methyl-phenyl)-ethyl]-3-(3-fluoro-phenyl)-urea and imidazo[1,2-a]pyridine-3-carboxylic acid following similar methods to those described in General Procedure B. The desired product was isolated by preparative HPLC and the two enantiomers were separated and isolated by chiral HPLC (16% yield over 2 steps). MS: $[M-H]^- = 430$. ^1H NMR (400 MHz, Me-d₃-OD): 9.52 (1H, d), 8.45 (1H, s), 7.74 (1H, d), 7.63-7.52 (1H, m), 7.41 (1H, d), 7.39-7.12 (5H, m), 7.00 (1H, dd), 6.73-6.63 (1H, m), 4.94 (1H, q), 2.33 (3H, s), 1.52 (3H, d).

Imidazo[1,2-a]pyridine-3-carboxylic acid [5-((S)-1-amino-ethyl)-2-methyl-phenyl]-amide

To a stirred solution of 5-((S)-1-amino-ethyl)-2-methyl-phenylamine (3.1 g, 13.9 mmol) in THF (30 mL) and pH 5 acetate buffer (1.0 M, 90 mL) was added imidazo[1,2-a]pyridine-3-carbonyl chloride (3.3 g, 18.1 mmol) and the mixture was stirred at room temperature for 1 hour. The volatiles were removed under reduced pressure and a mixture of water and sat. NaHCO_3 was added to the residue. The product was then extracted with 3:1 $\text{CH}_3\text{Cl}/\text{IPA}$ (x4). The organic phases were collected, washed with brine, dried over MgSO_4 , filtered and concentrated *in vacuo* to give the desired product as a pale yellow crystalline solid (60% yield). MS: $[M+H]^+ = 295$.

Compound 8

Imidazo[1,2-a]pyridine-3-carboxylic acid {2-methyl-5-[(S)-1-(3-trifluoromethyl-benzoylamino)-ethyl]-phenyl)-amide

To a solution of imidazo[1,2-a]pyridine-3-carboxylic acid [5-((S)-1-amino-ethyl)-2-methyl-phenyl]-amide (264 mg, 0.8 mmol) in DMF (5 mL) were added 3-trifluoromethyl-benzoic acid (152 mg, 0.8 mmol), DIPEA (400 μ L, 2.4 mmol) and HATU (310 mg, 1.0 mmol). The reaction was stirred at room temperature for 16 hours. The reaction was quenched with water and the product was extracted with EtOAc (2x). The organic phases were collected, dried over MgSO_4 , filtered and concentrated *in vacuo*. The residue was purified by preparative HPLC to give the desired product as a white solid (10% yield). MS: $[\text{M}+\text{H}]^+ = 467$. ^1H NMR (400 MHz, DMSO- d_6): 9.91 (1H, s), 9.45 (1H, d), 9.08 (1H, d), 8.54 (1H, s), 8.25 (1H, s), 8.20 (1H, d), 7.91 (1H, d), 7.81-7.69 (2H, m), 7.55-7.45 (1H, m), 7.39 (1H, s), 7.30-7.21 (2H, m), 7.21-7.11 (1H, m), 5.25-5.15 (1H, m), 2.23 (3H, s), 1.52 (3H, d).

5-Fluoro-3H-benzoxazole-2-thione

Potassium ethyl xanthogenate (1.3 g, 7.9 mmol) was added to a solution of 2-amino-4-fluorophenol (1.0 g, 7.9 mmol) in EtOH (20 mL). The reaction was stirred at reflux for 16 hours. The solvent was removed *in vacuo*, the residue was dissolved in water (30 mL) and the pH of the solution was adjusted to 4 with 2N HCl. The precipitate that formed was collected by filtration and dried *in vacuo* to give the desired product as a grey solid (83% yield). ^1H NMR (400 MHz, DMSO- d_6): 7.54 (1H, dd), 7.14 (1H, dd), 7.12-7.05 (1H, m).

2-Chloro-5-fluoro-benzoxazole

5-fluoro-3H-benzoxazole-2-thione (1.1 g, 6.5 mmol) was dissolved in neat thionyl chloride (5.7 mL, 78.0 mmol) and the solution was stirred at 70°C for 1 hour. The volatiles were removed *in vacuo* to give the desired product which was used in the next step without any further purification. ^1H NMR (400 MHz, CDCl_3): 7.48 (1H, dd), 7.40 (1H, dd), 7.18-6.98 (1H, m).

Compound 9

Imidazo[1,2-a]pyridine-3-carboxylic acid {5-[(S)-1-(6-fluoro-benzoxazol-2-ylamino)-ethyl]-2-methyl-phenyl}-amide

DIPEA (278 μ L, 2.0 mmol) was added to a solution of 2-chloro-5-fluoro-benzooxazole (155 mg, 0.9 mmol) and imidazo[1,2-a]pyridine-3-carboxylic acid [5-((S)-1-amino-ethyl)-2-methyl-phenyl]-amide (300 mg, 0.9 mmol) in NMP (3 mL). The reaction mixture was reacted in a microwave oven at 150°C for 30 min. The reaction was cooled to room temperature and poured into water. The precipitate that formed was collected by filtration, washed with water and petrol and dried *in vacuo*. The residue was purified by chromatography on silica gel (gradient 0-100% EtOAc in petrol) to give the desired product as a brown solid (30% yield over 2 steps). MS: $[M+H]^+ = 430$. ^1H NMR (400 MHz, DMSO- d_6): 9.92 (1H, s), 9.44 (1H, d), 8.67 (1H, d), 8.54 (1H, s), 7.77 (1H, d), 7.56-7.45 (1H, m), 7.39 (1H, s), 7.32 (1H, dd), 7.30-7.21 (2H, m), 7.21-7.11 (1H, m), 7.05 (1H, dd), 6.82-6.70 (1H, m), 4.99-4.89 (1H, m), 2.22 (3H, s), 1.51 (3H, d).