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Identification of false positives in "HTS hits to lead": The application of Bayesian models in HTS triage to rapidly deliver a series of selective TRPV4 antagonists

antagonist series. The hit-to-lead efforts in one such series, the hydroxypiperidines, will be described.

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# Herein, we describe the discovery and optimisation of a series of potent and selective TRPV4 antagonists. The application of a variety of computational techniques (including Bayesian modelling) at the HTS triage stage enabled the early deprioritisation of likely frequent hitters. The use of methods to positively prioritise compounds for follow-up screening allowed the rapid identification of a number of interesting TRPV4

# Introduction

TRPV4 is a member of the Transient Receptor Potential (TRP) superfamily of mammalian cation channels. It is expressed in a wide range of tissues<sup>1</sup> and is thought to play a role in a number of physiological responses such as osmoregulation,<sup>2,3</sup> thermosensation,<sup>4</sup> vascular regulation<sup>5,6</sup> and mechanosensation.<sup>2,7</sup> Indeed, it has been postulated that TRPV4 plays a crucial part in the mechanosensory pathway of the bladder by detecting changes in intravesical pressure.8,9 TRPV4 is activated by a range of stimuli including small molecules (e.g. anandamide and 5,6epoxyeicosatrienoic acid), temperature (27-34 °C), hypotonic solutions and mechanical stimuli.10,11

At the outset of the TRPV4 project, there were no known selective TRPV4 antagonists with which to build confidence in mechanism. A high through-put screening (HTS) campaign was therefore initiated on the Pfizer compound file and, in order to meet the through-put requirements of this campaign, a TRPV4 calcium flux assay using FLIPR Tetra technology was utilised.

### **Results and discussion**

#### HTS screening and triage

Compressed file screening generated a large number of hits (~2.5% of compounds had >40% inhibition of TRPV4 at 10  $\mu$ M) for confirmatory follow-up in a single-well, single-point TRPV4 assay. The single-point assay confirmed  $\sim 11$  k of these to be active in this assay (>40% inhibition at 10 µM), a 14% confirmation rate. It is, however, widely recognised that calcium flux assays can deliver false positives or 'frequent hitters' due to a compound being reactive, autofluorescent, cytotoxic or active at another target in the signal transduction pathway.12,13 A key goal of the HTS triage effort was therefore to identify and remove false positives at the outset, allowing efforts to be focussed on true TRPV4 antagonists.

The data from a number of prior Pfizer FLIPR HTS screening campaigns were used as a means of identifying likely false positives from the  $\sim 11$  K putative actives emerging from the TRPV4 single-point screen. Firstly, if a compound was active (>75% inhibition at 10 µM) in more than one of these HTS screens, it was deemed likely to be a false positive (provided the prior HTS was not directed at a target from the same gene family as TRPV4). Secondly, for each previous FLIPR HTS dataset, a Bayesian activity model14 was built to predict the likelihood of activity of a compound in that assay. If a compound was scored highly by more than one such model (Bayesian score >5), it was deemed likely to be a false positive. This second in silico approach offers the advantage that it does not require the compound itself to have been screened in HTS assays, because evidence of activity of its near neighbours is sufficient to suggest it could be a false positive. Indeed, this in silico method filtered out  $\sim$ 5 times as many compounds as the method based on in vitro data. An example of use of these Bayesian specificity models is shown in Fig. 1. Putative actives from the TRPV4 single point assay were trellised by molecular cluster, and % TRPV4 inhibition was plotted against molecular weight. Compounds were coloured by the number of times they were deemed "active" (i.e. scored >5) by any of the non-TRP FLIPR HTS Bayesian models (red = active according to  $\geq 2$  models, yellow = active according to 1 model, green = active according to 0 models). Compounds such as those found in Cluster 4 were retained, whereas compounds such as those found in Cluster 3 were discarded. A selection of compounds such as those found in Clusters 1 and 2 were also retained.

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**Fig. 1** TRPV4 activity vs. molecular weight, trellised by molecular cluster. Red = predicted active by  $\geq$ 2 HTS Bayesian models, yellow = predicted active by 1 model, green = predicted active by 0 models.

Compounds were also removed from the screening cascade if they possessed non-drug-like properties (molecular weight >600 Da, or clogP >6), or contained undesirable or reactive substructures e.g. nitro groups, aldehydes, etc. 15,16 In addition to the application of negative filters, a number of positive filters were applied to prioritise compounds for TRPV4 IC50 follow-up. Positive filters were utilised in order to select compounds with either a higher chance of repeating their activity, or that appeared synthetically tractable for follow-up in the event that they were active. Compounds with; a high level of inhibition in the TRPV4 single point assay (>75% inhibition at 10 µM), a high local hit rate (LHR) value (proportion of near neighbours that are active) with either a LHR >10% or a LHR greater than the background hit rate to the extent that the P value, as judged from a chi-squared test, was less than  $10^{-20}$ ,<sup>17</sup> or that scored highly in the TRPV4 Bayesian model built from the TRPV4 HTS data generated thus far (a Bayesian cut-off score of 15 giving a kappa value k = 0.09) were selected on the basis of their likelihood to retain activity. As can be seen in Fig. 2, the separation of active and inactive peaks in the Bayesian model is not



Fig. 2 Bayesian plot of single-point TRPV4 antagonist data.

significant; however compounds with a Bayesian score of >15 are likely to be enriched in actives.

Finally, compounds with little evidence supporting either inclusion or exclusion were analysed. These moderately active compounds (40–75% inhibition of TRPV4 at 10  $\mu$ M) with an unmeasurable local hit rate (0/0 active neighbours) were analysed by trellising TRPV4 activity plots by Library ID. Library compounds (*i.e.* compounds synthesised by parallel chemistry) were deemed synthetically tractable, and therefore prioritised for selection, along with further close-in library members not present in the original HTS screening set.

A total of 1050 compounds were selected for TRPV4 IC50 follow-up. The results from the IC50 screen were used to seed a second round of compound selection. Work focussed on closein mining of active compounds with the aim of deriving SAR around each hit. A number of fingerprint methods (BCI,18 ECFP619 and Atom Pair20 (in-house Tanimoto similarity algorithm)) were used to identify near neighbours around each selected molecule (the probe). Each neighbour was assessed by calculating its maximum common substructure (MCSS)<sup>21,22</sup> with the probe and only neighbours that differed from the probe by a single moiety were retained. For each probe, the number of neighbours per point of variation was restricted to ten to permit a diverse exploration of close-in neighbours around each probe. Tracking the relative occurrence of the attachment point for each probe also gave an indication of the chemical space occupied around it, suggesting gaps that could be explored by synthesis. The triage workflow is highlighted in Scheme 1.



Scheme 1 TRPV4 HTS triage workflow.



**Compound 1** hTRPV4 IC50 2,200 nM MW/clogP 433/2.7 LIPE/LE 3.3/0.3

Fig. 3 Structure and properties of compound 1.



LIPE/LE 4.00/0.44

Fig. 4 Structure and properties of compound 3c.

Table 1 In vitro TRPV4 data for compounds 2a-d and 3a-d

R2	R3	R4	hTRPV4 IC5	50(nM)	LE	LIPE
F	Cl	Cl	12.9		0.37	4.43
F	Cl	CN	49.1		0.33	5.13
Н	Cl	Cl	49.7		0.36	4.03
F	CN	CN	50.5		0.32	6.10
F	CN	Cl	3.4		0.41	5.39
Cl	CN	Cl	3.6		0.41	4.93
F	Cl	Cl	3.8		0.44	4.66
Cl	Cl	CN	4.0		0.41	5.18
	R2 F F F Cl F Cl Cl	R2 R3 F Cl F Cl H Cl F CN F CN Cl CN F Cl Cl Cl	R2   R3   R4     F   Cl   Cl     F   Cl   CN     H   Cl   Cl     F   CN   CN     F   CN   Cl     Cl   CN   Cl     Cl   CN   Cl     F   Cl   Cl     Cl   Cl   Cl     Cl   Cl   Cl	R2   R3   R4   hTRPV4 IC5     F   Cl   Cl   12.9     F   Cl   CN   49.1     H   Cl   Cl   49.7     F   CN   CN   50.5     F   CN   Cl   3.4     Cl   CN   Cl   3.6     F   Cl   Cl   3.8     Cl   Cl   CN   4.0	R2 R3 R4 hTRPV4 IC50(nM)   F Cl Cl 12.9   F Cl CN 49.1   H Cl Cl 49.7   F CN CN 50.5   F CN Cl 3.4   Cl CN Cl 3.6   F Cl Cl 3.8   Cl Cl CN 4.0	R2   R3   R4   hTRPV4 IC50(nM)   LE     F   Cl   Cl   12.9   0.37     F   Cl   CN   49.1   0.33     H   Cl   Cl   49.7   0.36     F   CN   CN   50.5   0.32     F   CN   Cl   3.4   0.41     Cl   Cl   3.8   0.44     Cl   Cl   CN   4.0   0.41

The TRPV4 HTS and rapid follow-up screening campaign generated a number of interesting TRPV4 series. One series of particular interest was the hydroxypiperidines, exemplified by compound **1** (Fig. 3). This series demonstrated encouraging levels of hTRPV4 inhibitory activity and binding efficiency (LIPE,  $LE^{\dagger}$ )<sup>15,23</sup> and was known to be straightforward to synthesise.

Table 2 In vitro selectivity data for compounds 2c and 3c

	2c	3c
hTRPV4 EC50 (nM)	>1000	>1000
rTRPV4 IC50 (nM)	1150	34.1
rTRPV4 EC50 (nM)	>1000	>1000
hTRPA1 IC50 (nM)	>12 500	>12 500
hTRPA1 EC50 (nM)	>12 500	>12 500
hTRPV1 IC50 (nM)	>3900	>16 000
hTRPV1 EC50 (nM)	>20 000	>20 000
hTRPM8 IC50 (nM)	4800	9580

	Table 3	In vitro ADME	and safety data	for compound 3c
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Compd	HLM $(\mu L \min^{-1} mg^{-1})$	$\begin{array}{c} \text{RLM} \\ (\mu L \ min^{-1} \ mg^{-1}) \end{array}$	DDI <sup>a</sup>	Log D	Dof. IC50 (nM)
3c	<8.1	87	<20%	3.2	9670

 $^a$  DDI (Drug–Drug Interaction). Potential for DDI at Cytochrome P450 1A2, 2C9, 2D6 and 3A4 was assessed at 3  $\mu M.$ 

#### Hit to lead follow-up

Hit-to-lead follow-up on the hydroxypiperidine series largely focussed on improving TRPV4 potency and efficiency (LIPE, LE). Any tool compound would need to be suitable for preclinical in vivo assessment so must also have an appropriate ADME profile<sup>15</sup> and orthologue (rat) pharmacology. Initial targets sought to replace the thio-ether linker with a more polar group to lower logP24 and increase the probability of achieving good metabolic stability.25 In addition, replacement of the 4-pyridyl group was desired to reduce the liability for drug-drug interactions (DDI).16,26 A small library of aryl ether hydroxypiperidines was synthesised and assessed in the hTRPV4 (human TRPV4) assay. Gratifyingly, a number of these compounds demonstrated improved TRPV4 potency and LIPE/ LE, and were devoid of the pyridyl structural alert.<sup>16</sup> Key compounds 2a-d are outlined in Table 1. Examples within the hydroxypiperidine series showed no TRPV4 agonist activity, were moderately potent inhibitors of rat TRPV4 (rTRPV4) and were selective over a number of other TRP channels. Compound 2c exemplifies the selectivity profile of this series (Table 2):

In order to further improve compound potency, a pairwise analysis was conducted on the piperidine linker.<sup>27</sup> In this analysis, a substructure search based method (named SWAP)<sup>27</sup> was used to interrogate the Pfizer database and identify pairs of molecules differing only in the replacement of a piperidine linker group. The biological activity (for a given biological assay) for each matched pair was recorded to generate a dataset that showed the performance of each isostere. Performance was assessed in terms of the likelihood of retaining, improving or losing potency with respect to the starting point. This analysis highlighted a number of potential isosteres, of which an azetidine replacement was calculated to have a  $\sim$ 70% chance of retaining or improving potency (as well as lowering logP).<sup>28</sup>

<sup>†</sup> LIPE (Lipophilic Ligand Efficiency) =  $pK_i - clogP$  (or log *D*). LE (Ligand Efficiency) =  $1.3643pK_i$ /number of heavy atoms.

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**Table 4**In vivo PK parameters of **3c** following IV (intravenous) and PO (oral)administration (n = 2, Sprague-Dawley rats)

3c	$1 \text{ mg kg}^{-1} \text{ IV}$	$2 \text{ mg kg}^{-1} \text{ PO}$
Half life (h)	2.2	
$Cl (mL min^{-1} kg^{-1})$	23.5	
$Cl_u (mL min^{-1} kg^{-1})$	756	
$Vd_{ss}$ (L kg <sup>-1</sup> )	4.3	
$C_{\rm max}$ (nM free)		16
$T_{\rm max}$ (h)		0.75
<i>F</i> %		73

A number of azetidine-linked compounds, utilising the sulfonamide and aryl-ether SAR established in the hydroxypiperidine series, were synthesised. Pleasingly, a number of these analogues such as compounds **3a–d** demonstrated improved hTRPV4 potency and an improved LIPE/LE profile (Table 1).

The hydoxyazetidines displayed similarly high levels of TRP selectivity as the hydroxypiperidines but gratifyingly delivered enhanced rTRPV antagonist activity. Compound **3c** exemplifies





**Scheme 3** Synthesis of hydroxyazetidines **3a–d**. *Reagents and conditions*: (i) Ph<sub>3</sub>PCH<sub>2</sub>Br, KOt-Bu, Et<sub>2</sub>O, RT, 61%; (ii) NBS, DMSO/H<sub>2</sub>O, RT, 0–25 °C, 83%; (iii) NaH, THF, 0–25 °C, 62%; (iv) (R<sub>1</sub>)(R<sub>2</sub>)Ar–OH, Cs<sub>2</sub>CO<sub>3</sub>, IPA/DMSO/H<sub>2</sub>O, 80 °C, 55–71%; (v) TFA, DCM, 25 °C, 85–97%; (vi) (R<sub>1</sub>)(R<sub>2</sub>)SO<sub>2</sub>Cl, NEt<sub>3</sub>, DCM, 0–25 °C, 16–90%.

the selectivity profile of the hydroxyazetidine series (Table 2). *In vitro* ADME<sup>‡</sup> end-points for compound **3c** were determined along with an assessment of hERG liability<sup>29</sup> utilising a Dofetilide binding assay<sup>30</sup> (Table 3). The *in vitro* ADME profile of **3c** was very promising, with low turnover in human liver microsomes and only a relatively weak activity signal in the Dofetilide assay. The pharmacokinetic properties of **3c** were also assessed. Compound **3c** demonstrated almost complete oral absorption and a ~2 hour half-life in the rat (Table 4). It was therefore deemed a suitable tool with which to conduct further rodent *in vivo* studies.

#### Chemistry

The synthetic route towards hydroxypiperidines **2a–d** is outlined in Scheme 2. Briefly, nucleophilic addition of phenol derivatives  $(R_1)(R_2)Ar$ -OH to epoxide **4** under basic conditions furnished *N*-Boc hydroxypiperdines **5a** and **b**. *N*-Boc deprotection of compounds **5a** and **b** was accomplished with TFA/ DCM to furnish amines **6a** and **b**. Addition of an appropriate sulfonyl chloride,  $((R_1)(R_2)ArSO_2Cl)$ , to amines **6a** and **b**, in triethylamine and dichloromethane (DCM), gave hydroxypiperidines **2a–d**.

The synthesis of hydroxyazetidines  $3\mathbf{a}-\mathbf{d}$  is described in Scheme 3. Ketone 7 was converted to alkene 8 *via* addition of Ph<sub>3</sub>PCH<sub>2</sub>Br and potassium *tert*-butoxide. Alkene 8 was further converted to a regioisomeric mixture of hydroxybromides (9a and 9b) through addition of NBS in DMSO and water. Addition of sodium hydride to a solution of 9a and 9b furnished epoxide 10 in good yield. Nucleophilic ring-opening of epoxide 10 with the appropriate phenol derivative, ((R<sub>1</sub>)(R<sub>2</sub>)Ar–OH), afforded *N*-Boc-hydroxypiperdines 11a and b, which were subsequently deprotected to their respective amines (12a and b) under TFA/ DCM conditions. A final sulfonylation step on amines 12a and bwith the appropriate sulfonyl chloride ((R<sub>1</sub>)(R<sub>2</sub>)SO<sub>2</sub>Cl), in triethylamine and DCM, furnished hydroxyazetidines 3a–d.

#### Conclusions

The application of computational techniques (including Bayesian modelling) in the triage of TRPV4 HTS data enabled the early deprioritisation of putative "actives" that were, in fact, likely to be "frequent hitters". In addition, the use of methods to positively prioritise compounds for follow-up screening allowed the rapid identification of a number of interesting TRPV4 series. Hit-to-lead work on the hydroxypiperidine series included the replacement of the sulfide linker and pyridyl group to generate potent and selective compounds such as 2a-d. Further optimisation, utilising a pairwise analysis protocol (SWAP) delivered a series of hydroxyazetine compounds. Compounds within this series, such as compound 3c, fulfil the TRPV4 potency (rat and human), selectivity and ADME criteria required for use as in vitro/in vivo tools. Pre-clinical in vivo TRPV4 pharmacology and safety data for compound 3c will be reported in due course.

<sup>‡</sup> ADME (Absorption Distribution Metabolism Excretion).

#### **Experimental section**

#### In vitro TRPV4 assay

Receptor-evoked changes in intracellular calcium were measured using calcium-selective fluorescent Ca<sup>2+</sup> NW dye (Molecular Devices, Sunnyvale, CA, USA) quantitated with a fluorometric imaging plate reader (FLIPR; Molecular Devices). Stably transfected CHOK1 cells expressing human and rat TRPV4 receptors have been used for this assay. On the day before the FLIPR experiment, frozen cryovials of cells were thawed, centrifuged and resuspended in DMEM medium (Invitrogen Cat. # 21063) containing glutamax, sodium pyruvate, non-essential amino acids and 10% FBS (Invitrogen Cat. # 10082-147). The cells were plated into black walled, clear bottom Greiner 384 poly-D-lysine plates at a density of 10 000 cells per well and incubated overnight in 5%  $\rm CO_2$  at 37  $^{\circ}\rm C.$  On the day of the experiment, media was removed and cells were loaded with dye loading buffer ( $0.5 \times Ca^{2+}$  NW-dye in HBSS with Ca<sup>++</sup>, Mg<sup>++</sup>, 2.5 mM Probenecid, 0.025% pluronic, 20 mM HEPES, pH 7.4) and incubated for 45 minutes at 37 °C. The test compounds and the reference compound (ruthenium red) were serially diluted in 100% DMSO followed by dilution in assay buffer (HBSS without Ca<sup>++</sup>, Mg<sup>++</sup>, 20 mM HEPES, 5.4 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.12% P104, pH 7.4) in order to achieve a final highest concentration of 10 µM in the assay plate. The plates were then placed in the FLIPR, and a baseline fluorescence measurement (excitation @ 488 nm and emission @ 510-570 nm) was obtained for 10 s before compound or vehicle addition. The assay was performed in dual mode: firstly, upon addition of compounds to the cells, any possible agonistic effect of the test compounds were monitored for 5 minutes after which the assay plate was incubated for further 15 minutes at room temperature. At the second step, EC75 of the agonist PF-04674114/ GSK1016790A was added and fluorescence was measured for an additional 5 minutes. Change in fluorescence values in response to the addition of PF-04674114 (in the absence and presence of test compounds) was measured and inhibition curves generated using nonlinear regression (Prism v.4, GraphPad Software, San Diego, CA, USA) employing a four parameter logistic equation (Y = bottom + (top - bottom)/(1 + top - bottom)) $10^{(logEC50 - X)}$ hillslope).

#### Chemistry

All commercially available chemicals and solvents were used without further purification. All temperatures are in °C. Flash column chromatography was carried out using Merck silica gel 60 (9385) or Redisep silica. NMR spectra were obtained on a Varian Mercury (400 MHz) or a Bruker Avance (400 MHz) using the residual signal of the deuterated NMR solvent as the internal reference. Chemical shifts are expressed in parts per million (ppm), multiplicity of the signals are indicated by lowercase letters (singlet s, doublet d, triplet t, quadruplet q, multiple m, broad singlet br s), and deuterated solvents are dimethylsulphoxide d6, methanol d4, and chloroform d1. Mass spectral date were obtained using Waters ZQ ESCI or Applied Biosystem's API-2000. General procedure for synthesis of *N*-Boc hydroxypiperidines 5a and b and *N*-Boc hydroxyazetidines 11a and b. To a stirred solution of epoxide (4 or 10) (0.47 mmol) and cesium carbonate (0.56 mmol) in IPA (3 mL), DMSO (0.5 mL) and water (0.5 mL), was added ( $R_1$ )( $R_2$ )Ar–OH (0.52 mmol) and the resulting mixture refluxed at 80 °C for 2.5 h. The reaction mixture was concentrated *in vacuo* and the residue partitioned between water and ethyl acetate. The organics were further washed with brine, dried over sodium sulphate, concentrated *in vacuo* and purified by silica-gel column chromatography.

4-(4-Cyano-3-fluoro-phenoxymethyl)-4-hydroxy-piperidine-1carboxylic acid *tert*-butyl ester (5a). Following the general procedure for the synthesis of *N*-Boc hydroxypiperidines 5a and b and *N*-Boc hydroxyazetidines 11a and b, and using 2-fluoro-4hydroxybenzonitrile and epoxide 4, the title compound was afforded as a white solid (80 mg, 49%). <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$ : 1.45 (s, 9H), 1.60–1.64 (m, 2H), 1.73 (m, 2H), 2.60 (m, 3H), 3.18 (m, 2H), 3.83 (s, 1H), 3.95 (m, 1H), 6.71–6.78 (m, 2H), 7.53 (t, 1H); *m*/z 351 [M + H]<sup>+</sup>.

4-(4-Cyano-phenoxymethyl)-4-hydroxy-piperidine-1-carboxylic acid *tert*-butyl ester (5b). Following the general procedure for the synthesis of *N*-Boc hydroxypiperidines **5a** and **b** and *N*-Boc hydroxyazetidines **11a** and **b**, and using 4-hydroxybenzonitrile and epoxide 4, the title compound was prepared as a white solid (9.25 g, 85%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 1.39 (s, 9H), 1.52 (m, 4H), 3.08 (d, 2H), 3.73 (d, 2H), 3.86 (d, 2H), 7.11 (d, 2H), 7.76 (d, 2H); *m/z* 333 [M + H]<sup>+</sup>.

General procedure for Boc deprotection of *N*-Boc hydroxypiperidines (5a and b) and *N*-Boc hydroxyazetidines (11a and b). To a stirred solution of 5a and b or 11a and b (0.92 mmol) in DCM (5 mL) was added TFA (1 mL) and the resulting mixture stirred at 25 °C for 2 h. The reaction mixture was then concentrated *in vacuo*, triturated with pentane–ether (5 mL) and used in the next step without further purification.

**2-Fluoro-4-(4-hydroxy-piperidin-4-ylmethoxy)-benzonitrile** (6a). Following the general procedure for Boc deprotection of *N*-Boc hydroxypiperdines (5a and b) and *N*-Boc hydroxyazetidines (11a and b), and using *N*-Boc hydroxypiperdine 5a, the title compound was a white solid (TFA salt) (251 mg, 75%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 1.74 (m, 3H), 3.09 (m, 2H), 3.17 (m, 2H), 3.95 (s, 2H), 5.20 (s, 1H), 6.90 (d, 1H), 7.15 (d, 1H), 7.84 (t, 1H), 8.23 (bs, 1H), 8.44 (bs, 1H); *m/z* 251 [M + H]<sup>+</sup>.

**4-(4-Hydroxy-piperidin-4-ylmethoxy)-benzonitrile** (6b). Following the general procedure for Boc deprotection of *N*-Boc hydroxypiperdines (**5a** and **b**) and *N*-Boc hydroxyazetidines (**11a** and **b**), and using *N*-Boc hydroxypiperdine **6a**, the title compound was a white solid (TFA salt) (300 mg, 79%). m/z 233 [M + H]<sup>+</sup>.

General procedure for sulfonamide formation (2a–e, 3a–d). To a stirred solution of amine (6a and b or 12a and b, 0.61 mmol) and triethylamine (1.55 mmol) in DCM (5 mL) at 0 °C was added the appropriate benzenesulfonyl chloride (0.61 mmol). The reaction mixture was allowed to warm to 25 °C and stir for 18 h. The reaction mixture was partitioned between water and DCM. The organics were further washed with brine, dried over sodium sulphate, evaporated *in vacuo* and purified *via* silica-gel column chromatography.

4-[1-(2,4-Dichloro-benzenesulfonyl)-3-hydroxy-azetidin-3ylmethoxy]-2-fluoro-benzonitrile (2a). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 6a and 2,4-dichlorobenzene-1-sulfonyl chloride, the title compound was prepared as a white solid (750 mg, 79%). <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$ : 1.74–1.79 (m, 4H), 3.15–3.22 (m, 2H), 3.73–3.76 (m, 2H), 3.84 (s, 2H), 6.70–6.77 (m, 2H), 7.36– 7.38 (dd, 1H), 7.53 (t, 1H), 7.90 (d, 1H), 7.98 (d, 1H); *m/z* 459 [M + H]<sup>+</sup>.

4-((1-((2-Chloro-4-cyanophenyl)sulfonyl)-4-hydroxypiperidin-4-yl)methoxy)-2-fluorobenzonitrile (2b). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 6a and 2-chloro-4-cyano benzenesulfonyl chloride, the title compounds was prepared as a white solid (47 mg, 38% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 1.64 (m, 4H), 3.06 (m, 2H), 3.58 (m, 2H), 3.91 (s, 2H), 4.92 (s, 1H), 6.95 (d, 1H), 7.13 (d, 1H), 7.81 (t, 1H), 8.04 (d, 1H), 8.11 (d, 1H), 8.35 (s, 1H); *m*/*z* 450 [M + H]<sup>+</sup>.

4-[1-(2,4-Dichloro-benzenesulfonyl)-4-hydroxy-piperidin-4ylmethoxy]-benzonitrile (2c). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 6b and 2,4-dichlorobenzene-1-sulfonyl chloride, the title compound was prepared as a white solid (579 mg, 56%). <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$ : 1.61–1.70 (m, 4H), 3.01 (m, 2H), 3.56 (m, 2H), 3.86 (s, 2H), 4.88 (s, 1H), 7.08 (d, 1H), 7.65 (d, 1H), 7.74–7.80 (m, 3H), 7.93 (d, 1H), 7.96 (dd, 1H); *m*/z 441 [M + H]<sup>+</sup>.

4-[4-(4-Cyano-3-fluoro-phenoxymethyl)-4-hydroxy-piperidine-1-sulfonyl]-isophthalonitrile (2d). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 6a and 2,4-dicyano benzenesulfonyl chloride, the title compound was prepared as a white solid (22 mg, 8%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 1.66 (m, 4H), 2.94 (m, 2H), 3.60 (m, 2H), 3.90 (s, 2H), 4.90 (s, 1H), 6.95 (d, 1H), 7.13 (d, 1H), 7.81 (t, 1H), 8.18 (d, 1H), 8.41 (d, 1H), 8.80 (s, 1H); *m/z* 441 [M + H]<sup>+</sup>.

3-Methylene-azetidine-1-carboxylic acid *tert*-butyl ester (8). To a stirred solution of Ph<sub>3</sub>PCH<sub>2</sub>Br (88.7 g, 248.4 mmol) in dry ether (400 mL) was added potassium *tert*-butoxide (27.9 g, 248.4 mmol) and the mixture stirred at 25 °C for 1 h. Ketone 7 (42.5 g, 248.4 mmol) in dry ether (400 mL) was added and the resulting mixture stirred at 25 °C for 18 h. The reaction mixture was filtered through Celite®. The filtrate was washed with water (100 mL), brine (100 mL), dried over sodium sulphate and concentrated *in vacuo* to afford the crude compound as a yellow oil (10.8 g, 61% yield), which was used without further purification. <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$ : 1.44 (s, 9H), 4.46 (s, 4H), 4.97 (s, 2H).

3-Bromomethyl-3-hydroxy-azetidine-1-carboxylic acid *tert*butyl ester and 3-bromo-3-hydroxymethyl-azetidine-1-carboxylic acid *tert*-butyl ester (9a and 9b). To a stirred solution of alkene 8 (2.9 g, 17.13 mmol) in DMSO (15 mL) at 0 °C was added NBS (6.10 g, 34.2 mmol) and water (617 mL, 34.2 mmol). The resulting mixture was allowed to warm to 25 °C and stirred for a further 18 h. The reaction mixture was partitioned between water (25 mL) and ethyl acetate (25 mL). The organics were washed with brine (10 mL), dried over sodium sulphate, concentrated *in vacuo* and purified by silica-gel column chromatography (eluting with ethyl acetate : hexane, 10 : 90) to afford the isomeric mixture of title compounds as a pale yellow oil (7.65 g, 83%). <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$ : 1.43 (s, 18H), 2.03–2.06 (t, 1H), 2.68 (s, 1H), 3.68 (s, 2H), 3.87–3.91 (m, 8H), 4.26–4.27 (dd, 2H).

1-Oxa-5-aza-spiro[2.3]hexane-5-carboxylic acid *tert*-butyl ester (10). To a stirred solution of isomeric mixture of 9a and 9b (4.6 g, 17.29 mmol) in THF (80 mL) at 0 °C was added sodium hydride (760 mg, 19.02 mmol, 60%) in one portion. The resulting mixture was allowed to warm to 25 °C and stirred for 7 h. The reaction mixture was quenched with saturated ammonium chloride (50 mL) and stirred at 25 °C for a further 18 h. The reaction mixture was extracted with ethyl acetate (100 mL) and the organic phase dried over sodium sulphate, concentrated *in vacuo*, and purified by silica-gel column chromatography (eluting with ethyl acetate : hexane, 10 : 90) to afford the title compound as a pale yellow oil (2.0 g, 62%). <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$ : 1.44 (s, 9H), 2.84 (s, 2H), 4.17–4.26 (m, 4H).

3-(4-Cyano-3-fluoro-phenoxymethyl)-3-hydroxy-azetidine-1carboxylic acid *tert*-butyl ester (11a). Following the general procedure for the synthesis of *N*-Boc hydroxypiperidines 5a and b and *N*-Boc hydroxyazetidines 11a and b, and using 2-fluoro-4hydroxybenzonitrile and epoxide 10, the title compound was afforded as a white solid (250 mg, 57%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 1.38 (s, 9H), 3.71 (d, 2H), 3.87 (d, 2H), 4.15 (s, 2H), 6.11 (s, 1H), 6.98 (d, 1H), 7.16 (d, 1H), 7.83 (t, 1H); *m*/*z* 323 [M + H]<sup>+</sup>.

3-(3-Chloro-4-cyano-phenoxymethyl)-3-hydroxy-azetidine-1carboxylic acid *tert*-butyl ester (11b). Following the general procedure for the synthesis of *N*-Boc hydroxypiperidines 5a and b and *N*-Boc hydroxyazetidines 11a and b, and using 2-chloro-4hydroxybenzonitrile and epoxide 10, the title compound was afforded as a white solid (250 mg, 55%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 1.38 (s, 9H), 3.71 (d, 2H), 3.87 (d, 2H), 4.17 (s, 2H), 6.10 (s, 1H), 7.10 (m, 1H), 7.36 (d, 1H), 7.87 (d, 1H); *m*/*z* 339 [M + H]<sup>+</sup>.

2-Fluoro-4-(3-hydroxy-azetidin-3-ylmethoxy)-benzonitrile (12a). Following the general procedure for Boc deprotection of *N*-Boc hydroxypiperdines (5a and b) and *N*-Boc hydroxyazetidines (11a and b), and using *N*-Boc hydroxypiperdine 11a, the title compound was a white solid (TFA salt) (200 mg, 79%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 3.94 (d, 2H), 4.03 (d, 2H), 4.19 (s, 2H), 6.59 (s, 1H), 7.05 (d, 1H), 7.22 (s, 1H), 7.88 (t, 1H), 8.74–8.86 (brs, 1H); *m*/z 223 [M + H]<sup>+</sup>.

2-Chloro-4-(3-hydroxy-azetidin-3-ylmethoxy)-benzonitrile (12b). Following the general procedure for Boc deprotection of *N*-Boc hydroxypiperdines (5a and b) and *N*-Boc hydroxyazetidines (11a and b), and using *N*-Boc hydroxypiperdine 11b, the title compound was a white solid (TFA salt) (200 mg, 77%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 3.92 (d, 2H), 4.03 (d, 2H), 4.20 (s, 2H), 6.58 (s, 1H), 7.17 (d, 1H), 7.44 (s, 1H), 7.93 (d, 1H), 8.71–8.82 (brs, 1H); *m*/z 239 [M + H]<sup>+</sup>.

4-({1-[4-Chloro-2-cyano-phenylsulfonyl]-3-hydroxyazetidine-3-yl}methoxy)-2-fluorobenzonitrile (3a). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 12a and 4-chloro-2-cyano-benzenesulfonyl chloride, the title compound was prepared as a white solid (50 mg, 16%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 3.77 (d, 2H), 3.96 (d, 2H), 4.12 (s, 2H), 6.34 (s, 1H), 6.90 (dd, 1H), 7.11 (dd, 1H), 7.83 (t, 1H), 8.05 (s, 2H), 8.47 (s, 1H); *m*/*z* 422 [M + H]<sup>+</sup>.

2-Chloro-4-({1-[4-chloro-2-cyano-phenylsulfonyl]-3-hydroxyazetidine-3-yl}methoxy) benzonitrile (3b). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 12b and 4-chloro-2-cyano-benzenesulfonyl chloride, the title compound was prepared as an off-white solid (52 mg, 16%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 3.78 (d, 2H), 3.97 (d, 2H), 4.14 (s, 2H), 6.33 (s, 1H), 7.03–7.05 (dd, 1H), 7.27 (d, 1H), 7.89 (d, 1H), 8.05 (s, 2H), 8.46 (d, 1H); *m/z* 438 [M + H]<sup>+</sup>.

4-[1-(2,4-Dichloro-benzenesulfonyl)-3-hydroxy-azetidin-3-ylmethoxy]-2-fluoro-benzonitrile (3c). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 12a and 4-chloro-2-chloro-benzenesulfonyl chloride, the title compound was prepared as a white solid (110 mg, 63%). <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$ : 4.04 (d, 2H), 4.12 (d, 2H), 4.20 (s, 2H), 6.77–6.81 (m, 2H), 7.38–7.41 (dd, 1H), 7.57 (t, 1H), 7.89 (d, 1H), 7.97 (d, 1H); *m/z* 431 [M + H]<sup>+</sup>.

2-Chloro-4-((1-((2-chloro-4-cyanophenyl)sulfonyl)-3-hydroxyazetidin-3-yl)methoxy)benzonitrile (3d). Following the general procedure for sulfonamide formation (2a–d, 3a–d), and using amine 12b and 4-cyano-2-chloro-benzenesulfonyl chloride, the title compound was prepared as a white solid (12 mg, 22%). <sup>1</sup>H NMR (400 MHz, DMSO-d6)  $\delta$ : 3.81 (d, 2H), 3.95 (d, 2H), 4.15 (s, 2H), 6.90 (s, 1H), 7.15 (d, 1H), 7.75 (t, 1H), 8.09 (d, 1H), 8.16 (d, 1H), 8.41 (s, 1H); *m*/z 438 [M + H]<sup>+</sup>.

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## Notes and references

- 1 B. Nilius, *et al.*, TRPV4 calcium entry channel: a paradigm for gating diversity, *Am. J. Physiol.*, 2004, **286**(2, Pt. 1), C195–C205.
- 2 W. Liedtke, *et al.*, Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in *Caenorhabditis elegans*, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**(suppl. 2), 14531–14536.
- 3 A. Mizuno, et al., Impaired osmotic sensation in mice lacking TRPV4, Am. J. Physiol., 2003, 285(1, Pt. 1), C96-C101.
- 4 H. Todaka, *et al.*, Warm temperature-sensitive transient receptor potential vanilloid 4 (TRPV4) plays an essential role in thermal hyperalgesia, *J. Biol. Chem.*, 2004, **279**(34), 35133–35138.
- 5 B. Nilius, G. Droogmans and R. Wondergem, Transient receptor potential channels in endothelium: solving the calcium entry puzzle?, *Endothelium*, 2003, **10**(1), 5–15.
- 6 R. N. Willette, et al., Systemic activation of the transient receptor potential vanilloid subtype 4 channel causes

endothelial failure and circulatory collapse: part 2, *J. Pharmacol. Exp. Ther.*, 2008, **326**(2), 443–452.

- 7 M. Suzuki, *et al.*, Impaired pressure sensation in mice lacking TRPV4, *J. Biol. Chem.*, 2003, 278(25), 22664–22668.
- 8 T. Gevaert, *et al.*, Deletion of the transient receptor potential cation channel TRPV4 impairs murine bladder voiding, *J. Clin. Invest.*, 2007, **117**(11), 3453–3462.
- 9 K. S. Thorneloe, *et al.*, *N*-((1*S*)-1-{[4-((2*S*)-2-{[(2,4-dichlorophenyl) sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: part I, *J. Pharmacol. Exp. Ther.*, 2008, **326**(2), 432–442.
- 10 S. F. Pedersen, G. Owsianik and B. Nilius, TRP channels: an overview, *Cell Calcium*, 2005, **38**(3–4), 233–252.
- 11 Transient Receptor Potential (TRP) Channels, in *Handbook* of *Experimental Pharmacology*, ed. V. Flockerzi and B. Nilius, 2007, vol. 179, p. 606.
- 12 S. D. Pratt, *et al.*, Identification and characterization of mGlu3 ligands using a high throughput FLIPR assay for detection of agonists, antagonists, and allosteric modulators, *Comb. Chem. High Throughput Screening*, 2011, 14(7), 631–641.
- 13 O. Roche, *et al.*, Development of a virtual screening method for identification of "frequent hitters" in compound libraries, *J. Med. Chem.*, 2002, **45**(1), 137–142.
- 14 M. Glick, *et al.*, Enrichment of extremely noisy highthroughput screening data using a naive Bayes classifier, *J. Biomol. Screening*, 2004, **9**(1), 32–36.
- 15 P. D. Leeson and B. Springthorpe, The influence of drug-like concepts on decision-making in medicinal chemistry, *Nat. Rev. Drug Discovery*, 2007, 6(11), 881–890.
- 16 A. F. Stepan, *et al.*, Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the united states, *Chem. Res. Toxicol.*, 2011, 24(9), 1345–1410.
- 17 B. A. Posner, H. Xi and J. E. J. Mills, Enhanced HTS hit selection *via* a local hit rate analysis, *J. Chem. Inf. Model.*, 2009, **49**(10), 2202–2210.
- 18 Barnard Chemical Information, S., http://www.digitalche mistry.co.uk.
- 19 Pipeline Pilot, V 6.1, Accelrys Inc., San Diego, CA.
- 20 M. Tu, Fingerprints generated by counting frequency with which particular pairs of atoms are separated by particular throughbond distances, unpublished results.
- 21 J. E. Mills, unpublished results.
- 22 J. W. Raymond, E. J. Gardiner and P. Willett, Heuristics for similarity searching of chemical graphs using a maximum common edge subgraph algorithm, *J. Chem. Inf. Comput. Sci.*, 2002, **42**(2), 305–316.
- 23 T. Ryckmans, *et al.*, Rapid assessment of a novel series of selective CB(2) agonists using parallel synthesis protocols: a Lipophilic Efficiency (LipE) analysis, *Bioorg. Med. Chem. Lett.*, 2009, **19**(15), 4406–4409.

- 24 R. Mannhold and R. F. Rekker, The hydrophobic fragmental constant approach for calculating logP in octanol/water and aliphatic hydrocarbon/water systems, *Perspect. Drug Discovery Des.*, 2000, **18**(Hydrophobicity and Solvation in Drug Design, Pt. II), 1–18.
- 25 C. E. Keefer, G. Chang and G. W. Kauffman, Extraction of tacit knowledge from large ADME datasets *via* pairwise analysis, *Bioorg. Med. Chem.*, 2011, **19**(12), 3739–3749.
- 26 J. L. Born and W. M. Hadley, Inhibition of *in vitro* cytochrome P-450-catalyzed reactions by substituted pyridines, *J. Pharm. Sci.*, 1980, **69**(4), 465–466.
- 27 J. E. J. Mills, *et al.*, SAR mining and its application to the design of TRPA1 antagonists, *MedChemComm*, 2012, 3(2), 174–178.
- 28 J. E. J. Mills and C. M. Barker, unpublished results.
- 29 M. C. Sanguinetti and M. Tristani-Firouzi, hERG potassium channels and cardiac arrhythmia, *Nature*, 2006, **440**(7083), 463–469.
- 30 M. Deacon, *et al.*, Early evaluation of compound QT prolongation effects: a predictive 384-well fluorescence polarization binding assay for measuring hERG blockade, *J. Pharmacol. Toxicol. Methods*, 2007, **55**(3), 238–247.