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## Discovery of novel hedgehog antagonists from cell-based screening: Isosteric modification of p38 bisamides as potent inhibitors of SMO

Bin Yang<sup>\*</sup>, Alexander W. Hird, Daniel John Russell, Benjamin P. Fauber, Les A. Dakin, Xiaolan Zheng, Qibin Su, Robert Godin, Patrick Brassil, Erik Devereaux, James W. Janetka

AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, MA 02451, USA

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## ABSTRACT

Cell-based subset screening of compounds using a Gli transcription factor reporter cell assay and shh stimulated cell differentiation assay identified a series of bisamide compounds as hedgehog pathway inhibitors with good potency. Using a ligand-based optimization strategy, heteroaryl groups were utilized as conformationally restricted amide isosteres replacing one of the amides which significantly increased their potency against SMO and the hedgehog pathway while decreasing activity against p38 $\alpha$  kinase. We report herein the identification of advanced lead compounds such as imidazole **11c** and **11f** encompassing good p38 $\alpha$  selectivity, low nanomolar potency in both cell assays, excellent physiochemical properties and in vivo pharmacokinetics.

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The Hedgehog pathway affects numerous biological processes, such as cell differentiation and proliferation, and particularly embryogenesis, where the pathway regulates embryo patterning. During embryogenesis, Hedgehog pathway typically is activated by the secreted Hedgehog ligands, which directly bind to their receptor protein, 12-pass transmembrane protein Patched1 (Ptch1),<sup>1</sup> and inhibit its repression of the G protein-coupled receptor-like protein Smoothened (SMO).<sup>2</sup> Recently it has been reported that primary cilia plays a pivotal role in Hedgehog pathway.<sup>3</sup> Under basal conditions, Ptch1 is localized to the primary cilium and SMO is sequestered in endosomes;<sup>4</sup> Hedgehog ligands induce Ptch1 movement out of and consequently SMO trafficking into primary cilium. The pathway activation ultimately leads to the modulation of Gli zinc-finger transcription factors, permitting transcription activation of Hedgehog-responsive genes whose expression is crucial for tissue patterning, growth and differentiation and tissue homeostasis.

The linkage of the Hedgehog pathway to diseases, such as cancer, is established, and a number of different oncogenetic mechanisms related to the Hedgehog pathway have been identified. Autocrine or paracrine Hedgehog signaling was found in certain neoplasms, such as small-cell lung cancers and pancreatic adenocarcinomas.<sup>5</sup> Ligand-independent Hedgehog target gene expression can also lead to tumorigenesis, exemplified by Gorlin's syndrome patients who are heterozygous for Ptch1mutations and susceptible to basal cell

carcinomas, medulloblastomas, and rhabdomyosarcomas.<sup>6</sup> Oncogenic mutations in SMO and Su(fu) have also been identified.<sup>7</sup> Pharmacological inhibitors of the Hedgehog pathway should have great therapeutic value. Particularly, the SMO antagonist cyclopamine is known to block tumor progression in a variety of mouse cancer models.<sup>8</sup> More recently, known SMO antagonist Vismodegib (GDC-0449) demonstrated good response rate in basal cell carcinoma patients and has since been approved by FDA to treat metastatic basal cell carcinoma and locally advanced basal cell carcinoma.<sup>9</sup>

To identify Hedgehog pathway inhibitors, we screened a subset of over 40,000 compounds against a cell-based Gli-Luciferase assay<sup>10</sup> and a selection of active compounds from the Gli-Luciferase assay in a C3H10T1/2 Hedgehog-dependent differentiation assay.<sup>11</sup> A series of bisamide compounds, including compound **1a** (Fig. 1), were among the initial hits identified. In both Hedgehog pathway assays, this series of compounds offered attractive potency and



Figure 1. Structure of a bisamide hit identified from the cell-based Gli-Luciferase assay.

<sup>\*</sup> Corresponding author. Tel.: +1 781 839 4135; fax: +1 781 839 4230. *E-mail address:* bin.yang@astrazeneca.com (B. Yang).

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#### Table 1

Activities of bisamide compounds against Hedgehog pathway and p380



preliminary SAR. Herein we report our lead optimization of this series to improve its potency and selectivity against p38-type kinases.

The synthesis of the bisamide compounds (e.g., 1a) was reported previously.<sup>12</sup> Compound **1a** offered considerable potency in the firefly reporter assay against the Hh pathway with  $IC_{50} = 0.45 \mu$ M, and it appeared to be selective over the renilla firefly  $assay^{13}$  (IC<sub>50</sub> >30  $\mu$ M). The biophysical properties of this series are poor in general; aqueous solubility of compound **1a** is  $\sim 1 \,\mu M$ and its strong protein binding in plasma gave smaller than 1% free fraction. In addition, one of the main potential concerns as a Hedgehog pathway inhibitor is that the bisamide series are known as potent p38 MAP kinases inhibitors (p38 $\alpha$  IC<sub>50</sub> = 0.025  $\mu$ M for compound **1a**). The initial screening results in the firefly reporter assay for this series indicated a wide tolerance of various functionalities on the phenyl ether side of the molecule, but those structural variations did not meaningfully attenuate p38\alpha activity. The data for a selection of compounds and their activities in Hh pathway firefly reporter and p38 $\alpha$  kinase assays are summarized in Table 1.

It is noteworthy that the 2-pyridyl group on the right side of the molecule appeared to be optimal for Hh pathway inhibition, and other heteroaryls or alkyl groups reduced potency. Further installation of solubilizing basic amine groups on the right hand side of the molecule failed to attenuate their activities on p38 kinase, and it further reduced their potency on Hh pathway inhibition. Some improvement on solubility was observed, although with limited significance (data not shown).

Structural optimization efforts focused on the modifications of C-5 substitution on the middle phenyl group. It was reasoned that reducing the molecular weight from the left hand side of molecule could potentially improve both potency and the physical properties of this series of compounds.



Scheme 1. Synthesis of benzimidazole amide compound 8. Reagents and conditions: (a) 2-(bromomethyl)pyridine, K<sub>2</sub>CO<sub>3</sub>, acetonitrile/water (80%); (b) NaOH (1 N), MeOH, reflux (90%); (c) 3-amino-4-methylbenzoic acid methyl ester, HATU, DIPEA, DMF (99%); (d) NaOH (1 N), MeOH, reflux (91%); (e) 1,2-diaminobenzene, HATU, DIPEA, DMF (93%); (f) AcOH, reflux (99%).

## Table 2

Nitrogen-containing heterocycles as amides isosteres increased Hh pathway potency and reduced p38 $\alpha$  potency

# 

	11								
	R	Firefly $IC_{50}$ ( $\mu M$ )	$shh \ EC_{50} \ (\mu M)$	BST p38α IC <sub>50</sub> (μM)	Solubility (µM)	Hu PPB (% free)			
8		0.005	0.03	30	<1	<1			
11a	HN,	<0.04	0.0392	1.6	90	5.1			
11b		<0.016	0.018	15	190	5.1			
11c		<0.006	0.011	100	350	2.2			
11d	CF3 N, '	0.0024	0.022	12	<1	1.2			
11e	NH N	0.0026	0.015	5.9	19	ND			
11f	NH NH	<0.012	0.013	2.7	25	5.6			
11g	N I	0.005	0.089	19	80	9.5			
11h	HN HN	0.007	0.007	36	15	3.2			
11i	NH	0.009	0.021	100	120	6.3			
11j	N N	0.021	0.027	100	46	5.6			
11k	N N N	0.012	0.008	100	5	1.8			
111	F <sub>3</sub> C N.N.,	0.009	0.029	100	<1	<1			
11m	N N S	0.042	>0.145	30	22	3.3			
11n	N N	0.044	0.028	14	<1	<1			
110		>0.45	ND	NV	<1	<1			
11p		0.021	0.017	15	<1	1.1			
11q	N N	>0.40	0.04	22	2	1.8			
11r	H <sub>2</sub> N N	<0.019	0.008	15	7	5.2			
11s	NH	0.061	0.115	3.5	ND	ND			



Scheme 2. Syntheses of heterocyclic amides using Suzuki coupling reactions. Reagents and conditions: (a) HATU, DIPEA, DMF, rt (50%); (b) heterocyclic halides, Pd(PPh<sub>3</sub>)<sub>4</sub>, dioxane/water, 100–150 °C.

Nitrogen-containing heterocycles are regularly used as isosteres for amide groups in medicinal chemistry. Interestingly, using heterocycles as amide replacements to improve SMO antagonist activity is recently reported.<sup>14</sup> Particularly, some heteroaryls groups, such as benzimidazoles, widely appear in numerous SMO inhibitors.<sup>15</sup> To explore the structure-activity relations of C-5 substitution on compound 1a, it was reasoned that heterocyclic moieties could be potential surrogates for the C-5 amide group in compound **1a**.<sup>16</sup> In addition, X-ray crystal structure revealed hydrogen bondings between the C-5 amide group of the bisamide compounds and p38 $\alpha$  protein residues Glu<sub>71</sub> and Asp<sub>168</sub>. We envisioned that amide isosteres could potentially disrupt these hydrogen bondings and hence attenuate their p38 activity. Aiming to improve Hh pathway inhibition potency and to limit p38 inhibition activity associated with both bisamide series, benzimidazole group was first introduced as an amide isostere, and its synthesis is shown in Scheme 1.

Phenol **2** was directly alkylated under mild basic conditions in acetonitrile. Notably, it was found that a small fraction of water presence in the solvent significantly facilitated the reaction rate. Subsequent hydrolyses on methyl esters and amides coupling steps using HATU generated bisamide **7**, which was cyclized upon refluxing in acetic acid to produce the benzimidazole, **8**.<sup>17</sup> Satisfactorily, compound **8** significantly improved the potency in both firefly reporter assay (4.7 nM) and the differentiation assay ( $EC_{50} = 30$  nM). Even more encouragingly, compound **8** diminished the activity against p38 kinases, given that its observed IC<sub>50</sub> for p38 $\alpha$  kinase is greater than 100  $\mu$ M (Table 2). However, the solubility of compound **8** at pH 7.4 is less than 1  $\mu$ M, and it also suffers from high plasma protein binding, with free fraction in human plasma less than 1%.

Given the improved potency and reduced p38 activity of the benzimidazole compound **8**, and to further address its poor physical properties, a variety of heterocycles were next explored as amide isosteres at the C5 position of compound **1a**. Suzuki coupling reaction was used as the key step to introduce a large set of structurally diverse heterocycles, and their syntheses are outlined in Scheme 2.

Benzoic acid **4** was directly coupled with anilino boronic acid **9** using HATU to produce the boronic acid intermediate **10**, setting the stage for the installation of a variety of heterocycles by Suzuki coupling. As shown in Table 3, a large array of nitrogen-containing heterocycles was successfully introduced as C-5 substituents, including imidazole, pyrazole, thiazole, pyridine, and purine. The yields of the coupling reactions ranged from 30% to 70%. The analogs shown in Table 3 were characterized by LCMS and <sup>1</sup>H NMR.

Table 3	
In vivo CD-1 murine DMPK properties for selected heteroaryls	

	Compound	Dose <sup>a</sup> IV	(mg/kg) PO	Cl (mL/min/kg)	PO AUC (µg*h/L)	Vss (L/kg)	IV Half life (h)	%F	
	11c 11f	3.0 3.0	10.0 7.5	210 8.3	100 12535	12 0.5	0.9 1.0	22 83	
-									-

<sup>a</sup> The compounds were formulated using DMA/TEG/saline 40/40/20%.

As illustrated in Table 2, the majority of the heterocyclic analogs either retained or improved the potency to inhibit Hh pathway in both cell assays, with some of the compounds in Table 2 reaching close to the lowest testing concentration of both the firefly reporter assay and the differentiation assay. Substitution of C-5 amide group by imidazoles was systematically explored (compound **11a–11i**). Between the unsubstituted 2-imidazole (**11a**)<sup>18</sup> and 5imidazole (**11f**).<sup>19</sup> similar inhibition activities against Hh pathway were observed, and both showed diminished activities against p38 kinase. While the monosubstituted analogs on both imidazoles (11b, 11d, 11e, 11g, 11h, and 11i) significantly further reduced p38 inhibition potency, the disubstituted imidazole analogs  $(11c,^{20} 11j)$  showed no p38 $\alpha$  inhibition at the maximum testing concentration (100 µM). Pyrazole and thiazole analogs (11k, 11l, **11n**) inhibited Hh pathway at comparable potency to imidazols, but these analogs also gave less than desirable solubility and higher plasma protein binding. Notably, installation of a morpholine group as a solubilizing group on a thiazole ring (11m) was detrimental to the potency in the cell differentiation assay. All three pyridine (110, 11p, 11q) analogs were synthesized. Compared to 3 and 4 substituted pridines (110, 11q), 2-substituted pyridine analog (11p) demonstrated superior potency in both cell assays, consistent with what others have previously reported.<sup>14,21</sup> The amino-pyridazine analogs offered superior potency, but at the expense of solubility. Purine as a heterocycle with more structural complexity was also installed, only to be proved with inferior potency, particularly in the differentiation assay (11s).

To further confirm the diminished p38 activity of this new series of hetercyclic analogs, compound **11f** was tested in a whole blood cell assay for its activity against p38. Satisfactorily, no inhibitory activity against p38 was observed at the maximum concentration tested ( $50 \mu$ M). Moreover, to determine whether the inhibitors acted specifically on SMO, we used a SMO binding assay and this series of compounds were found to competitively displace BODIPY labeled cyclopamine in HeLa cells engineered to express either human or mouse SMO.<sup>22</sup> Based on their overall profile, compounds **11c** and **11f** were chosen for in vivo pharmacokinetic measurements in mouse. As illustrated in Table 3, the dimethyl substitution on imidazole ring in compound **11c** demonstrated enormous impact on in vivo pharmacokinetics. Compared to its analog **11c**, compound **11f** has significantly lower clearance and higher overall exposure, and it also demonstrates excellent oral bioavailability.

In conclusion, structural modifications on a series of bisamide compounds led to the discovery of a novel class of heterocyclic amide compounds as potent Hh pathway inhibitors. Using nitrogen-containing heterocycles as amide isosteres, the heterocylic amide compounds demonstrated superior potency against Hh pathway in both the Gli reporter cell assay and shh stimulated cell differentiation assay, and significantly reduced p38 inhibition activity associated with the bisamide compounds. While a large set of structurally diverse heterocycles can be tolerated for their potency against Hh pathway, they also offer distinctly different profiles in physical properties and in vivo pharmacokinetics. Imidazole compounds were selected as the front runners for more advanced studies due to their excellent potency and physical properties. A balance of Hh pathway potency, physical properties, and in vivo pharmacokinetic properties make **11f** a suitable tool for in vivo pharmacodynamic and efficacy studies of Hh pathway inhibition. Further reports on the optimization of the heterocyclic amides series are forthcoming.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.104.

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cells per well in DMEM supplemented with 10% FBS and 1% L-glutamine overnight at 37 °C. The following day, the media was exchanged for a 50% CM + shh (DMEM with 0.5% FBS, 1% L-glutamine + shh ligand). Compounds were solubilized in 100% DMSO to a concentration of 10 mM and then serially diluted threefold also in a 100% DMSO solution. The highest concentration on the cell plate was 33  $\mu$ M and the lowest was 0.00045  $\mu$ M. The compounds were dosed onto the cells and the plates were incubated for 20 h. The cell plates were then assayed for luciferase and renilla activity by using the Promega Dual-Glo Luciferase Assay System (Cat #2590) per manufacturer's instructions. Plates were read on the Tecan Ultra at 50 ms integration time per well.

- 11. Differentiation assay: C3H10T1/2 cells are plated into 384-well plates at a concentration of 5000 cells/well in DMEM/10% FBS. The following day the media is changed to 20% CM (low serum media DMEM/2% FBS + Shh ligand). Compounds are solubilized in 100% DMSO to a concentration of 10 mM and then serially diluted three fold in 100% DMSO. The highest concentration in the cell plate is 30  $\mu$ M and the lowest is 3 nM. The compounds are then added to the cells. Cell plates are incubated with compound for 72 h and then assayed for alkaline phosphatase production using pNp as a substrate. Briefly, after 72 h of incubation the media is aspirated from the cells and washed with 30  $\mu$ L of PBS. PBS is aspirated off the cells and 15  $\mu$ L of 1 × RIPA cell lysis buffer is added on to the cells. The plates are then thawed back to room temperature. The substrate solution containing pNp at 1 mg/mL in diethanolamine buffer pH 9.8 is then added onto the lysed cells. The plates are incubated at 30 °C overnight for color development and read at absorbance of 405 nm.
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- Experimental characterization of compound 8: <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ ppm 2.33 (s, 3H), 5.30 (s, 2H), 7.20 (d, *J* = 8.85 Hz, 2H), 7.26 (dd, *J* = 5.84, 3.01 Hz, 2H), 7.38 (m, 1H), 7.49 (d, *J* = 7.91 Hz, 1H), 7.60 (m, 3H), 7.86 (m, 1H), 8.01 (m, 3H), 8.21 (s, 1H), 8.61 (d, *J* = 4.52 Hz, 1H), 9.93 (s, 1H); LCMS (M+H) = 435.
- 18. Experimental characterization of compound **11a**: <sup>1</sup>H NMR (300 MHz, DMSOd<sub>6</sub>)  $\delta$  ppm 12.54 (s, 1H), 9.83 (s, 1H), 8.59 (d, J = 4.6 Hz, 1H), 7.98 (d, J = 8.8 Hz, 2H), 7.90 (d, J = 1.5 Hz, 1H), 7.85 (td, J = 7.7, 1.8 Hz, 1H), 7.72 (dd, J = 8.0, 1.6 Hz, 1H), 7.54 (d, J = 7.8 Hz, 1H), 7.36 (dd, J = 7.1, 5.1 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.16 (d, J = 8.8 Hz, 2H), 7.11 (s, 2H), 5.28 (s, 2H), 2.23 (s, 3H); LCMS (M+H) = 385.
- Experimental characterization of compound 11f: <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ ppm 12.54 (s, 1H), 9.83 (s, 1H), 8.59 (d, *J* = 4.6 Hz, 1H), 7.98 (d, *J* = 8.8 Hz, 2H), 7.90 (d, *J* = 1.5 Hz, 1H), 7.85 (td, *J* = 7.7, 1.8 Hz, 1H), 7.72 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.54 (d, *J* = 7.8 Hz, 1H), 7.36 (dd, *J* = 7.1, 5.1 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.16 (d, *J* = 8.8 Hz, 2H), 7.11 (s, 2H), 5.28 (s, 2H), 2.23 (s, 3H). LCMS (M+H) = 385.
  Experimental characterization of compound 11c: <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>)
- 20. Experimental characterization of compound **11c**: <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>) δ ppm 2.33 (s, 3 H), 2.65 (s, 3 H), 3.68 (s, 3 H), 5.37 (s, 2 H), 7.19 (d, J = 8.85 Hz, 2 H), 7.32 (m, 1 H), 7.49 (m, 2 H), 7.57 (s, 1 H), 7.68 (d, J = 7.91 Hz, 1 H), 7.74 (s, 1 H), 8.01 (d, J = 8.85 Hz, 3 H), 8.67 (br s, 1 H), 9.94 (s, 1 H), 14.47 (br s, 1 H); LCMS (M+H) = 413.
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