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Discovery of *N*-[(1-aryl-1*H*-indazol-5-yl)methyl]amides derivatives as smoothened antagonists for inhibition of the hedgehog pathway

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ABSTRACT

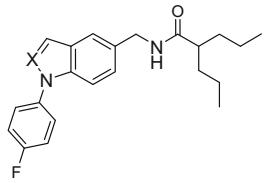
We report the synthesis and biological evaluation of *N*-[(1-aryl-1*H*-indazol-5-yl)methyl]amide derivatives as Smoothened antagonists and inhibitors of the Hedgehog pathway. Identification of the lead structure **1** by HTS, followed by SAR study on the amide and aryl portions led to the discovery of antagonists with nanomolar activity.

The Hedgehog (Hh) signaling pathway is essential for cell differentiation and organ formation in embryogenesis and is involved in growth and survival of various cells and tissues.[1,2] The Hh signaling cascade is initiated by Hh binding to Patched (Ptch) protein on the cell surface. In the absence of the ligand, Ptch represses the activity of Smoothened (Smo), a G-protein coupled receptor (GPCR)-like protein. Binding of Hh to Ptch relieves the inhibition of Smo and triggers a cytoplasmatic signal transduction cascade leading to the activation of Gli transcription factors that regulate Hh target gene expression.[3,4]

While the loss of Hh signaling at the embryonic stage has been demonstrated to cause developmental defects,[4] aberrant activation of this pathway in adults has been documented to be involved in the formation of basal cell carcinoma (BCC), medulloblastoma and rhabdomyosarcoma. Aberrant pathway activation was also reported for pancreatic adenocarcinoma, lung cancer, prostate, breast, and digestive tract tumors.[5] Thus the Hh pathway is an important pharmacological target for the development of new treatments for cancer.[2]

Recent studies have demonstrated that the natural product cyclopamine inhibits Hh pathway activation by binding directly to Smo[6] and is able to inhibit tumor cell proliferation in mouse models,[7] thus validating Smo as a therapeutic target in the treatment of Hh related diseases. Recently several small molecule Smo antagonists have been developed[8] in fact clinical proof of concept has been demonstrated for use of a Smo antagonist in BCC.[9]

In our company a research program was initiated with the aim of identifying agents useful for the treatment of Hh dependent malignancies. An HTS campaign was conducted on Shh-light 2 cells (NIH 3T3 cells stably incorporating a Gli-dependent firefly luciferase).[10] Indazole **1** was shown to be an inhibitor of the pathway with IC_{50} = 150 nM and displayed no cytotoxicity at 5 μM. The compound was confirmed to bind to Smoothened in a whole cell Smo binding assay,[11] where **1** could compete with the binding of bodipy-cyclopamine, displaying IC_{50} = 130 nM. In a phenotypic assay **1** was able to inhibit the growth of irradiated Ptch +/− medulloblastoma cells with a CC_{50} = 3 nM[12] (see Fig. 1).



1 (X= N) **2** (X=C)

Smo Binding	IC_{50} = 0.13 μM	2.0 μM
Light 2	IC_{50} = 0.15 μM	2.8 μM
Medulloblastoma Proliferation	CC_{50} = 0.003 μM	

Figure 1.

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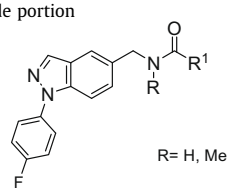
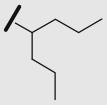
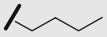
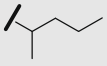
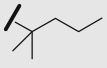
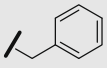
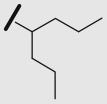
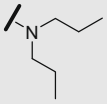
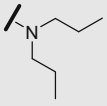
Table 1
SAR study around the amide portion



Compds	R	R^1	Smo binding 2% FCS IC_{50}[a,b] (μM)	Light 2 IC_{50}[c] (μM)
1	H		0.13	0.16
3	H		0.72	0.46
4	H		10.0	N.D.[d]
5	H		10.0	6.6
6	H		10.0	3.5
7	H		1.2	0.84
8	H		0.36	0.23
9	H		0.33	0.15
10	H		10.0	N.D.
11	H		>10.0	N.D.
12	Me		3.0	1.7
13	H		0.31	0.49
14	Me		1.3	>5.0

[a] The affinity of compounds for Smo receptor is measured as reported in Ref. 11.
[b] Values are means of at least two experiments. SD are ±30% of the mean value.
[c] The Light 2 activities were measured as reported in Ref. 10.
[d] Not determined.

Encouraged by this result a SAR study based on the lead structure was initiated to identify compounds with improved activity over **1**. The modification of structure **1** to the corresponding indole **2** resulted in a more than 10-fold drop in activity in both Smo binding and light 2 assays, thus the study was focused on the indazole scaffold. Initial SAR from the HTS campaign suggested that the 2-propylpentanamide was important for activity so initial exploration was focused on the modifications of the alkyl-amide portion (Table 1).

Shortening of the hydrocarbon chain of **1** proved to be detrimental for activity, as demonstrated by compound **3**, which was 6-fold less active in the Smo binding assay (IC_{50} = 0.72 μM), and by the isopropyl derivative **4**, that displayed an even more marked drop in potency, showing micromolar binding affinity. Constraining of the alkyl chains of **3** into a cyclohexyl ring (**5**) produced more than a 10-fold drop in activity. The same loss of activity was also observed by introduction of bulkier *tert*-butyl (**6**) group. Activity could be partially regained by introducing a long linear chain as in **7**, although **7** proved to be 10-fold less potent than the lead **1**. The presence of an α-methyl as in **8**, or an α-*gem*-dimethyl as in **9**, was beneficial for activity, affording submicromolar compounds (IC_{50} = 0.36 and 0.33 μM, respectively), albeit both 3-fold less active than **1**.

The aliphatic side chain was crucial for activity since substantial loss of activity was observed for phenyl and benzyl analogues **10** and **11**. The importance of the amide hydrogen for activity was verified as its replacement with a methyl group resulted in a 30-fold drop in activity with respect to **1**, with **12** showing IC_{50} = 3.0 μM.

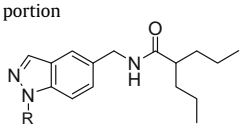
The replacement of the amide with a urea functionality proved to be well tolerated, thus urea derivative **13** (Table 1) showed only a slight drop in activity with respect to amide **1**. The N-methylated analogue **14** was characterized by 4-fold drop in activity with respect to **13**, confirming the importance of the N–H moiety.

The most active compounds in Table 1 (**3**, **8**, **9** and **13**) maintained the antagonism as seen with **1** in the Light 2 assay, confirming the inhibitory activity on the pathway, and they were devoid of toxicity at 5 μM.

We then turned our efforts to the exploration of the phenyl portion of structure **1** (Table 2). Replacement of the fluorine in **1** with a chlorine (compound **15**) proved detrimental and led to a 10-fold drop in binding affinity (IC_{50} = 1.2 μM). The isomeric 2-fluoro and 3-fluoro derivatives **16** and **17** were weaker binders than the 4-fluoro compound **1**. The unsubstituted phenyl derivative **18** displayed comparable binding affinity to **1** (IC_{50} = 0.2 μM). The effect of a methyl group was also evaluated in all the positions of the phenyl ring. While the 4-methyl derivative **19** showed a decrease in activity with respect to **1**, 2- and 3-methyl substitution produced antagonists **20** and **21**, displaying similar levels of activity to **1**, although the 2-methyl analogue **20** was very active in the functional assay. Combination of the two methyl groups was tolerated, although no additional improvement in affinity was observed as 2,3-dimethyl analogue **22** and tetrahydronaphthyl derivative **24** displayed IC_{50} = 0.12 and 0.15 μM respectively in the Smo binding assay. This is in contrast to the 2,6-dimethyl analogue **23** which lost around 7-fold activity. Interestingly, introduction of the methoxy group into all the positions was well tolerated (**25–27**). In particular the ortho and para isomers **25** and **27** displayed both high affinity with IC_{50} = 5 and 40 nM and in the functional assay they inhibited the pathway with IC_{50} = 5 and 10 nM, respectively. Introduction of electron withdrawing groups, such as ketone in **28** and ester in **29**, in the 4-position was characterized by a significant loss in potency.

We then evaluated replacement of the phenyl moiety with a heteroaromatic ring (Table 3). While the 2-pyridine isomer **30**

Table 2
SAR study around the aryl portion



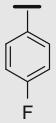
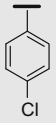
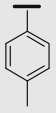
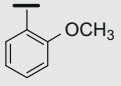
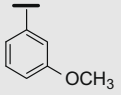
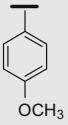
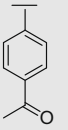
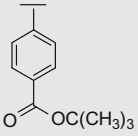
Compds	R	Smo binding 2% FCS IC_{50}[a,b] (μM)	Light 2 IC_{50}[c] (μM)
1		0.13	0.16
15		1.2	0.98
16		0.9	0.15
17		0.68	1.4
18		0.2	0.12
19		0.9	3.0
20		0.2	0.04
21		0.07	0.17
22		0.12	0.14
23		0.95	0.41
24		0.15	0.11
25		0.005	0.005
26		0.10	0.11

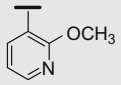
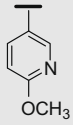
Table 2 (*continued*)

Compds	R	Smo binding 2% FCS IC_{50}[a,b] (μM)	Light 2 IC_{50}[c] (μM)
27		0.04	0.01
28		1.4	1.5
29		1.8	5.9

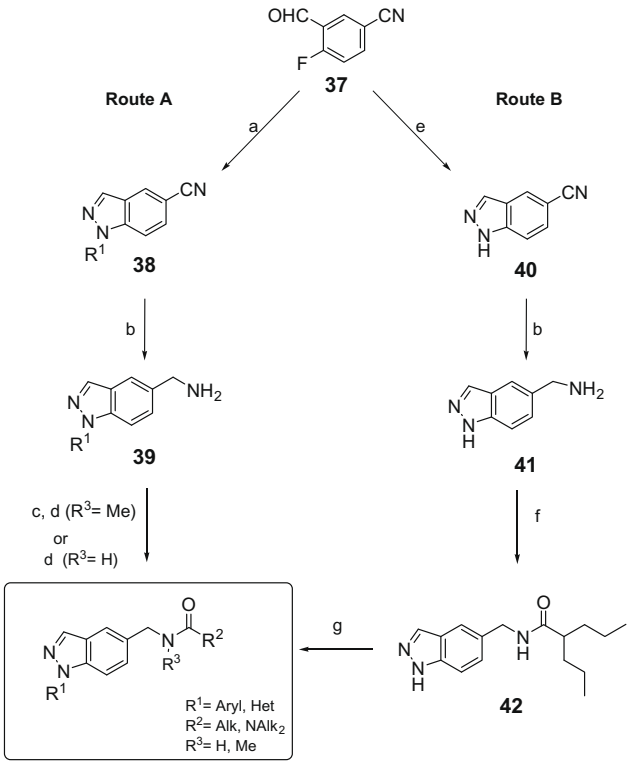
[a–c] See footnotes in Table 1.

was devoid of activity, both the 3- and 4-isomers **31** and **32** maintained micromolar activity. The activity of **31** could be enhanced, however, by the introduction of a methoxy-group in positions 2 or 4 (**33** and **34**, Smo binding IC_{50} = 0.34 and 0.13 μM, respectively), as already observed for phenyl derivatives **18** and **27** (Table 2). Both compounds retained antagonistic activity in the light 2 cells. In contrast introduction of a pyridazine (**35**) and pyrimidine (**36**) proved to be detrimental for activity.

Table 3
Heterocyclic replacement

Compds	R	SMO binding IC_{50}[a,b] (μM) 2% FCS	Light 2 IC_{50}[c] (μM)
30		>10	N.D.[d]
31		1.83	0.87
32		4.5	2.6
33		0.34	0.39
34		0.13	0.32
35		>25	N.D.
36		6.3	N.D.

[a–d] See footnotes in Table 1.



Scheme 1. Synthesis of compounds in Tables 1–3. Reagents and conditions: (a) (1) $ArNHNH_2$, μW (80 °C, 5 min); (2) K_2CO_3, μW (150 °C, 20 min) ($Y = 90\%$); (b) $LiAlH_4$, THF, reflux, 15 min; (c) (1) 2,2,2-trifluoroethylformate, K_2CO_3, THF; (2) BH_3·DMS, THF; (d) R_2COCl or dipropylcarbamoyl chloride, TEA, DMF; (e) $NH_2NH_2·H_2O$, μW (80 °C, 25 min); (f) valproyl chloride, TEA, DMF; (g) ArI or ArBr, *N,N′*-dimethylcyclohexane-1,2-diamine, CuI, K_3PO_4, dioxane, 100–110 °C, 12 h.

For the synthesis of the compounds described in Tables 1–3, two different routes were developed, with the aim to introduce diversity in the final step. Compounds reported in Table 1 were prepared following route A (Scheme 1): condensation of 5-cyano-2-fluoro-benzaldehyde **37** with 4-fluorophenyl hydrazine produced **38** (R^1 = 4-fluorophenyl), that was smoothly reduced with $LiAlH_4$[13] to the corresponding amine **39**, then acylated with the appropriate acyl chlorides to yield the final products of Table 1. Formylation of **39** followed by formyl reduction and acylation were performed to prepare methyl amide **12**. This route was also followed for the preparation of the two urea derivatives **13** and **14**. The aminomethyl indazole **39** was reacted with dipropylcarbamoyl chloride to obtain **13** while formylation followed by formyl reduction and urea formation were performed for preparation of **14**.

Most of the compounds reported in Tables 2 and 3 were prepared following route B (Scheme 1). Indazole nitrile intermediate **40**, obtained by condensation of **37** with hydrazine, was reduced to amine **41**,[13] that was acylated with valproyl chloride to provide **42**.[14] The indazole intermediate **42** was then functionalized with aryl or heteroaryl iodides or bromides via copper catalyzed coupling reaction.[15,16]

Unfortunately, the 2-substituted aryl halides did not react under copper catalyzed conditions, thus they were prepared following route A, using the appropriate aryl hydrazines in the condensation step.[17]

In summary, we have reported the synthesis and biological evaluation of *N*-1-aryl-indazole-5-yl-methylamide derivatives as inhibitors of the Hedgehog pathway. Our work focused on the improvement of the Smo binding affinity over the lead compound **1**, identified by HTS. The alkyl chain of the amide moiety of **1** appeared to be important for activity. On the other hand a SAR study around the aryl portion has revealed that a range of aryl groups are tolerated and has resulted in the identification of **25** and **27** as nanomolar Smo antagonists.

Acknowledgments

The authors acknowledge Danielle McMaster for the first synthesis of **1**.

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10. Shh Light II cells (ATCC catalog No. CRL-2795) were seeded into a 384-well Matrix white microplate at an initial concentration of 15,000 cells/well in 20 μL of assay medium (DMEM without Phenol Red complemented with 2% FCS, 0.1 mg/mL Penicillin-Streptomycin, 2 mM L-Glutamine) and serial dilutions of antagonists in DMSO/H_2O (0.25% DMSO, 2 μL) were added. The plate was incubated for 16 h at 37 °C under 10% CO_2, and then agonist Purmorphamine (Calbiochem catalog No. 540220) was added at a final concentration of 3 μM. The plate was then incubated for 30 h at 37 °C under 10% CO_2, and then Gli-dependent firefly luciferase measured with Bright-Glo Kit and cell viability measured using CellTiter Blue Kit on View Lux. The IC_{50} and CC_{50} value was determined based on the residual Gli-dependent transcriptional activity, and cell viability.
11. Human HEK293 Flag-Smo cells were seeded into a 384-well Matrix black microplate (Poly-lys) at an initial concentration of 10,000 cells/well in 20 μL of assay medium (DMEM without Phenol Red complemented with 2% FCS, 0.1 mg/mL Penicillin-Streptomycin, 2 mM L-Glutamine). The plate was incubated for 16 h at 37 °C under 5% CO_2, and then Smo antagonists in DMSO/H_2O were added with serial dilutions points (0.25% DMSO, 2 μL). The plate was incubated for 2 h at 37 °C under 5% CO_2, and then BODIPY-cyclopamine (Toronto Research Chemical Catalog No. B674800) was added at a final concentration of 15 nM. The plate was then incubated for 3 h at 37 °C under 5% CO_2, and then the cells were fixed 10 min in 20 μL/well of 7% formaldehyde. After washing five times with PBS, cell nuclei were stained with 0.5 μM Propidium Iodide, and the plate was read using an Acumen Explorer (488 nm laser λ_{ex}, λ_{em} 535–618 nm). The IC_{50} value was determined based on the residual fluorescence normalised for PI values.
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14. *1H-Indazole-5-carbonitrile* (**40**). A solution of 5-cyano-2-fluorobenzaldehyde (10.0 mmol) and hydrazine hydrate (101 mmol) in DMF (33.5 mL) was heated under microwave irradiation at 80 °C for 25 min. After aqueous work up, the residue was purified through a SCX cartridge. ($Y = 83\%$). ^1H NMR (300 MHz, DMSO-d_6) δ 13.56 (br s, 1H), 8.39 (s, 1H), 8.25 (s, 1H), 7.74–7.60 (m, 2H). MS (ES) $C_8H_5N_3$ requires 143, found 144 $(M+H)^+$.
1-(1H-Indazol-5-yl)methanamine (41): Obtained by $LiAlH_4$ reduction of **40**, as described in Ref. 13.
N-(1H-Indazol-5-ylmethyl)-2-propylpentanamide (**42**): To a solution of 1-(1*H*-indazol-5-yl)methanamine **41** (1.02 mmol) in DMF (5 mL) 2-propylpentanoyl chloride (1.12 mmol) and TEA (1.12 mmol) were added. The reaction was stirred at room temperature for 2 h. Aqueous work up provided the title compound ($Y = 57\%$). ^1H NMR (300 MHz, DMSO-d_6) δ 8.29 (m, 1H), 7.99 (s, 1H), 7.57 (s, 1H), 7.47 (d, J = 8.6 Hz, 1H), 7.23 (d, J = 8.6 Hz, 2H), 4.33 (d, J = 5.8 Hz, 2H), 2.19 (m, 1H), 1.54–1.38 (m, 2H), 1.31–1.1 (m, 6H), 0.81 (t, J = 7.0 Hz, 6H). MS (ES) $C_{16}H_{23}N_3O$ requires 273, found 274 $(M+H)^+$.
15. Antilla, J. C.; Baskin, J. M.; Barder, T. E.; Buchwald, S. L. *J. Org. Chem.* **2004**, *69*, 5578.
16. As an example, synthesis of *N-[1(3-methoxyphenyl)-1H-indazol-5-ylmethyl]-2-propylpentanamide* (**26**). Indazole **42** was arylated with 1-iodo-3-methoxybenzene according to ref. 15 ($Y = 59\%$). ^1H NMR (300 MHz,

DMSO-d_6) δ 8.37 (m, 1H), 8.32(s, 1H), 7.82 (d, J = 8.7 Hz, 1H), 7.70 (s, 1H), 7.54–7.21 (m, 4H), 6.96 (dd, J_1 = 8.2, J_2 = 1.8 Hz, 1H), 4.38 (d, J = 5.8 Hz, 2H), 3.84 (s, 3H), 2.21 (m, 1H), 1.55–1.37 (m, 2H), 1.31–1.1 (m, 6H), 0.81 (t, J = 7.0 Hz, 6H). MS (ES) $C_{23}H_{29}N_3O_2$ requires 379, found 380 $(M+H)^+$.

All the compounds prepared as described for **26** were obtained in comparable yields (>50%).

17. Compounds synthesized acconding to route A were obtained in 25–50% yields from **37**. Compound **25** was obtained in 35% yields.