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Identification and structure–activity relationships of *ortho*-biphenyl carboxamides as potent Smoothened antagonists inhibiting the Hedgehog signaling pathway

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ABSTRACT

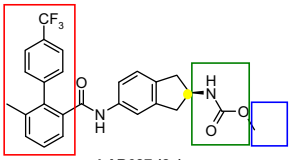
Ortho-biphenyl carboxamides, originally prepared as inhibitors of microsomal triglyceride transfer protein (MTP) have been identified as novel inhibitors of the Hedgehog signaling pathway. Structure–activity relationship studies for this class of compounds reduced MTP inhibitory activity and led to low nanomolar Hedgehog inhibitors. Binding assays revealed that the compounds act as antagonists of Smoothened and show cross-reactivity for both the human and mouse receptor.

The Hedgehog (Hh) signal transduction pathway plays critical roles in the development and homeostasis of many organs and tissues.[1] In the resting state, the 12-pass transmembrane protein Patched (Ptch) inhibits activity of the membrane receptor Smoothened (Smo) which resembles G-protein coupled receptors (GPCR) in general topology. The Hh family protein ligands Sonic Hedgehog, Desert Hh, and Indian Hh can all bind to Ptch, which releases the repression of Smo activity. Active Smo signals via a cytosolic complex of proteins leading to activation of the Gli family of transcription factors. Active Gli1 signaling can cause cell proliferation and differentiation. Genetic activation of the Hh pathway at or upstream of Smo is linked to tumorigenesis in several cancers such as basal cell carcinoma and medulloblastoma.[2]

Treatment with small molecule Hh inhibitors such as HhAntag and the natural product cyclopamine, both binding to Smo, induced tumor remission in a genetic mouse model of medulloblastoma.[3,4] We therefore became interested in identifying small molecule inhibitors of the Hh pathway with cross-reactivity to both the human and mouse Smo receptor to be of potential use in a therapeutic application.[5]

In this study we describe potent inhibitors of the Hh pathway which act as antagonists of the Smo receptor. Screening of part of our compound collection provided us with *ortho*-biphenyl carboxamides such as LAB687 (Fig. 1) as micromolar inhibitors of the Hh signaling pathway. In addition, binding assays showed weak binding of this compound to the mouse and human Smo receptors (Table 1). LAB687 was originally prepared as an antagonist of the microsomal triglyceride transfer protein (MTP) inhibiting the secretion of apoB from Hep G2 cells with subnanomolar activity (Table 1).[6,7] Our initial aims were to identify compounds with improved Hh and Smo potency over LAB687 and significantly reduced ability to inhibit the secretion of apoB. Herein, we describe the structure–activity relationships obtained from studying the effect of substituents on the biaryl unit (Fig. 1, red box), the stereochemistry of the aminoindane moiety (yellow dot), the nature of the linker (green box), and the western substituent (blue box).[8]

Synthesis: The preparation of the *ortho*-biphenyl carboxamides **4**–**26** follows the route described in Ksander et al. (Scheme 1).[6] *Ortho*-biphenyl carboxylic acids **1** were converted into their acid chlorides and coupled with either racemic or the enantiomerically



LAB687 (2a)

Figure 1. Structure of LAB687, a Hedgehog inhibitor screening hit.

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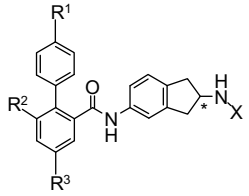
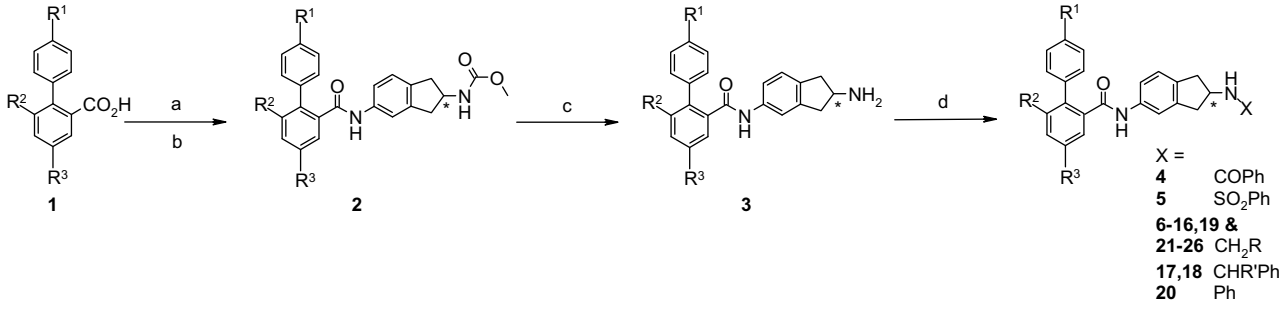
Table 1
In vitro activity in the Hedgehog cell assay, the Smoothened mouse (mu), and human (hu) receptor binding assay and the apoB secretion assay (IC_{50}s, nM)[9]



Compound	Chirality	R^1	R^2	R^3	X	Hh assay	Smo mu binding	Smo hu binding	apoB assay
2a (LAB687)	R	CF_3	CH_3	H	COOMe	1200	2480	3420	0.9
2b	S	CF_3	CH_3	H	COOMe	2150	5090	7520	4.3
2	rac	CF_3	CH_3	H	COOMe	1330	2960	2700	
4	rac	CF_3	CH_3	H	COPh	600			
5	rac	CF_3	CH_3	H	SO_2Ph	1250			
6	rac	CF_3	CH_3	H	CH_2Ph	93			
7	rac	CF_3	CH_3	H	CH_2-2-pyridyl	100	110		
7a	R	CF_3	CH_3	H	CH_2-2-pyridyl	4890	8770		3.5
7b	S	CF_3	CH_3	H	CH_2-2-pyridyl	100	57	120	2350
8a	R	CF_3	CH_3	H	CH_2-2-thiazolyl	4270	1190	770	0.6
8b	S	CF_3	CH_3	H	CH_2-2-thiazolyl	25	10	30	140
9	rac	CF_3	H	CH_3	CH_2-2-pyridyl	6800	8680		
10	rac	CF_3	H	OCH_3	CH_2-2-pyridyl	>5000	>5000		
11	rac	CF_3	H	H	CH_2-2-pyridyl	200	490	790	
12	rac	Cl	H	H	CH_2-2-pyridyl	4950	2710		
13	rac	H	H	H	CH_2-2-pyridyl	1000	1420	1150	



Scheme 1. Reagents and conditions: (a) oxalyl chloride, rt, 16 h; (b) rac, *R* or *S* (5-amino-indan-2-yl)-carbamic acid methyl ester, pyridine, CH_2Cl_2, 0 °C, 1 h; (c) TMSI, CH_3CN, rt, 16 h; (d) for **4**: PhCOCl, DIPEA, CH_2Cl_2, 0 °C, 2 h; for **5**: $PhSO_2Cl$, DIPEA, CH_2Cl_2, 0 °C, 2 h; for **6–16** and **21–26**: RCHO, sodium triacetoxy borohydride, CH_2Cl_2, rt, 16 h; for **17**, **18**: PhCOR′, $Ti(^iPrO)_4$, THF, 100–150 °C, 30 min, then $NaBH_4$, EtOH, rt, 2 h; for **20**: bromo-benzene, Pd_2dba_3, Xantphos, Cs_2CO_3, toluene, 130–140 °C, microwave synthesizer, 4 h.

pure *S* and *R* diaminoindane methylcarbamates[7] to yield compounds **2**. Removal of the methylcarbamate with iodotetramethylsilane (TMSI) produced the *ortho*-biphenyl aminoindanes **3**. Reaction of **3** with either benzoyl chloride or phenylsulfonyl chloride provided the desired N-substituted 2-aminoindanes **4** and **5**. *N*-Alkyl derivatives **6**–**16**, **19**, and **21**–**26** were prepared by reductive amination of the free amines **3** with the appropriate aldehydes in the presence of sodium triacetoxy borohydride as reducing agent. On the other hand, *N*-alkyl derivatives **17** and **18** required pre-formation of the imines followed by reduction with $NaBH_4$. Synthesis of *N*-phenyl compound **20** was best achieved using Buchwald–Hartwig amination of bromo-benzene with amine **3** and microwave heating at 130–140 °C.

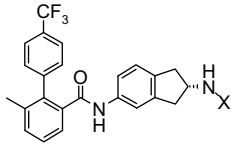
Results and discussion: Table 1 shows the structure–activity relationship (SAR) for representatives of the biphenyl-2-carboxylic acid (2-amino-indan-5-yl)-amide series. A comparison of compounds **4**, **5**, and **6** which differ only in the functional group connecting the aminoindane to the phenyl substituent suggests that benzylamines are superior to carboxamides or sulfonamides in terms of Hh inhibitory activity. Key for increasing Hh potency and simultaneously decreasing MTP activity proved to be the stereochemistry of the aminoindane moiety: A comparison of the enantiomeric compound pairs **7a**,**b** and **8a**,**b** demonstrates that the Hh activity of the *N*-benzyl amines resides predominantly ($\geqslant$50:1) in the *S*-enantiomer whereas the *R*-enantiomers (**7a, 8a**) are significantly more potent in inhibiting apoB secretion. Enantiomeric purity was determined by chiral chromatography to be >99.9% ee for both **8a** and **b** practically excluding that the weak Hh activity observed with the *R*-enantiomer **8a** is caused by contamination with small amounts of the *S*-enantiomer. This diverging SAR for Hh and apoB activity is characteristic for the *N*-benzylamines and not observed to the same degree for the other series, for example, the carbamates (**2a,b**). We next explored the influence of the biphenyl substituents R^1, R^2, and R^3 on Hh potency. Moving the methyl group from the *ortho* to the *para* position on the lower phenyl ring (compounds **7** and **9**) resulted in a drastic decrease of activity. Other substituents such as the methoxy group in compound **10** in the R^3-position were not tolerated either. Replacing the *ortho* methyl substituent with a hydrogen yielded compound **11** with a slight drop in activity compared to the corresponding analogue **7**. Finally, substituents R^1 play a crucial role for activity, too: The trifluoromethyl substituent is the best substituent identified so far; both chloro and hydrogen in this position (compounds **12** and **13**) resulted in lower activity.

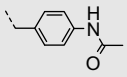
For all compounds tested in Table 1, Hh inhibition and binding to the mouse Smo receptor correlates well and suggests that the observed Hh activity observed in the reporter-gene cell assay is driven by Smo antagonism. Furthermore, the compounds show good cross-reactivity between the human and mouse Smo receptor with IC_{50} values for both receptors typically within a factor of two.

As the *S*-enantiomers of the *N*-benzyl amines provided the best potency and selectivity, the SAR of this compound series was further explored in more detail (Table 2). Replacing the phenyl substituent (**6b**) with either a 2-pyridyl or 4-pyridyl substituent (**7b**, **14b**) resulted in a ~5- to 7-fold drop in potency in the Hh assay and a stronger drop in binding affinity to the mouse Smo receptor. Substitutions on the phenyl ring were not well tolerated and the activity of compounds **15b** and **16b** with *para*-substituents on the phenyl group dropped significantly. The same was true by replacing the pyridyl group with a bicyclic aromatic group such as the 3-quinolinyl moiety in compound **19b**. Substitution on the methylene unit, including the methyl and trifluoromethyl substituents in compounds **17b** and **18b** were well tolerated and resulted in only slightly less potent Hh inhibitors compared to compound **6b**. The methylene unit can be omitted attaching the phenyl ring directly to the nitrogen (**20b**) producing a slight 2-fold reduction in inhibitory activity. It was found that 5-membered heterocycles at the benzylic position (**8b**, **21b, 22b**) are among the best substituents with IC_{50}s in the low double-digit nanomolar range for Hh pathway inhibition. An additional methyl substituent in the distal position as in **23b** and **25b** maintained or even slightly increased potency whereas a methyl group in the *ortho*-position resulted in a compound (**24b**) with significantly less activity. Aryl substituents on the methylene unit are vital for excellent potency: Replacement of the aryl group with an alkyl group, as exemplified by the tetrahydrofuranyl substituent in **26b** resulted in a compound with only moderate micromolar inhibitory activity. As observed with compounds in Table 1 Hh pathway inhibitory activity and affinity to the human and mouse Smo receptor track very well over the full range of potency. The most potent inhibitors in Table 2 show a ~5-fold higher activity in the Hh cell assay and significantly higher affinity to the Smo receptors than the natural product cyclopamine.

In conclusion, we described the optimization based on SAR studies of the micromolar hit LAB687 to afford low nanomolar inhibitors (e.g., **21b**, **23b**) of the Hh signaling pathway which act as antagonists for both the human and mouse Smo receptor. During this process the activity against the original target MTP of this compound class was significantly reduced and the most potent Hh inhibitors (e.g., **8b**, **21b**, **22b**) have a ratio on-target/off-target of ~10:1. Therefore, these compounds have potential as novel lead structures in the search for therapeutic agents for the treatment of Hh dependent cancers.

Table 2
In vitro activity in the Hh cell assay, the Smo mouse (mu), and human (hu) receptor binding assay and the apoB secretion assay (IC_{50}s, nM)[9]



Compound	X	Hh assay	Smo mu binding	Smo hu binding	apoB assay
Cyclopamine		46	1200	280	
6b	CH_2-phenyl	21	8	14	
7b	CH_2-2-pyridyl	100	57	120	2350
14b	CH_2-4-pyridyl	150	190	240	
15b	CH_2-(4-methoxy)-phenyl	360	93	120	
16b		450	360	340	
17b	CH(Me)-phenyl	88	29	32	180
18b	$CH(CF_3)$-phenyl	33	57	55	
19b	CH_2-3-quinolinyl	1290	350	210	1400
20b	Phenyl	41	82	130	
8b	CH_2-2-thiazolyl	25	10	30	140
21b	CH_2-2-thienyl	10	9	7	140
22b	CH_2-2-furanyl	23	14		150
23b		17	4	7	
24b		160	120	170	
25b		8	6	10	
26b		3700	16,700		

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9. *Hh cell assay*: This assay measures the end stage of the Hh signaling pathway, that is, the transcriptional modulation of Gli, using Luciferase as readout (Gli-Luc assay). Test compounds were prepared for assay by serial dilution in DMSO and then added to empty assay plates. TM3Hh12 cells (TM3 cells containing Hh-responsive reporter gene construct pTA-8xGli-Luc, kind gift of Xu Wu, Genomics Institute of the Novartis Foundation, La Jolla, CA) were cultured in F12 Ham's/DMEM (1:1) containing 5% horse serum, 2.5% fetal bovine serum (FBS), and 15 mM Hepes, pH 7.3. Cells were harvested by trypsin treatment, resuspended in F12 Ham's/DMEM (1:1) containing 5% FBS and 15 mM Hepes pH 7.3, added to assay plates and incubated with test compounds for approximately 30 min at 37 °C in 5% CO_2. 1 nM Hh-Ag 1.5 (Frank-Kamenetsky, M.; Zhang. X. M.; Bottega, S.; Guicherit, O.; Wichterle, H.; Dudek, H. et al. *J. Biol.* **2002**, *1*, 10) was then added to assay plates and incubated at 37 °C in the presence of 5% CO_2. After 48 h, either Bright-Glo (Promega E2650) or MTS reagent (Promega G258B) was added to the assay plates and luminescence or absorbance at 492 nm was determined. IC_{50} values, defined as the inflection point of the logistic curve, were determined by non-linear regression of the Gli-driven luciferase luminescence or absorbance signal from MTS assay vs log10 (concentration) of test antagonist using the R statistical software package.*Smo binding assay*: Smo membranes were prepared from CHO-K1 cells which were stably transfected with HA-tagged cDNA encoding human or mouse Smo. Test compounds were prepared for assay by serial dilution in DMSO and then added to empty assay plates. Smo membranes (10 μg total protein) were added to these assay plates and incubated with 1.5 nM [^{3}H]-Hh-Ag 1.5 in binding buffer (50 mM Tris–HCl, 10 mM EDTA, and 5 mM magnesium chloride, pH 7.2) for 48 h at 37 °C. 96 well Unifilter GF/B filter plates (Perkin Elmer) were prepared by 60 min incubation in binding buffer containing 0.5% w/v polyethyleneimine (Acros) and 0.1% (w/v) bovine serum albumin (Jackson Immuno Research) followed by three rinses with 2% beta-hydroxy propyl cyclodextrin (HPCD, Acros) in distilled water. Assay plates were harvested into filter plates using a Unifilter-96 cell harvester (Perkin Elmer). Loaded filter plates were washed three times with 2% HPCD buffer to remove unbound [^{3}H]-Hh-Ag 1.5, dried in a 60 °C oven for 30 min and then cooled. The bottom of the plate was sealed, 50 μL of Microscint-O (Perkin Elmer) was added to each well, the top of the plate was sealed and the plate was incubated 100 min to overnight. The amount of bound [^{3}H]-Hh-Ag 1.5 was determined by scintillation counting on a TopCount (Perkin Elmer). The data were analyzed for saturation binding using global fitting with Graphpad Prizm software.*apoB secretion assay*: This assay monitors inhibition of the microsomal triglyceride transfer protein (MTP) by measuring Apolipoprotein B (ApoB) production from HepG2 cells by ELISA. HepG2 cells (ATCC HB-8065) were cultured in MEM media with Earle's salts and L-glutamine containing 10 mM non-essential amino acids, 100 mM sodium pyruvate, and 10% fetal bovine serum (complete media). Cells at 80%

confluence were harvested with trypsin–EDTA 0.05%, resuspended in complete media, and 20,000 cells per well were added to assay plates (Corning 3716) and incubated with test compounds for 16 h at 37 °C in 5% CO_2. Concentration of ApoB secreted by the cells was determined using total human apolipoprotein B (Apo B) ELISA Assay kit (Alerchek A70102) according to manufacturer's instructions, with the following modifications. The HepG2 cell supernatant was diluted 1:2 with the sample diluent provided in the kit. 100 μL of the diluted sample was added to the ELISA microplate and incubated for 120 min at room temperature. Washes, development, and termination of assay were performed according to the manufacturer's protocol and absorbance was measured at 450 nm. IC_{50} values, defined as the inflection point of the logistic curve, were determined by non-linear regression of the absorbance signal from ApoB ELISA vs log10 (concentration) of test compound using the R statistical software package.