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Identification of MK-5710 ((8*aS*)-8*a*-methyl-1,3-dioxo-2-[(1*S*,2*R*)-2-phenylcyclopropyl]-*N*-(1-phenyl-1*H*-pyrazol-5-yl)hexahydroimid azo[1,5-a]pyrazine-7(1*H*)-carboxamide), a potent smoothened antagonist for use in Hedgehog pathway dependent malignancies, Part 1

Savina Malancona *, Sergio Altamura, Gessica Filocamo, Olaf Kinzel, Jose Ignacio Martin Hernando, Michael Rowley, Rita Scarpelli, Christian Steinkühler, Philip Jones *

IRBM, Merck Research Laboratories Rome, Via Pontina km 30,600, Pomezia, 00040 Rome, Italy

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ABSTRACT

The Hedgehog (Hh-) signaling pathway is a key developmental pathway which controls patterning, growth and cell migration in most tissues, but evidence has accumulated showing that many human tumors aberrantly reactivate this pathway. Smoothened antagonists offer opportunities for the treatment of malignancies dependent on the Hh pathway, and the most advanced clinical candidates are demonstrating encourage initial results. A novel series of [6,5]-bicyclic tetrahydroimidazo[1,5-*a*]pyrazine-1,3(2*H*,5*H*)-dione smoothened antagonists has been identified, and the series has been extensively explored to ascertain the key detriments for activity, demonstrating that the *trans*-2-phenylcyclopropyl and hydantoin ring systems are critical for potency, while a variety of urea substituents can be tolerated. The combination of these optimal groups gives smoothened antagonists with activity in the low nanomolar range.

The Hedgehog (Hh-) signaling is essential for development, but in recent years, evidence has accumulated showing that many human tumors aberrantly reactivate this developmental pathway and that interfering with it may provide a new strategy for the development of novel anti-cancer therapeutics.[1–4]

The Hh signaling pathway controls patterning, growth and cell migration in most tissues and organs throughout development.[5] Hedgehog was first identified in the early 1980s in a screen for genes required for embryonic patterning in Drosophila and received its name from the peculiar phenotype arising in deficient embryos.[6] In vertebrates three related orthologs named Sonic (Shh), Desert (Dhh) and Indian (Ihh) Hedgehog have been identified. Hedgehog initiates its signaling cascade by interacting with the 12-pass transmembrane protein patched (Ptch). In the absence of ligand, this protein exerts an inhibitory action, by a poorly understood mechanism, on the G-protein-coupled receptor (GPCR)-like, 7-span transmembrane protein smoothened (Smo).[1,3,7] Binding of Hh to Ptch relieves this inhibition, and triggers a series of intracellular events culminating in the nuclear translocation and activation of Gli 1–3 transcription factors with the initiation of the Hh-transcriptional program, upregulating a number of proteins important for differentiation, proliferation and survival.

In line with its primary role as a developmental morphogen, Hh signaling ceases on completion of development, but remains activatable in very few tissues after birth (such as CNS, hair follicles, bone and testes). In adult tissues the Hh pathway may be reactivated during processes such as wound healing and tissue repair. In fact, in all highly regenerative tissues such as the hematopoietic system, intestine or skin, a subpopulation of adult stem cells usually residing in a stem cell niche is maintained to control homeostasis and repair tissues after possible injuries. Given the need of proliferation, stem cells reactivate development pathways, including the Hh signaling cascade.

In the past decade several studies have demonstrated that aberrant reactivation of Hh signaling occurs in a variety of human tumors,[1–3] the best characterized being basal cell carcinoma (BCC),[8] but there is strong evidence of alterations in the pathway in medulloblastoma, as well as small cell lung, gastric, colorectal, prostate and pancreatic cancers.[1–3]

Smoothened is a G-protein coupled receptor-like protein that is essentially involved in Hedgehog signal transduction, and small molecule Smo antagonists have started to show antitumor activity in preclinical models, and are being developed clinically. The plant steroidal alkaloid cyclopamine (**1**) was first identified as an inhibitor of Hh signaling due to its teratogenicity in sheep.[9,10]

* Corresponding authors. Tel.: +1 617 582 9648.
E-mail addresses: savinamal@libero.it (S. Malancona), philip_jones@dfci.harvard.edu (P. Jones).

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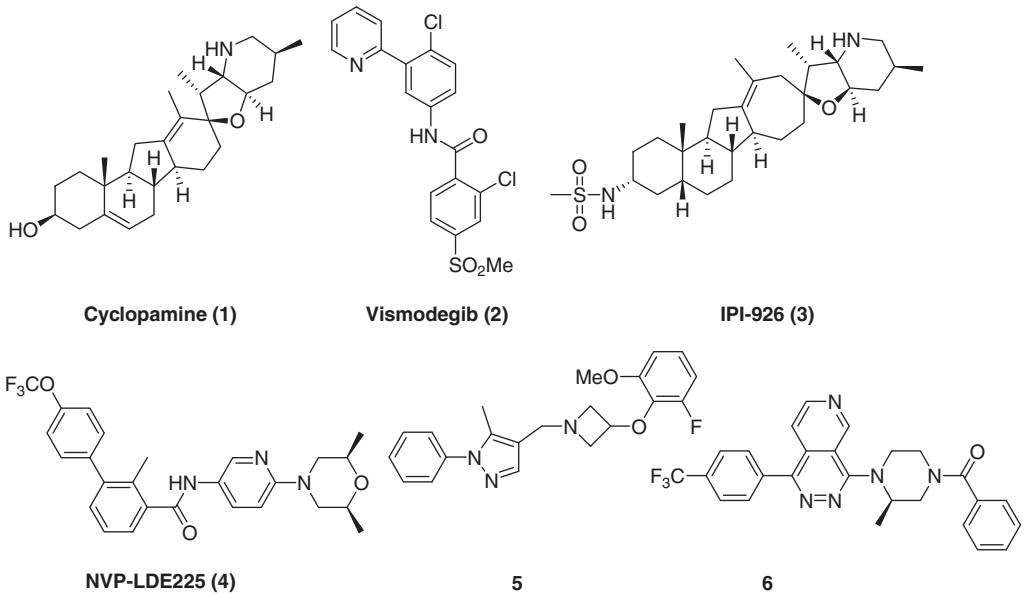
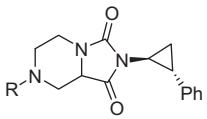
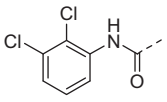
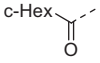
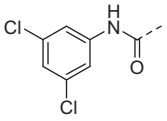
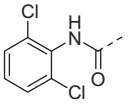
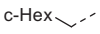
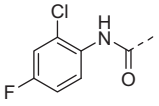
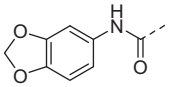
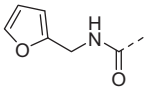
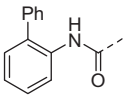
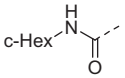
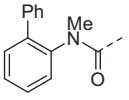
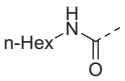
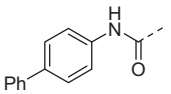


Figure 1. Known Smo antagonists.

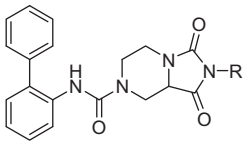
Table 1
SAR study of the left hand side chain[a]

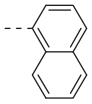
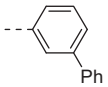
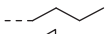
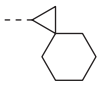
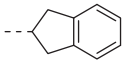


Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)	Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)
7		ND[b]	>1.5 μM	16		1900	920
8		ND	>1.5 μM	17		340	220
9		ND	>1.5 μM	18		780	280
10		4500	4000	19		140	280
11		ND	>2.5 μM	20		1100	1100
12		ND	>2.5 μM	21		2700	700
13		ND	>2.5 μM	22		84	94
14		600	250	23		1600	1000
15		1500	380	24		2000	890

[a] Values are means of at least two experiments with standard deviations <30% of the mean value.
[b] ND = Not determined.

Table 2
SAR study of the right hand side chain[a]



Compds	R	Light2 IC_{50}[a] (nM)	Smo binding IC_{50}[a] (nM)
22		84	94
25		>2.5 μM	>2.5 μM
26		5000	2000
27		ND[b]	>2.5 μM
28		ND	>2.5 μM
29		2500	>2.5 μM
30		ND	>2.5 μM
31		>2.5 μM	>2.5 μM
32		130	440
33		320	880

<30% of the mean value.
[a] Values are means of at least 2 experiments with standard deviations.
[b] ND = Not determined.

Cyclopamine was later shown to directly bind and inhibit Smo, and inhibit the proliferation of medulloblastomas and BCC. Subsequent research has identified a number of small molecule Smo antagonists which are advancing in clinical trials (Fig. 1).[3,4] The most advanced of these is Vismodegib (GDC-0449) (**2**),[11] which has demonstrated encouraging results in phase I studies in advanced BCC,[12] and is currently in phase II trials in BCC, colorectal, ovarian and pancreatic cancer. Other clinical candidates include a semi-synthetic cyclopamine analogue IPI-926 (**3**),[13] and NVP-LDE225 (**4**),[14] as well as the structurally undisclosed BMS-833923 (XL-139), PF-04449913, TAK-441 and LEQ506.[15] Recently, a number of publications have appeared describing other classes of Smo antagonists, such as pyrazole (**5**) and phthalazine (**6**).[16,17]

Our program began with an uHTS campaign to identify small molecules useful for the treatment of Hh dependent malignancies. Compounds were screened for their ability to inhibit the Hh pathway in Shh-Light2 cells expressing a Gli-dependent reporter gene, simultaneously monitoring for cytotoxicity.[18] Hits were then profiled in a whole cell Smo binding assay, measuring the ability of compounds to displace a Bodipy-labeled cyclopamine derivative.[10] Screening identified a series of bicyclic hydantoins as Smo antagonists, and inhibitors of the Hh-pathway. The characterization of these hits and exploratory SAR work is the subject of this article.

A key aspect of this hit cluster from the initial screen was the presence of the [6,5]-bicyclic tetrahydroimidazo-[1,5-*a*]pyrazine-1,3(2*H*,5*H*)-dione scaffold (Tables 1 and 2), and the majority of the active hits conserved the *trans* 2-phenylcyclopropyl fragment in the right hand region (Table 1). The preliminary result presented in this article focus in the investigation of a library of racemic bicyclic hydantoins, it should be highlighted that these are mixtures of two diastereomeric racemates, 4 compounds in total, and that the stereochemical aspects are discussed in the accompanying article.[19]

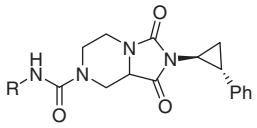
Initial SAR was determined from interrogation and follow up of related compound present in the sample collection. Maintaining the key [6,5]-bicyclic hydantoin scaffold and the *trans* 2-phenylcyclopropyl fragment, the left hand side of these molecules was initially investigated (Table 1). The simple methyl carbamate (**7**) displayed activity greater than 1.5 μM, as were simple amides such as acetamide (**8**) and cyclohexylamide (**9**). Only when additional lipophilic groups were added, like the *t*-butyl group in **10**, was weak micromolar activity observed. Alkyl amines, such as cyclohexylmethylamine (**11**) displayed only weak activity. In contrast, the urea fragment was deemed beneficial for activity, as seen with derivatives **12–24**. While the simple dimethyl urea (**12**) displayed only marginal activity at 2 μM, more elaborate fragments improved Smo affinity, and demonstrated encouraging levels of Hh pathway inhibition in the absence of cytotoxicity at 2.5 μM. The introduction of cyclohexyl urea generated **14** with sub-micromolar activity in Smo binding and Light2 assays (IC_{50} = 600 and 250 nM respectively). The corresponding acyclic derivatives (**15** and **16**) were slightly less active, also highlighting the importance of the N–H in the urea for activity.

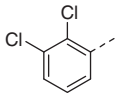
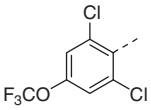
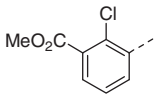
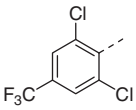
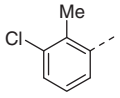
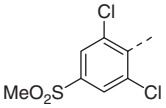
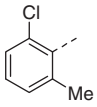
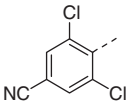
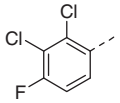
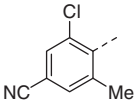
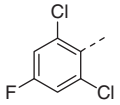
Further investigation of these urea derivatives focused in the phenyl series (**17–24**). Several compounds from this subseries demonstrated submicromolar Smo antagonism, notably **17–19** and **22**. Disubstitution with two lipophilic groups appeared to be beneficial on activity, with the 2,3- and the 2,6-dichlorophenyl fragments (**17** and **19**) displaying activity in the low nanomolar range in Smo binding assay (IC_{50} = 220 and 280 nM respectively) and inhibiting the Hh pathway in Light2 cells (IC_{50} = 340 and 140 nM respectively). Another interesting derivative was the 2-biphenylurea **22**, which displays double digit nanomolar activity in both Smo binding (IC_{50} = 94 nM) and Light2 (IC_{50} = 84 nM) assays. As previously mentioned the substitution at NH of urea appeared detrimental for the activity, and similarly alkylation of **22** to give the methylated analog **23** resulted in an 11-fold drop in potency. The position of the phenyl group was critical for activity, as the isomeric 4-biphenyl **24** was 10-fold less active.

Having demonstrated the striking Smo antagonism of **22**, attention focused on exploration of the right hand side of these compounds, and investigation of the SAR around the *trans* 2-phenylcyclopropyl fragment (Table 2). Strikingly it was demonstrated that the regiochemistry of cyclopropyl ring was critical for activity, and while *trans*-isomer **22** displayed activity in the low nanomolar range, the *cis*-isomer **25** showed only modest micromolar Smo binding affinity (IC_{50} >2.5 μM). Replacement of the *trans* 2-phenylcyclopropyl with a 2-phenethyl group as in **26** resulted in only micromolar activity. Other more radical changes such as replacement of the *trans* 2-phenylcyclopropyl group with benzyl, naphthyl, *meta*-biphenyl and butyl derivatives (**27–30**) were not tolerated. Similarly, spiro fused analogue **31** displayed only micromolar activity on the Hh-pathway. Two structural changes which were tolerated, although resulting in several fold loss in activity, were the replacement with 3-phenylcyclobutane (**32**) and indane (**33**). These two derivatives displayed Smo binding affinities of 440 and 880 nM respectively, compared to 94 nM for **22**.

Compounds **17** and **22** were selected for further characterization, both compounds were able to inhibit the growth of irradiated Ptch +/- medulloblastoma cells with CC_{50} = 3.3 and 12 nM respectively.[20] Profiling in liver microsomes revealed that whilst the dichlorophenyl urea **17** display medium turnover in rat microsomes (Cl_{int} = 180 μL/min/mgP), the more potent 2-biphenyl analogue **22** displayed inferior stability (Cl_{int} >300 μL/min/mgP).

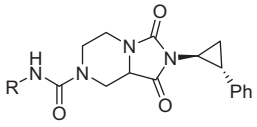
Table 3
SAR monophenyl series[a]

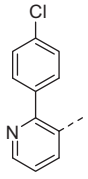
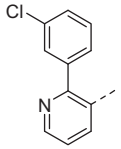
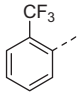
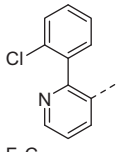
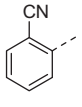
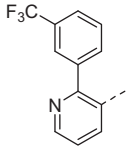


Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)	Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)
17		340	220	**39**		140	130
34		2200	1100	**40**		330	280
35		370	460	**41**		53	77
36		530	770	**42**		120	62
37		510	480	**43**		150	84
38		220	310				

[a] Values are means of at least two experiments with standard deviations <30% of the mean value.

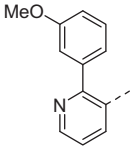
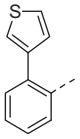
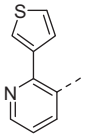
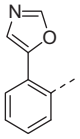
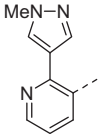
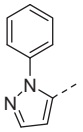
Table 4
SAR biphenyl series[a]



Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)	Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)
22		84	94	**52**		890	680
44		180	210	**53**		18	19
45		1100	890	**54**		210	140
46		2400	>2.5 μM	**55**		120	160

(*continued on next page*)

Table 4 (*continued*)

Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)	Compds	R	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)
47		2400	1700	**56**		82	47
48		210	220	**57**		240	88
49		2000	1800	**58**		1200	620
50		28	12	**59**		16	13
51		89	88				

[a] Values are means of at least two experiments with standard deviations <30% of the mean value.

Encouraged by these results a more extensive SAR study was conducted, aiming to further improve Smo activity and increase metabolic stability. We decided to optimize the activity in the racemic series, preparing mixtures of four isomers with achiral *trans* 2-phenylcyclopropyl, and racemic [6,5]-bicyclic hydantoin, relying on robust chemistry to quickly prepare analogues.

In the optimization work around **17** attempts were made to modulate potency by variation of the substituents on the phenyl group (Table 3). When one of the chlorine atom in the 2,3-disubstitution pattern is replaced with methyl ester (**34**) or methyl (**35**) groups a drop in potency was observed. Maintaining the dichloro substitution pattern attempts were made to modulate the compound properties introducing a further substituent on the phenyl ring. The introduction of a fluorine in the *para*-position of the 2,3-dichloro (**37**) and the 2,6-dichloro systems (**38**) resulted in a small drop in binding affinity compared to the corresponding des-fluorinated analogues. However, the addition of stronger electron withdrawing groups in the *para*-position of the 2,6-dichlorophenyl motif gave some interesting derivatives (**39–42**). In particular, the 4-methylsulfonyl (**41**) and 4-cyano (**42**) were very active compounds. The former was one of the most potent compounds displaying IC_{50} = 53 nM in the Light2 assay, and IC_{50} = 77 nM in the Smo binding assay. These two derivatives illustrate that it is possible to derive potent Smo antagonists without the need to resort to the bulky biphenyl urea.

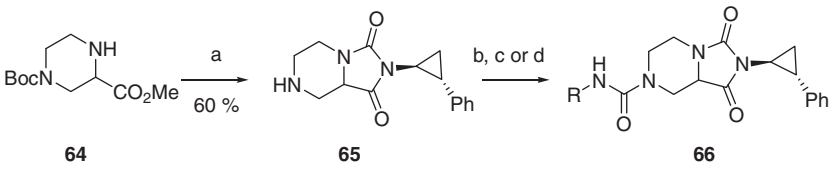
Meanwhile, the results for the optimization of 2-biphenyl urea **22** are reported in Table 4. Replacement of the *ortho*-phenyl ring with small substituents (**44**–**47**) was initially attempted, due to suspicions that this group could be the cause for the high intrinsic clearance, however, trifluoromethyl, cyano and methoxy groups were detrimental for affinity. The larger isopropyl substituent (**44**) retained some activity, Smo binding IC_{50} = 210 nM. Compounds where the phenyl had been replaced by five-membered heterocycles were also prepared, and while thiophene (**48**) maintained activity, the polar 1,3-oxazol-5-yl derivative (**49**) was poorly tolerated, resulting in a 20-fold loss of activity. In contrast, the introduction of heteroatoms in the internal phenyl ring was tolerated, and the pyridine analog **50** demonstrated very high activity in both assays (Smo binding IC_{50} = 12 nM, Light2 IC_{50} = 28 nM). Although less active, the regioisomer **51** presents comparable activity to **22**, displaying IC_{50} <100 nM. Given the superior activity of **50** additional substituents were introduced on the distill phenyl ring (**52–54**), and it was discovered that *meta*-substitution gave the most active compound (IC_{50} <20 nM). While the *ortho*- and *para*-substituents were poorly tolerated losing 10- and 30-fold activity respectively. Further *meta*-substitutions were examined, like trifluoromethyl (**55**) and methoxy (**56**), which both showed good activity, although less active than **53**. Replacement of the terminal aromatic ring with more polar heterocycles like in **58** was detrimental for the activity. Finally, the biphenyl fragment and 2-arylpyrid-3-yl motif was exchanged for the *N*-phenylpyrazole moiety in **59** giving one of the most potent compounds identified in this series, in fact **59** displays Smo binding IC_{50} = 13 nM.

Some exploratory SAR work into alternatives core scaffolds was also conducted (Table 5), which demonstrated the critical importance of the bicyclic hydantoin system. The corresponding phthalimide **60** resulted in a 34-fold loss in potency compared to **22**. Similarly, removal of one carbonyl groups of the hydantoin as in **61** was also detrimental for the activity, resulting in a 15-fold loss of activity. Manipulations of the hydantoin ring by ring expansion to 6-membered rings (**62** and **63**) resulted in an even more substantial loss in affinity.

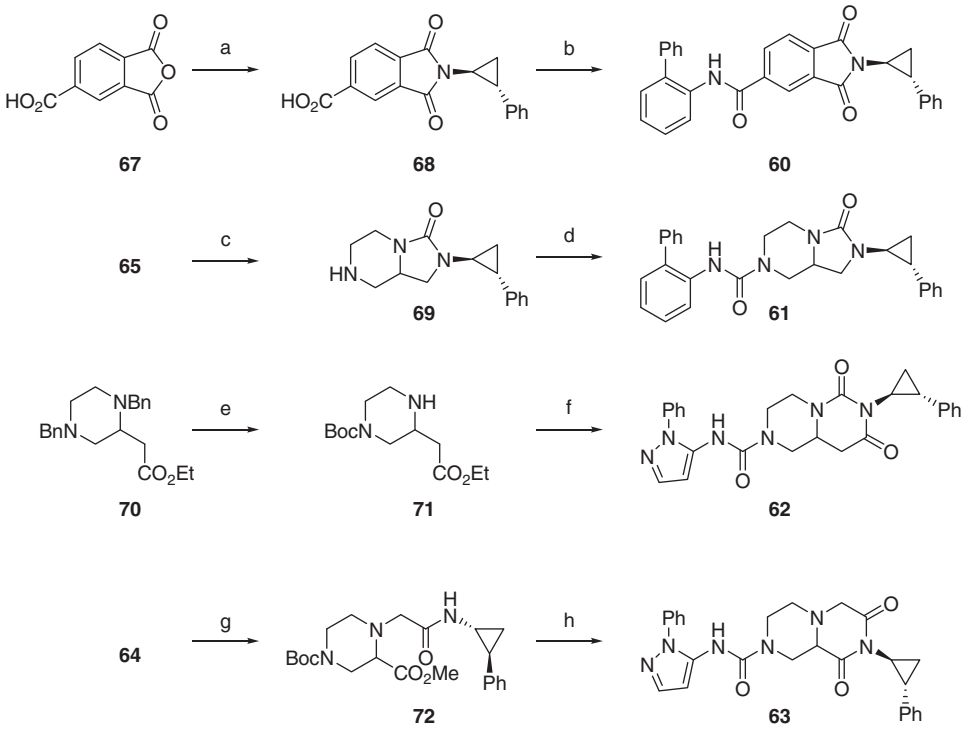
Table 5
Core structure modifications[a]

Compds	Light2 IC_{50} (nM)	Smo binding IC_{50}[a] (nM)
60	4600	3200
61	3000	1400
62	ND	7800
63	ND	5100

[a] Values are means of at least two experiments with standard deviations <30% of the mean value. ND = not determined.



Scheme 1. General hydantoin synthesis. Reagents and conditions: (a) (i) *trans* 2-Phenylcyclopropyl isocyanate, toluene, rt; (ii) DIPEA, reflux; (iii) TFA, DCM; (b) R-NCO, DIPEA, DCM; (c) RNH_2, CDI, TEA, MeCN; (d) RNHC(O)OPh, DIPEA, DCE.



Scheme 2. Core structure modifications. Reagents and conditions: (a) *trans* 2-Phenylcyclopropyl amine, DIPEA, μW (150 °C, 1 h); (b) Biphenyl-amine, HATU, DIPEA, DMF; (c) BH_3.THF, THF, Δ; (d) RNHC(O)OPh, DCM, TEA; (e) H_2, Pd/C, HCl, MeOH then K_2CO_3, BOC-ON; (f) (i) *trans* 2-Phenylcyclopropyl isocyanate, DCM; (ii) 4 N HCl in dioxane; (iii) RNHC(O)OPh, DCM, TEA; (g) (i) $BrCH_2CO_2Bn$, K_2CO_3, acetone; (ii) H_2, Pd/C, MeOH; (iii) *trans* 2-Phenylcyclopropylamine, HATU, DIPEA; (g) (i) K_2CO_3, toluene; (ii) TFA, DCM; (iii) RNHC(O)OPh, DCM, TEA.

The bicyclic hydantoins were synthesized according to Scheme 1, whereby the required isocyanate was reacted with known **64** at RT in toluene.[21] After initial urea formation, DIPEA was added and the reaction heated at reflux to afford the Boc-protected bicyclic hydantoin, which after deprotection gave **65**. The urea side chains were installed by reaction with isocyanates, prepared when necessary from the corresponding amine by reaction with triphosgene or via Curtius rearrangement from the carboxylic acid. Alternatively, **65** was reacted with either an intermediate formed *in situ* from the corresponding amine and CDI, or with a phenyl carbamate intermediate derived from the amine and phenyl chloroformate. The alternative ring systems were prepared according to established procedures (Scheme 2), either: by condensation with phthalic anhydride (**67**); from ethyl (1,4-dibenzylpiperazin-2-yl)acetate (**70**) using similar chemistry; or by alkyation of **64** with benzyl bromoacetate, followed by deprotection, and coupling to phenylcyclopropylamine and cyclization.

In summary, a novel series of [6,5]-bicyclic tetrahydroimidazo[1,5-*a*]pyrazine-1,3(2*H*,5*H*)-dione smoothened antagonists has been identified, with potential for the treatment of malignancies dependent on the Hh-pathway. This series of compounds has been extensively explored to ascertain the key detriments for activity, demonstrating that the *trans*-cyclopropyl phenyl and hydantoin ring systems are critical for potency, while a variety of urea substituents can be tolerated. The combination of these optimal groups gives smoothened antagonists with activity in the low nanomolar range. Further optimization of this series is the subject of the accompanying paper.[19]

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