Macrocyclic Hedgehog Pathway Inhibitors: Optimization of Cellular Activity and Mode of Action Studies

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S Supporting Information

ABSTRACT: Macrocyclic Hedgehog (Hh) pathway inhibitors have been discovered with improved potency and maximal inhibition relative to the previously reported macrocycle robotnikinin. Analogues were prepared using a modular and efficient build-couple-pair (BCP) approach, with a ring-closing metathesis step to form the macrocyclic ring. Varying the position of the macrocycle nitrogen and oxygen atoms provided inhibitors with improved activity in cellular assays; the most potent analogue was **29** (BRD-6851), with an IC_{50} of 0.4 μM against C3H10T1/2 cells undergoing Hh-induced activation, as measured by *Gli1* transcription and alkaline phosphatase induction. Studies with Patched knockout ($Ptch^{-/-}$) cells and competition studies with the Smoothened (Smo) agonists SAG and purmorphamine demonstrate that in contrast to robotnikinin, select analogues are Smo antagonists.

lipophilic aryl or alkyl substituent required
(*R*)-stereochem. required

improved potency
with inverted amino alcohols

IC_{50} = 0.4 uM

lipophilic side chain required

KEYWORDS: macrocycle, diversity-oriented synthesis (DOS), Sonic Hedgehog pathway, Smoothened antagonist, C3H10T1/2
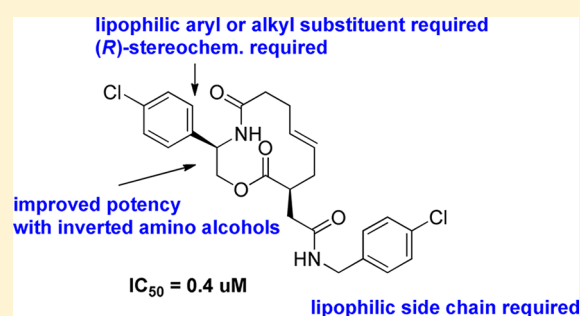
The Hedgehog (Hh) signaling pathway regulates cell growth and migration during embryonic development. It is normally dormant in adult cells, but elevated activity of this pathway is associated with cancers such as medulloblastoma and basal cell carcinoma.[1] In recent years, antagonism of different members of this pathway has been investigated as a novel mode of cancer chemotherapy, as described in several reviews.[2−6] Inhibition of Smoothened (Smo), a seven-pass transmembrane receptor with similar topology to G-protein-coupled receptors (GPCRs), shows promise for the treatment of cancers driven by activating mutations to the Hh pathway and also for a subset of epithelial cancers that may require Hh for growth via a paracrine mechanism.[7−10] Several Smo antagonists have proceeded to advanced clinical trials,[11−13] including GDC-0449 (vismodegib),[14] which was recently approved by the Food and Drug Administration (FDA) for treatment of advanced basal cell carcinoma.

In 2009, a macrocyclic compound derived from a diversity-oriented synthesis (DOS) library [robotnikinin (**1**)] was described that inhibits the Hh signaling pathway.[15,16] Hit compounds were identified by their interaction with Sonic Hedgehog (Shh) in a small-molecule microarray screen. We aimed to build upon this work by developing analogues with improved activity in cellular assays. We ultimately used an assay based on the differentiation of murine mesenchymal C3H10T1/2 cells, induced by the Shh protein.[17] The

differentiation of these cells results in the expression of alkaline phosphatase, which is readily quantified with fluorescent substrates; *Gli1* expression levels (mRNA) provide another readout of Hh pathway activation. Our primary objective was thus to find analogues with improved potency and maximal inhibition in these assays.[18]

The preparation of macrocyclic analogues of robotnikinin used a build/couple/pair[19] strategy related to previous reports[15,16,20] that affords rapid access to diverse analogues. Our general strategy is illustrated in Scheme 1, with only one of numerous accessible stereoisomers depicted. Amino alcohols and diamines were coupled with successive alkenoic acid building blocks, and the resulting dienes were paired in a ring-closing metathesis (RCM) step. Many compounds underwent further elaborations at the functional handles included with the carboxylic acid building blocks.

One focus of our medicinal chemistry studies was the determination of the optimal linker joining the alkenoic acids. To this end, a variety of amino alcohols and diamines were obtained or prepared, and these building blocks were incorporated into different macrocyclic products. A selection of these compounds is depicted in Chart 1, along with their

Scheme 1. General Strategy for Macrocycle Synthesis
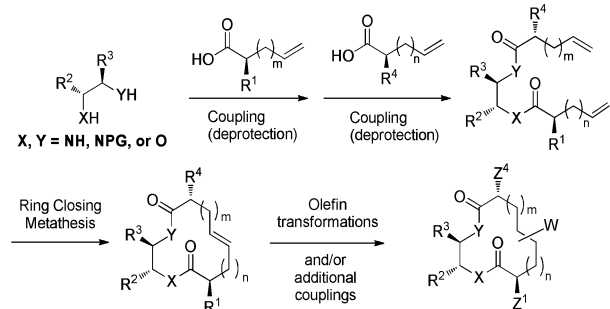
Chart 1. Analogues with Alternative Amino Alcohol Linkers

robotnikinin 1
IC$_{50}$ > 25 μM
max inhib. = 47%

2 IC$_{50}$ > 25 μM

3 IC$_{50}$ = 15 μM
max inhib. = 77%

4 IC$_{50}$ = 11 μM
max inhib. = 69%

5 IC$_{50}$ = 15 μM
max inhib. = 77%

6 IC$_{50}$ = 5 μM
max inhib. = 86%

7 IC$_{50}$ > 25 μM
max inhib. = 40%

Chart 2. Analogues Probing Key SARs

8 IC$_{50}$ = 8 μM
max inhib. = 78%

9 IC$_{50}$ = 20 μM
max inhib. = 95%

10 IC$_{50}$ = 8 μM
max inhib. = 78%

11 IC$_{50}$ = 6 μM
max inhib. = 106%

12 IC$_{50}$ > 25 μM
max inhib. = 41%

13 IC$_{50}$ = 4 μM
max inhib. = 97%

14 IC$_{50}$ = 11 μM
max inhib. = 59%

15 IC$_{50}$ > 25 μM

Chart 3. Analogues with Alternative Side Chains at the 6-Position

16 IC$_{50}$ > 25 μM

17 IC$_{50}$ > 25 μM

18 IC$_{50}$ > 25 μM

19 IC$_{50}$ = 4 μM
max inhib. = 28%

20 IC$_{50}$ = 7 μM
max inhib. = 60%

21 IC$_{50}$ = 8 μM
max inhib. = 104%

22 IC$_{50}$ = 5 μM
max inhib. = 50%

23 IC$_{50}$ > 25 μM
max inhib. = 47%

half-maximal inhibitory concentrations in the Shh-induced[21] C3H10T1/2 alkaline phosphatase assay, and their maximal activity relative to the prototypical Shh pathway inhibitor cyclopamine.

Cyclopamine produced an half-maximal inhibitory concentration (IC$_{50}$) of 0.6 μM and reduced the alkaline phosphatase activity to levels measured in the cells without Shh treatment. As previously reported, robotnikinin (**1**) proved to be only weakly active in this assay.[18] Removal of the 2-phenyl substituent from the macrocycle of **1** obviated all activity (compound **2**). Norephedrine-based compound **3** and norpseudoephedrine-based **4** had improved maximal activity over **1** and slightly improved potency in the C3H10T1/2 assay, as did the prolinol derivative **5**. A significant improvement was observed with compound **6** (IC$_{50}$ = 5 μM), where the positions of the macrocyclic oxygen and nitrogen are reversed. Indane **7** was also prepared, but its potency and maximal activity were poor.

We systematically explored several other structure–activity relationships (SARs) (Charts 2 and 3 and Table 1). Methylation of the macrocyclic nitrogen (compound **8**) gave a slight improvement versus **3**, but inversion of stereochemistry at the 2-position of **6** (compound **9**) decreased potency. Substitution at the 11-position was well-tolerated; methyl (**10**) and benzyl-substituted (**11**) analogues of **6** maintained potency with good to excellent maximal activity. The 11-isopropylamino-substituted analogue **12** was weakly active. Certain modifications of the olefin were also tolerated. For example, compound **13**, possessing a Z-olefin derived from the minor product of a metathesis reaction, was more potent than the analogous E-olefin **10**. A number of hydrogenated analogues showed activity comparable to the parent olefin series; for example, ephedrine derivative **14** showed moderate potency but low maximal activity. Noncyclic dienes such as **15** were inactive.
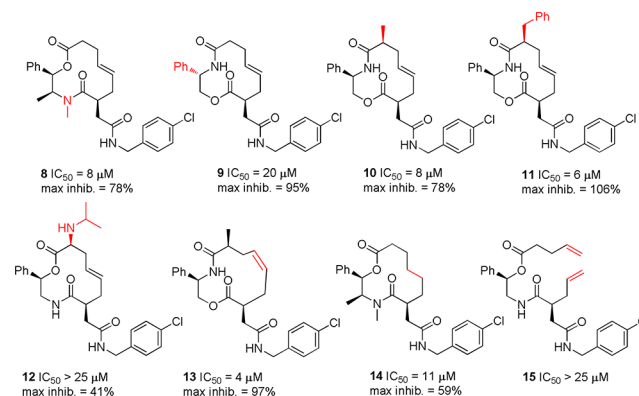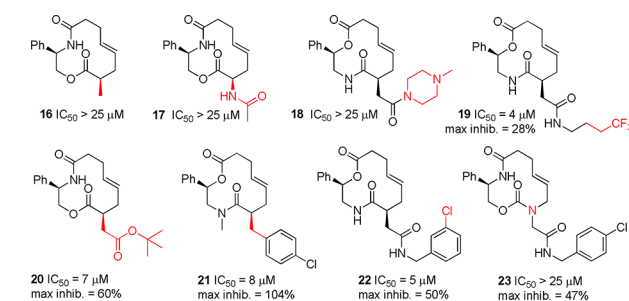
Table 1. Activity of 2-Substituted Macrocycles in Shh-Induced C3H10T1/2 Cell Differentiation

Compound	R	IC$_{50}$ (μM)	Max. inhib.[a]
6	phenyl	5[b]	86%[b]
24	cyclohexyl	5[b]	75%[b]
25 (BRD-0607)	Ph–CH$_2$	4[b]	90%[b]
26	n-butyl	>10[b]	53%[b]
27	isobutyl	23[b]	54%[b]
28	4-F-phenyl	5[b]	90%[b]
29 (BRD-6851)	4-Cl-phenyl	0.6,[b] 0.4[c]	105%,[b] 100%[c]
30	5-Cl-pyridin-2-yl	4.3[c]	85%[c]

[a]Relative to cyclopamine (100%). [b]Measured by alkaline phosphatase activity. [c]Measured by Gli1 levels. See the Supporting Information for details.
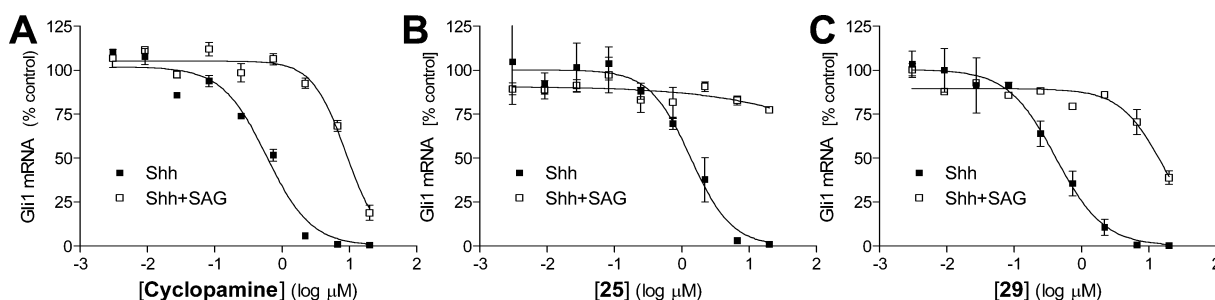
Figure 1. Use of Smo agonists and antagonists demonstrates Shh pathway specificity of macrocyclic inhibitors. Shh-induced activation of C3H10T1/2 cells is inhibited by cyclopamine (A), **25** (B), and **29** (C). Pathway inhibition is rescued by the Smo agonist SAG (20 nM). *Gli1* mRNA levels were measured by qPCR using *Actb* as an internal control.

We next modified the substituent at position 6 (Chart 3) of the various scaffold variants. Truncated analogues such as **16** and **17** were inactive in the cell assay or were only partial inhibitors. Amides such as **18** possessing solubilizing groups had poor activity, suggesting that a lipophilic chain is necessary at position 6. Compound **19**, possessing a trifluorobutyl group in place of the 4-chlorobenzylamide, showed a dose–response in this assay, but with poor maximal inhibition. Compounds **20** (IC$_{50}$ = 7 μM) and **21** (IC$_{50}$ = 8 μM) demonstrate that the amide moiety is not critical for activity. Interestingly, movement of the aromatic chloride of **1** from the para to the meta position (**22**) gave improved potency in this assay relative to **1** (IC$_{50}$ = 8 μM), although the moderate maximal inhibition was not improved and reached only 50%. The macrocyclic carbamate **23** was prepared to remove the chiral center at the 6-position and because it would be expected to have improved plasma stability. Unfortunately, it showed poor activity and decreased maximal inhibition relative to **6**.

Using **6** as a lead compound, we reexamined the SAR at the 2-position of the scaffold (Table 1). The potency was maintained when the arene was replaced with a cyclohexyl (**24**) or benzyl group (**25**); however, replacement with a *n*-butyl group gave weak, difficult-to-quantify activity (**26**), and the isopropyl analogue **27** had significantly weaker activity than **6**. Potency and maximal inhibition were unaffected by introduction of a fluorine to the para position of the arene of **6** (compound **28**). A log gain in activity [IC$_{50}$ = 0.6 μM (alkaline phosphatase readout); 0.4 μM (*Gli1* expression)] was observed with the introduction of a 4-chloro substituent (**29**), and this compound also attained the maximal inhibition of cyclopamine. The synthesis of **29** is depicted in the Supporting Information. Heteroarene **30** had lower activity, pointing to the importance of a hydrophobic aromatic ring at the 2-position of the scaffold.

To confirm specificity of the new macrocyclic inhibitors for the Shh pathway, a SAG rescue test was performed, in which inhibition of Shh-induced *Gli1* expression in C3H10T1/2 cells was measured in the presence of the Smo agonist SAG[22,23] for two of the most potent compounds, **25** and **29**. We used SAG at 20 nM concentration as it was the minimal concentration that produced a nearly maximal effect in this assay (see the Supporting Information). *Gli1* mRNA transcript levels were measured using real-time polymerase chain reaction (PCR) (Figure 1). Similar to cyclopamine (Figure 1A), both macrocycles showed a significant rightward shift of the dose–response curve in the presence of 20 nM SAG, resulting in 18- and 32-fold increases in the IC$_{50}$ values for **25** and **29**,

respectively (Figure 1B,C). These findings suggest that both compounds act at or above Smo in the Shh-signaling pathway.

To narrow the range of possible targets of **25** and **29**, we used constitutively active *Ptch*$^{-/-}$ mouse embryonic fibroblast (MEF) cells carrying Gli-responsive β-galactosidase (β-gal) reporter.[17] Because Patched functions upstream of Smo and acts as its repressor, inhibition of the Shh pathway at or upstream from Patched is prevented in this cell line. Measuring both β-gal activity and *Gli1* transcription levels, we found that both **25** [IC$_{50}$ = 8.8 μM (*Gli1* readout); 8.1 μM (β-gal readout)] and **29** [IC$_{50}$ = 3.5 μM (*Gli1* readout); 2.0 μM (β-gal readout)] behave similar to cyclopamine [IC$_{50}$ = 1.5 μM (*Gli1* readout); 1.9 μM (β-gal readout)] and fully inhibit Shh pathway activity. This indicates that in contrast to robotnikinin, part or all of their activities are derived from interactions downstream from Patched. As in the studies with the wild-type (*Ptch*$^{+/+}$) cell line, these activities are abrogated by the addition of 20 nM SAG. A representative dose–response curve for **25** is shown in Figure 2. Additional evidence of interaction with Smo was provided by BODIPY-cyclopamine competitive binding assays[15,23] (see the Supporting Information).
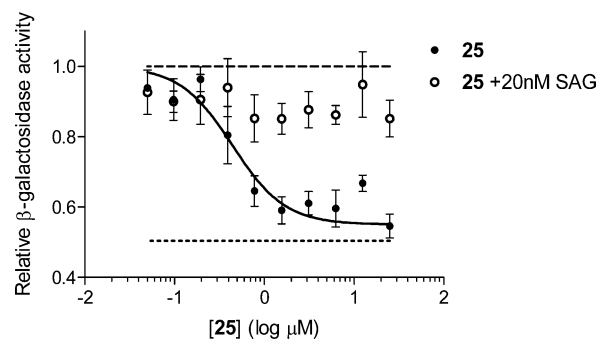

Figure 2. Dose–response curves of **25** in *Ptch*$^{-/-}$ MEF cells carrying β-gal reporter gene. The activity in this assay is evidence for action of the inhibitor downstream of Patched. Pathway inhibition is rescued by the Smo agonist SAG (20 nM). β-Gal activity levels in untreated cells (dashed line) and in cells treated with 100 nM KAAD-cyclopamine (complete inhibition, dotted line) are indicated.

To obtain further mechanistic insight, a Schild type analysis[24] was undertaken using double titration experiments with the Smo agonist SAG (Figure 3). We measured downstream *Gli1* expression induced by different concentrations of SAG in C3H10T1/2 cells in the presence of different concentrations of the inhibitors **1** (robotnikinin), **25** and **29**, as well as the Smo antagonists cyclopamine and GDC-0449 (vismodegib). Cyclopamine (Figure 3A) and GDC-0449 (Figure 3B) both induced
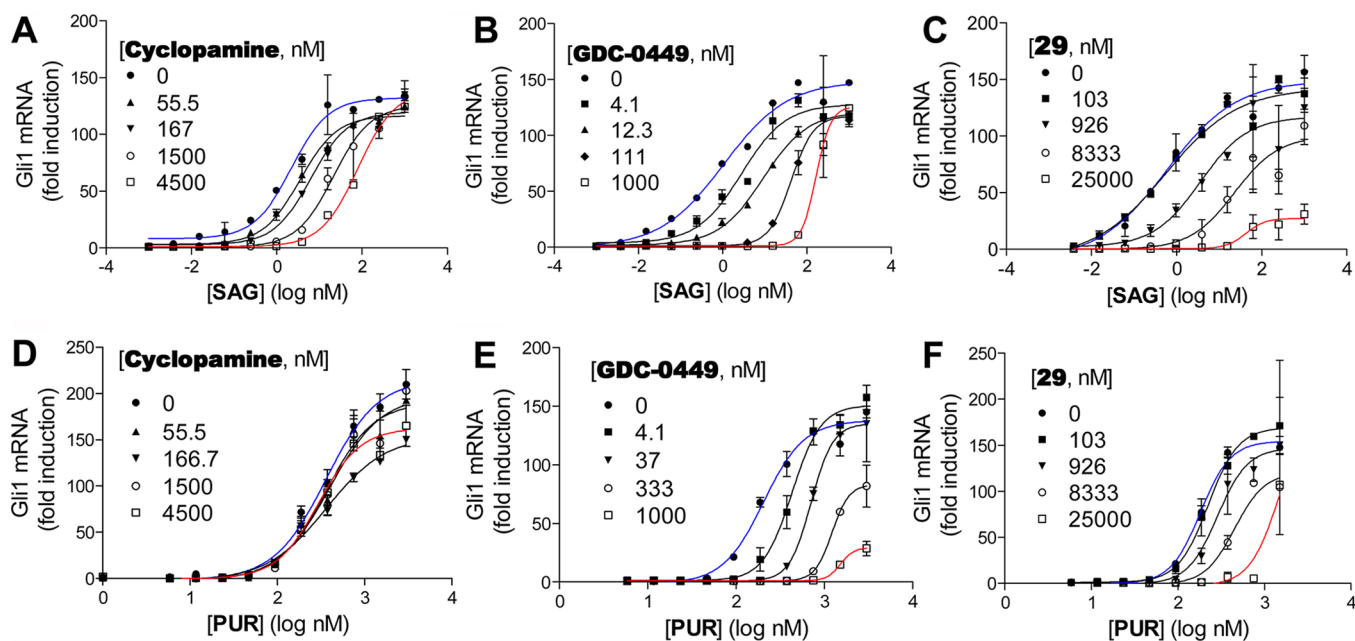
Figure 3. Double-titration experiment with the Smo agonists SAG and purmorphamine (PUR) in the presence of cyclopamine (A, D), GDC-0449 (vismodegib) (B, E), and **29** (C, F). *Gli1* mRNA levels were measured by qPCR using *Actb* as an internal control.

rightward shifts in the dose−response curves without affecting the maximal induction by SAG, consistent with their actions as competitive inhibitors of SAG at Smo. In contrast, robotnikinin (**1**) showed only marginal effects on the induction of *Gli1* transcription by SAG (see the Supporting Information). Interestingly, the related macrocycles **25** (see the Supporting Information) and **29** (Figure 3C) behaved differently by strongly decreasing maximal activity of SAG and only weakly (**25**) or moderately (**29**) increasing its EC_{50} value. This is consistent with a mechanism that involves allosteric inhibition of SAG at Smo.[25]

These double-titration studies were repeated with the alternative Smo agonist purmorphamine.[26,27] In contrast to its effects with SAG, cyclopamine had a very weak effect on purmorphamine-induced *Gli1* expression (Figure 3D), and GDC-0449 affected *Gli1* expression strongly but in a manner consistent with allosteric inhibition[25] (Figure 3E). Robotnikinin showed little effect on purmorphamine-induced Gli-expression (see the Supporting Information), consistent with its weak inhibition in the original C3H10T1/2 screen (Chart 1). Interestingly, the responses to increasing concentrations of **25** (see the Supporting Information) and in particular to **29** (Figure 3F) were more pronounced. This resulted in the reduction of purmorphamine EC_{50} without a strong effect on its maximal induction, thus supporting a competitive interaction between **25** and **29** and purmorphamine. It should be emphasized that although the Gli mRNA levels may be modulated by binding events at the Smo receptor, it is impossible to rule out additional interactions with downstream pathway components, so interpretations should be made cautiously.

The studies described in Figure 3 support a two-site binding model at Smo, whereby SAG and purmorphamine bind to unique sites on the receptor. Related competition studies with alternative Smo antagonists that also provide evidence for allosteric binding modes have been reported by Rominger[24] and Tao.[28] Such novel allosteric inhibitors of Smo could show important utility for the treatment of cancers with mutated

forms of Smo, such as the D473H mutation characterized after clinical treatment with GDC-0449 (vismodegib).[29]

In summary, several novel macrocyclic compounds are reported that appear to block the Shh pathway by inhibiting the membrane protein Smo. These compounds were assembled with a modular build/couple/pair synthetic strategy using different olefin-containing carboxylic acid and amino alcohol building blocks. SAR studies determined that a lipophilic side chain at the 6-position is required for activity. Several amino alcohol linkers provided decent activity; interestingly, improved potency was observed when the macrocycle nitrogen and oxygen of **1** were reversed. With this scaffold, maximal activity was observed with an aromatic moiety at the 2-position with (*R*) stereochemistry. The most potent compound was the 4-chlorophenyl analogue **29** (IC_{50} = 0.4 μM). Competition studies with the Smo agonists SAG and purmorphamine, as well as activity in a *Ptch*$^{-/-}$ cell line, suggest that **25** (BRD-0607) and **29** (BRD-6851), in contrast to robotnikinin, act predominantly as Smo antagonists. It is interesting to note that the subtle structural changes between robotnikinin (a direct binder of Shh) and compounds such as **29** (characterized here as a Smo antagonist) apparently lead to an additional mode of Shh pathway inhibition. These studies also provided evidence for a two-site binding model at Smo. Compound **29** is particularly interesting for its activity as an allosteric inhibitor of SAG but a competitive inhibitor of purmorphamine-induced *Gli1* expression. Its activity in cell lines carrying Smo mutations is presently under investigation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Assay protocols, structures of tool compounds, double-titration experimental data with **25**, BODIPY-cyclopamine competitive binding assay data, and protocols for the preparation of **25** and **29**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions
§These authors contributed equally to this work.

■ ABBREVIATIONS

β-gal, β-galactosidase; DOS, diversity-oriented synthesis; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FDA, Food and Drug Administration; GPCR, G-protein-coupled receptor; Hh, Hedgehog; IC_{50}, half-maximal inhibitory concentration; MEF, mouse embryonic fibroblasts; NPG, nitrogen with protecting group; PCR, polymerase chain reaction; RCM, ring-closing metathesis; SAR, structure–activity relationship; Shh, Sonic Hedgehog; Smo, Smoothened

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