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Design of Hedgehog pathway inhibitors for cancer treatment

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Abstract

Hedgehog (Hh) signaling is involved in the initiation and progression of various cancers and is essential for embryonic and postnatal development. This pathway remains in the quiescent state in adult tissues but gets activated upon inflammation and injuries. Inhibition of Hh signaling pathway using natural and synthetic compounds has provided an attractive approach for treating cancer and inflammatory diseases. While the majority of Hh pathway inhibitors target the transmembrane protein Smoothened (SMO), some small molecules that target the signaling cascade downstream of SMO are of particular interest. Substantial efforts are being made to develop new molecules targeting various components of the Hh signaling pathway. Here, we have discussed the discovery of small molecules as Hh inhibitors from the diverse chemical background. Also, some of the recently identified natural products have been included as a separate section. Extensive structure-activity relationship (SAR) of each chemical class is the focus of this review. Also, clinically advanced molecules are discussed from the last 5 to 7 years. Nanomedicine-based delivery approaches for Hh pathway inhibitors are also discussed concisely.

Keywords

cancer; cyclopamine; GDC-0449; Hedgehog inhibitor; nanomedicine; SMO; Smoothened; structure-activity relationship (SAR)

1 | INTRODUCTION

Hedgehog (Hh) signaling plays an important role in cell growth and differentiation. Activation of Hh pathway results in tumorigenesis, malignancy, and metastasis. This is one of the most widely studied signaling pathways and is a crucial effector of early embryonic development.¹ Sonic Hedgehog (Shh) is a morphogen, whose distribution in developing embryo determines the cell fate, and organogenesis. Hh signaling is significantly reduced in adults while retaining its functional role in stem cell preservation,^{2,3} and tumorigenesis of basal cell carcinoma (BCC),⁴ medulloblastoma (MB),⁵ pancreatic cancer,⁶ prostate cancer,⁷ and many more.⁸ The Hh pathway is also associated with cancer stem cell (CSC) biology

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CONFLICTS OF INTEREST

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and multi-drug resistance (MDR).^{9,10} Therefore, devising strategies to modulate the Hh pathway is of great interest for treating cancer.

The three-mammalian ligands of Hh gene named Shh, desert Hedgehog (Dhh), and Indian Hedgehog (Ihh) have been identified. After secretion, Hh ligands are converted to their active form by cleavage of the C-terminal domain and conjugation of a cholesterol moiety at the cleaved site. Subsequently, palmitoylation on the N-terminal occurs. Upon activation, Hh ligands bind to a 12-transmembrane protein called Patched1 (PTCH1). The function of PTCH1 is to keep a 7-transmembrane protein Smoothened (SMO) in an inactive form.¹¹ Shh binds to PTCH1 receptor and reduces its SMO inhibition activity, resulting in the activation and nuclear localization of glioma (Gli)-associated transcription factors. PTCH2 is another PTCH1 analog receptor, which is highly expressed and active in spermatocytes and mediate Dhh activity in germ cell development, thus has an entirely different biological role. Several intracellular components strictly control Hh pathway activation. In the cytoplasm, suppressor of fused (SuFu), a downstream kinase protein of SMO, negatively regulates the Hh pathway by two distinct mechanisms. SuFu can directly bind to the Gli transcription factor and prevent its nuclear accumulation. In another mechanism, SuFu forms a repressor complex that can interact with DNA-bound Gli1 and reduce Gli1 activity¹² (Figure 1). Gli is the only transcription factor known downstream of Hh.

In mammals, three Gli transcription factors, Gli1, Gli2, and Gli3 have been identified that are differentially expressed in several tissues. Gli1 is a full-length transcription activator, whereas Gli2 and Gli3 act as either a positive or a negative regulator based on protein kinases.^{13,14} Gli acts simultaneously in a context-dependent fashion while Gli1 only has transcriptional activator role, Gli2 and Gli3 have both activator and repressor activities.¹⁵ In the absence of Hh ligands, Gli1 is transcriptionally repressed, whereas Gli3 and possibly Gli2 are truncated or degraded resulting in pathway silencing. In the presence of Hh ligands, the Gli1 transcription is activated whereas the processing of Gli2 and Gli3 is inhibited, leading to their full-length form's accumulation and activation-repression of Hh-Gli target specific genes (Ptc1, Gli1).¹⁶ The targets of Glis can overlap or can be distinct from each other. Fibroblast growth factor (FGF) induces the expression of Gli2 and Gli3 and subsequently the Gli proteins induce the expression of effector pathways such as Wnt, insulin-like growth factor 2 (IGF2), and platelet-derived growth factor receptor- α (PDGFR α).¹⁷ Several nuclear factors can also modulate the activity of transcription factor Gli. A transcriptional coactivator cAMP-response element binding (CREB) protein can activate Gli3 protein. Phosphorylated Gli3 then binds directly to the Gli1 domain and induces transcription of Gli1 targeted genes. Another protein, dual-specificity tyrosine phosphorylation-regulated kinase-1 (Dyrk1) can potentiate Gli1-dependent gene transcription by enhancing its transcription activity and by promoting its nuclear accumulation.¹⁸ Furthermore, canonical activation of Gli1 by transforming growth factor- β (TGF β),¹⁹ mitogen-activated protein kinase (MAPK),²⁰ and phosphatidylinositol 3-kinase (PI3K)/Akt signaling²¹ is also known. Gli mediates transcription of genes involved in Hh pathway regulation (*Gli1*, *PTCH1*), proliferation (*MYC*, *Cyclin-D1*),^{22,23} apoptosis (*Bcl-2*),²⁴ angiogenesis (*ANG1/2*),²⁵ epithelial-to-mesenchymal transitions (*SNAIL*),²⁶ and stem cell self-renewal (*NANOG*, *SOX2*).²⁷

Activation of Hh pathway includes multiple steps providing an opportunity for regulating its activity. Basic understanding of Hh activation in specific cancer may help in selecting the appropriate Hh pathway inhibitor. Hh pathway mutation-driven tumors, such as BCC or MB, could be treated with single-agent Hh inhibitor acting downstream of the activating mutation, whereas cancer driven by autocrine or paracrine (prostate, gastric) activated Hh signaling, can be effectively treated with a combination of Hh antagonists and cytotoxic drugs. Large molecules can also inhibit the noncanonical Hh pathway, such as anti-Shh monoclonal antibody 5E1 (5E1). 5E1 was generated with mouse hybridoma technology and widely used for studying the effects of Hh signaling in developmental biology and cancer.²⁸ Despite the extensive use of 5E1 and RNAi in biological assays, their production costs, poor tissue penetration, and induction of immune response limits their widespread use as therapeutics.

The majority of natural and synthetic compounds developed as Shh pathway inhibitors including cyclopamine, vismodegib (GDC-0449), and sonidegib (LDE-225) target SMO receptor. Cyclopamine, a natural compound, has demonstrated high efficiency through SMO inhibition but failed in the clinics due to its poor drug-like properties.²⁹ Acyltransferase inhibitors (such as RU-SKIs) decrease the maturation of the Shh molecule and affect Hh activation. However, mutations in SMO and *SuFu* have been identified, and cancers originated from direct activation of Hh pathway independent of PTCH1/SMO or with mutated SMO, remained unaffected by these small molecules.³⁰ Therefore, inhibitors acting downstream of these proteins have attracted significant attention. Gli1 activity can be suppressed directly by small molecules such as GANT58, GANT61,³¹ or indirectly by natural products such as zerumbone, arcyriaflavin C, and physalin H.³² GANT61 is known to modulate the DNA-binding activity of Gli1, whereas arcyriaflavin C and physalin F antagonize Gli functions indirectly through PKC/MAPK pathway blockade. Besides these, another natural product forskolin can nonselectively inhibit Hh signaling by activating adenylate cyclase and consequently protein kinase A (PKA).^{33–35}

The major challenge for currently used Hh pathway targeting drugs include the development of resistance toward SMO inhibitors, unfavorable solubility, and poor pharmacokinetic (PK) profile, need of combination with standard chemotherapy, therapy-induced side effects and lack of predictive values of Hh ligands, SMO, or PTCH1 messenger RNA (mRNA) in the disease stage. Nanoformulation approach has been extensively applied by various research groups to overcome the majority of these issues. Nanomedicine can offer the advantage of improving the PK and safety profiles and the possibility of combination with other drugs. Examples of formulations with Hh inhibitors have also been discussed in this review.

Hh signaling pathway inhibitors have been reviewed from time to time by many research groups highlighting their mode of actions and stages of clinical development.^{36–46} Understanding the medicinal chemistry of various small molecules Hh pathway inhibitors can accelerate the development of new and potent drug candidates. In this review, we have discussed different chemical classes of small molecules (including clinically used drugs) capable of inhibiting the Hh pathway at various molecular targets. The focus of this review is to critically discuss the structure-activity relationship (SAR) for each class of compounds.

Also, the effects of structural modifications on PK and pharmacodynamics (PD) of some selected compounds have also been discussed briefly.

We have carefully selected the vital literature reports from the last 5 to 7 years resulting in the identification and evolution of small molecules inhibiting the Hh pathway at various stages including upstream and downstream targets of Hh signaling pathway. Delivery of small molecules to the specific site proved challenging. However, the use of polymeric carriers for their delivery and targeting is promising. In this perspective, we have discussed the reports dealing with the delivery of Hh pathway inhibitors to specific organs. We have also discussed the strategies for further improving their therapeutic effects and efficient delivery to the tumors. For better understanding, we have divided this section into subsections based on the chemical structures of the compounds (Figure 2).

2 | RECENT ADVANCES IN DESIGN OF HH PATHWAY INHIBITORS

Substantial development has been witnessed in the last few years for the development of various classes of Hh inhibitors including small molecules as well as natural products. We have grouped small molecules based on their chemical structures, and discussed the detailed SAR of each chemical class in the following subheadings.

2.1 | Thiophenes

Thiophenes (thiofurans) are sulfur-containing heterocyclic compounds and are widely used as building blocks in pharmaceuticals, biomaterials, and agrochemicals. Thiophenes are observed as a bioisostere of the benzene ring and used extensively in drug discovery. This section highlights some selected reports where thiophene analogs have been identified as inhibitors of Hh pathway.

Eggmanone **3a** (EGM1) (Figure 3A) was identified as Hh signaling inhibitor functioning by selective antagonism of phosphodiesterase (PDE4), which plays a key regulatory role in Hh signaling downstream of SMO. However, EGM1 has low aqueous solubility and modest potency. Thus, Williams et al⁴⁷ investigated new modifications in EGM1 by synthesizing a series of thiophenes **3b** (Figure 3A) of EGM1. First, replacement of the methyl allyl group (region-A) in EGM1 with small alkyl and cycloalkyl substituents were well tolerated and improved the lipophilicity. However, the phenyl analog was sterically disfavored. Substitution in the side chain at R² (region-B) with (hetero) aromatic or cycloalkyl amides in EGM1 were not favored and decreased potency. Only 2-methylpiperidine or a phenyl ring as a substituent were acceptable functionalities. Further, bioisosteric replacement of thiophene with a phenyl ring yielded a moderately active derivative. Analogs incorporating a polar atom (Y = O, S, NH) into the cyclohexyl ring (region-C) decreased the activity. Replacement of the cyclohexyl thiophene core with benzothiophene or benzo pyrimidinone led to a complete loss of Hh inhibitory activity, due to disfavored conformational and aromatic effects compared with EGM1. Thienopyrimidine **3c** (Figure 3A), bearing cyclopropyl ring at R¹ and phenyl at R², yielded the first analogs with better potency over EGM1 (EC₅₀ = 0.082 ± 0.01 μM) along with a satisfactory increase in lipophilicity efficiency (LipE) (3.84). Further optimization of the attached linker by reducing the ketone to secondary or tertiary alcohol led to moderately low activity. Interestingly, the thioether linker was essential for

biological activity as analogs bearing ether and secondary amine were completely inactive. Mechanistic study of the active compounds revealed that noncyclohexyl thiophene analogs inhibited the Hh signaling via a PDE4-independent mechanism that functions in between SMO and Sufu, whereas cyclohexyl thiophene analogs inhibited Hh signaling via a PDE4-dependent mechanism downstream of Sufu (Figure 3A).⁴⁸

Petrova et al⁴⁹ screened a large molecular library using a peptide-based assay to monitor inhibitory action on Hh acyltransferase (Hhat)–mediated Shh palmitoylation. Four compounds, RU-SKI 39 **3d**, RU-SKI 41 **3e**, RU-SKI 43 **3f**, and RU-SKI 50 **3g** (Figure 3A), were identified in this study as potent inhibitors. These small molecules inhibit the activity of Hhat in vitro and blocks Hhat-mediated Shh palmitoylation in cells. RU-SKI 43 was specific for the Hh pathway in cells and decreased Gli1 activation in an Shh-driven cell-based reporter system. RU-SKI 43 had no effect on the Shh signaling in *Sufu*^{−/−} cells, and did not affect the ability of SMO agonist (SAG) or Shh (C24II) to activate Gli1 in Shh-Light 2 cells. These findings revealed that Hhat inhibition is a new way of inhibiting the Hh signaling (Figure 3A). In the 5-acyl-6,7-dihydrothieno[3,2-*c*] pyridine core of RU-SKI, the amide linker adopted two conformations as *E*-**3h** and *Z*-**3i**. The conformational preference was affected by noncovalent interactions between the amide carbonyl and adjoining substituents. The potent compound RU-SKI 41 adopted *E/Z* conformation in the ratio of 1:1 (Figure 3A).^{50,51}

Rodgers et al⁵² have observed that RU-SKI 43 possessed cytotoxicity unrelated to canonical Shh signaling. RU-SKI 43 exhibited nonspecific off-target cytotoxicity, hiding its effect on Hhat-dependent signaling. Interestingly, another potent compound from this series, RU-SKI-201 **3j** (Figure 3A) showed targeted cytotoxicity and induced Hhat-dependent and Hh-dependent inhibition in tumor cell lines. This compound selectively inhibited the Hh palmitoylation over a panel of >100 palmitoylated substrates in cells. Thus, RU-SKI-201 was a superior small-molecule as an inhibitor of Hhat catalytic function. However, none of the compounds from RU-SKI series is under clinical trials till date.⁵³ More dedicated efforts are required to further modify the PK and PD properties of thiophene compounds.

Inspired from the interesting Hh signaling antagonist potency of pyrimidine-indole hybrid (PIH) structure,⁵⁴ Khatra et al⁵⁵ have designed a small compound library of PIH series **3k** (Figure 3B) and identified their distinct modes of action as Hh inhibitors. All the new compounds were screened in Shh-Light 2 cells at 1.0 mM concentration. These compounds inhibited Hh signaling pathway by blocking Gli-dependent signaling by depolymerizing microtubules and abrogating ciliogenesis at nanomolar concentration. Compounds in this series were mechanistically distinct from nocodazole (tubulin depolymerizer), known ciliogenesis antagonists, CA1 and CA2, ciliobrevin (cytoplasmic dynein antagonist), and JK-184 (microtubule inhibitor) as PIH-H **3i** (Figure 3B) did not perturb γ -tubulin localization and did not induce nuclear fragmentation or abnormal cilia morphologies. In silico studies suggested high-binding affinity of these compounds for the colchicine-binding site of tubulin. SAR study of this series revealed that analogues bearing a phenyl ring or a hydrogen atom in region-A resulted in a small loss in activity indicating tolerance of bulky groups at this position. Similarly, substitution with methyl group on both nitrogen (*R*¹) and region-A was also allowed, however, with slight potency loss. However, PIH without

hydrazine (region-B) was inactive indicating the important role of hydrazine linker for potency. Further, PIH-H **3i** showed significant downregulation of the polymerized form of tubulin compared with other derivatives.

2.2 | Pyridines

Pyridines are six-membered heterocyclic compounds containing a single nitrogen atom and found widely in many naturally occurring bioactive compounds, pharmaceuticals, and chiral ligands. As Hh pathway inhibitors, pyridine represents an important chemical class providing some Food and Drug Administration (FDA) approved drug candidates such as GDC-0449.

Robarge et al⁵⁶ have reported an optimization study of pyridine containing benzimidazole **4a** (Figure 4A), which is an outcome of hit-to-lead discovery program, possessing attractive efficacy toward inhibiting the Hh pathway in Luciferase activity assay. Benzimidazole **4a** was optimized to increase the metabolic stability and aqueous solubility at physiological pH. In this optimization, the two-point modification in compound **4a** was selected. First, the benzimidazole part (region-A) was optimized by replacing with a variety of heterocyclic as well as opened ring amide systems. The best replacement for benzimidazole was found to be a 2-pyridyl functional group. This optimization study revealed that the basic nitrogen adjacent to the ring fusion point was necessary for potency as it helped to prevent tautomerization in the molecule. Second, the amide side chain (region-B) of biaryl **4b** (Figure 4A) was optimized by exploring a variety of heterocyclic and aromatic rings. Among these, 2-chloro-4-methyl sulfone phenyl was best in activity with improved clog P. The resulting compound **4c** (GDC-0449) (Figure 4A) exhibited enhanced potency (mouse S12 = 13.0 nM, human HEPM = 3.0 nM), solubility (3.5 mg/mL at pH 1), and metabolic stability (microsomal clearance in human = 4.56 ml/min/kg) relative to compound **4a** or **4b**. This compound was found potent (2.8 nM) in human Gli-luciferase assay using in human embryonic palatal mesenchyme (HEPM) cells. This study provided a new Hh antagonist GDC-0449 (Figure 4A).

Following the discovery of GDC-0449, Castanedo et al⁵⁷ optimized the core structure of GDC-0449 and one of its potent analogs **4d** (Figure 4B). The 4-position of the carboxamide aryl ring (region-A) was modified, and the 2-chloro group in the structure was kept intact as it was critical for the desired solubility. A variety of polar functional groups such as extended sulfone and sulfonamides were tolerated in the 4-position, while inclusion of simple amides with methyl and ethyl groups decreased potency relative to GDC-0449. From this series, compound **4e** (Figure 4B) was the most potent compound with acceptable solubility (4.3 mg/mL at pH 1.0), improved Hh inhibition (mouse S12 = 4.0 nM, human HEPM = 0.4 nM), and improved cytochrome (CYP) inhibition (CYP 3A4/2Cp 20/4.5 μ M). SAR study of the second lead compound, 2-pyridyl biphenyl amide **4d**, revealed that the nicotinic amide (region-B) accommodates substitutions at the 6-position including amines as well as *N*-linked heterocycles. Most of the new compounds were more potent than the compound **4d** in the reporter gene assay derived from HEPM cells. In this series, the acyl-piperazine containing analog **4f** was 2-fold more potent in Gli1 suppression relative to

compound **4d**. Both compounds (**4e** and **4f**) were potential backups for GDC-0449 (Figure 4B).

Yang et al⁵⁸ screened a library of over 40 000 compounds and selected a bisamide compound **4g** (Figure 4C) as a potential lead compound using the firefly reporter assay against the Hh pathway. However, this compound had low aqueous solubility and strong protein binding in plasma, which reduced its bioavailability significantly. In addition, the bisamide compounds are well-known for their potent p38 MAP kinase inhibition (p38 α IC₅₀ = 25 nM for compound **4g**). Therefore, bisamide **4g** was optimized to improve its potency for Hh inhibition and to reduce its selectivity for p38-type kinase. The 2-pyridyl (region-A) was optimal for the Hh pathway inhibition, as other heteroaryl or alkyl groups gave reduced potency. The inclusion of solubilizing groups (eg, basic amine) on region-B did not attenuate their activity on p38 kinase and resulted in reduced potency. Modification of C-5 substitution in compound **4g** was envisioned to prevent hydrogen bonding between the C-5 amide of bisamide compounds and p38 α protein residues Glu71 and Asp168. Most heterocyclic analogs tried at the C-5 position either retained or improved the potency as the Hh pathway inhibitor. The unsubstituted 2-imidazole or 5-imidazole containing analogs were potent inhibitors of Hh pathway with diminished activity against p38 kinase. The monosubstitution on imidazole analogs significantly reduced the p38 α inhibition, whereas the disubstituted imidazole analogs were completely inactive for p38 α inhibition. Other heterocycles such as pyrazole, thiazole, morpholine, pyridine, amino-pyridazine, and purine also gave potent derivatives but at the expense of undesirable physicochemical properties. The most active analog **4h** bearing unsubstituted 2-imidazole was inactive for p38 tested in a whole blood cell assay. This compound has excellent oral bioavailability (F = 83%) with low clearance (Cl = 8.3 ml/min/kg) resulting in higher overall exposure (PO, AUC = 12,535 μ g h/L) in mouse (Figure 4C).

In 2015, FDA approved a pyridine derivative Sonidegib **4i** (Figure 4C) to treat patients with advanced BCC followed by surgery or radiation therapy and for patients when surgery or radiation therapy is not possible. In clinical Phase I study, Sonidegib has confirmed its tolerable safety profile and antitumor activity in various malignancies, including MB, lung cancer BCC, and advanced solid tumor.⁵³

In clinical trials on MB patients, GDC-0449 was found ineffective due to a point mutation in SMO protein resulting in patient's insensitive to the treatment. Further, abnormal expression of zinc finger transcription factor Gli, PI3K signaling pathway along with low solubility of GDC-0449 limit its use. Prompted from these limitations, Kumar et al⁵⁹ have reported new analogs, MBD series **4j** (Figure 4D) of GDC-0449. SAR analysis of this small series of compounds revealed that 2-pyridyl biphenyl (region-A) was essential for the potency as its replacement with 2-pyridylmethyl provided an inactive compound. The central phenyl ring (region-B) seemed to be important for activity as replacement with electron-deficient pyridine ring reduced the potency significantly. Further, substitution with halogen, particularly chlorine, at the 3-position of the phenyl ring improved the potency. Interestingly, bulky groups on amide nitrogen (region-C) played an important role in the activity as well as in regulating the hydrophobicity of the compounds. Thus, 2-pyridyl biphenyl (region-A) and 2-pyridylmethyl (region-C) were optimal for potency. Further, the addition of two 2-

pyridylmethyl improved the potency and gave the most potent compound MBD5 **4k** (Figure 4D) of the series. However, insertion of additional phenyl ring between the amide nitrogen and the 2-pyridyl ring led to significant reduction in potency (Figure 4D).

Poor aqueous solubility of GDC-0449 is attributed to its planar molecular structure and presence of all the carbons in sp²-hybridization state, except terminal methyl group. Thus, efforts were also made to disturb the planarity of the molecule by introduction of sp³ hybridized carbons utilizing the scaffold hopping strategy. Applying this strategy, Lu et al⁶⁰ have developed a series of compounds using 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine-based scaffold **4l** (Figure 4E) exhibited decent inhibition of Hh pathway signaling in NIH3T3-GRE-Luc reporter gene assay in the presence of 10 nM SAG as the Hh pathway agonist. SAR study around the terminal position of this scaffold (region-A) using various substitutes such as substituted esters, amides, reverse amides, and carboxamides were explored. Among these, esters bearing small substitutes such as ethyl, isopropyl, or cyclopropyl resulted descent potency. Similarly, simple amides bearing small substitutes were moderately active whereas, secondary amine-based amides such as with pyrrolidine, provided the potent compounds. The carboxamides derived from anilines provided potent derivatives. Among them, 3-F-aniline bearing carboxamide **4m** (Figure 4E) being best in the series. Compounds bearing reversed amides were also moderately active.

Extending this approach, Ma et al⁶¹ have developed tetrahydrothiazolo[5,4-*c*]pyridine-based templet **4n** (Figure 4E) and screened new compounds for Hh signaling pathway inhibition using a cell-based NIH3T3-GRE-Luc reporter gene assay (with 10 nM SAG as the Hh pathway agonist). SAR study of this new series of compounds around the tetrahydrothiazolo[5,4-*c*]pyridine revealed that various functional groups such as amine, amide, ether, carbamate, urea, and aniline bearing a variety of substitutions were well tolerated and provided potent compounds compared to vismodegib. Among them, analogue bearing propionamide **4o** (Figure 4E) and 4-hydroxy-piperidine **4p** (Figure 4E) were found most potent. Activity of these compounds was further confirmed by boron-dipyrromethene (BODIPY)-cyclopamine displacement assay in the U2OS cells overexpressing human SMO. The improved potency of these compounds over 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine was believed to be due to the larger size of the thiazole system resulted in favorable orientation of the substitutions to interact with SMO. Compound **4o** showed quick absorption when given orally (10 mg/kg, PO) and showed $T_{\max}$ of 0.25 hours, clearance (16.6 mL/min/kg), good steady-state volume ($V_{d,ss}$) of 1.7 L/kg, and half-life ($t_{1/2}$) of 3.3 hours with overall bioavailability ($F\%$) of 34% ($AUC_{0-24\text{ hours}} = 3455\text{ ng h/mL}$), whereas compound **4p** was absorbed slowly when given orally (10 mg/kg, PO) with a $T_{\max}$ of 2.0 hours and had slow clearance (9.3 mL/min/kg), a good steady-state volume ($V_{d,ss}$) of 1.5 L/kg, half-life ($t_{1/2}$) of 3.3 hours, and overall bioavailability ($F\%$) of 77% ($AUC_{0-24\text{ hours}} = 13,131\text{ ng h/mL}$).

Lu et al⁶² further optimized this scaffold where tetrahydrothiazolo[5,4-*c*]pyridine was replaced with tetrahydropyrido[4,3-*d*]pyrimidine by applying computation approach to design a novel series **4q** (Figure 4E) as SMO antagonist. SAR analysis around the region-A revealed that small alkyl groups on the terminal nitrogen were well tolerated. Secondary amines, with cyclic or acyclic structure were also well tolerated, where the 4-

hydroxypiperidine as substituent in this region being the best. Further attempts to improve the potency by incorporating substituents as ethers, sulfonamide, or aromatic groups on to the terminal nitrogen did not improve the activity. The SAR around region-B revealed that the biaryl ring system was needed in the tilted position for good interaction with SMO receptor as modifications in this region by removing ortho-methyl group from the terminal pyridine (R^2) resulted in significant potency loss. Other modifications (at the terminal pyridine, R^2) such as inclusion of strong electron-withdrawing group at *para*-position, introduction of additional nitrogen in the ring, or bioisosteric replacement of the pyridine ring (R^2) did not improve the potency. Further modifications in this region by replacing pyridine ring with a phenyl significantly enhanced the inhibitory potency. From this SAR study, compounds **4r** and **4s** (Figure 4E) were identified as potent leads and their selective SMO antagonistic activity was confirmed by Wnt pathway reporter gene assay and BODIPY-cyclopamine displacement assay in the U2OS cells overexpressing human SMO. Both compounds (**4r** and **4s**) moderately inhibited the CYP 3A4, 1A2, 2C9, and 2C19 at 10 μ M concentration. Compound **4s** has a lower melting point and high solubility compared with vismodegib, showed low inhibition for hERG potassium channel (IC_{50} = 38 μ M) and was highly permeable to Caco-2 cells. When compound **4s** was given to Sprague-Dawley (SD) rats at the dose of 10 mg/kg, PO, it exhibited T_{max} of 0.5 hours, C_{max} of 2180 ng/mL and clearance (Cl) of 18.6 mL/(min kg). It displayed steady-state volume ($V_{d,ss}$) of 1.7 L/kg, $t_{1/2}$ of 2.3 hours, AUC of 5541 ng h/mL and oral bioavailability ($F\%$) of 62%. Compound **4s** crossed the BBB with an exposure of 831 ng/g within 1 hour after oral dosing of 10 mg/kg, resulting in a brain/plasma ratio of 0.4. The biodistribution of compound **4s** after oral administration in ICR mouse at 10 mg/kg, and its PK parameters were calculated as the following: T_{max} of 0.25 hours, C_{max} of 4132 ng./mL, moderate clearance (Cl) of 20 mL/(min kg), $V_{d,ss}$ of 1.0 L/kg, $t_{1/2}$ of 1.0 hours and AUC of 4183 ng h/mL with bioavailability ($F\%$) of 49%. Further, PK profiles of compound **4s** in beagle dogs at oral dose of 5 mg/kg were the following: T_{max} = 1 hour, C_{max} = 1300 ng/mL, Cl = 8.1 mL/(min kg), $V_{d,ss}$ of 1.8 L/kg, $t_{1/2}$ = 5.4 hours, and AUC of 7656 ng. h/mL, resulting in a bioavailability ($F\%$) of 72%. In a dose-escalating toxicity study in mouse (100, 250, and 500 mg/kg, daily oral gavage), compound **4s** was found well tolerated at all doses with slight increase in liver weight at higher dose. Antitumor efficiency of compound **4s** was assessed by a subcutaneous xenograft model of Ptch1[±] mouse MB at a dose of 50 mg/kg. Significant inhibition in tumor growth (up to 94%) was observed without any behavioral and body weight changes of mice. Compound **4s** resulted in 94% inhibition of Gli1 mRNA expression whereas vismodegib inhibited 86%. Further, antitumor efficiency of compound **4s** was accessed in MB of Math1-cre: SMOM2 mice. Treatment with compound **4s** for 2 weeks significantly reduced the tumor volume in cerebellum and improved the physical condition of the mouse.

Chen et al⁶³ further examined the Hh inhibition and apoptosis induction potential of compound **4r** (Hh003) (Figure 4E). Such dual-acting compounds have high therapeutic significance for cancer treatment. Compound **4r** activated specifically the caspase 8-dependent extrinsic apoptosis pathway besides inhibiting the Hh signaling pathway. Compound **4r** significantly inhibited the tumor colony formation in HCT116 and Panc-1 cells compared to vismodegib. Further, in nude mouse xenograft model of colorectal cancer,

compound **4r** significantly inhibited the tumor growth, whereas vismodegib had no effect on tumor growth at the dose of 60 mg/kg. Compound **4r** acts as a potent Hh inhibitor and an effective apoptosis inducer with great potential for future anticancer therapy.

In an effort to improve the metabolic stability of vismodegib, Wang et al⁶⁴ synthesized deuterated analogues of vismodegib and determined its biodistribution after oral administration in mice. This approach was based on the fact C-D bonds cleaved at a slower rate compared with C-H bonds, making the deuterium-labeled drugs more stable. The new vismodegib analogues were deuterated at the metabolically active sites like pyridine or the middle benzene ring to slow down the rate of metabolism. Among the new deuterated derivatives, compound **4t** SKLB-C2211 (Figure 4E) was found superior to vismodegib when administered orally in female CD-1 mouse of 5 mg/kg. Compound **4t** SKLB-C2211 exhibited improved PK profiles compared with vismodegib: $AUC_{0 \rightarrow \infty}$ (17 147.31 vs 7657.68 $\mu\text{g/L h}$), $t_{1/2}$ (8.55 vs 5.66 hours), and C_{max} (1870 vs 854 ng/mL). These results proved that SKLB-C2211 is a long-acting inhibitor of Hh signaling pathway compared to its prototype Vismodegib.

Recently, Ma et al⁶⁵ developed a potent molecule **4u** (Figure 4E) that inhibited the Wnt and Hh pathway at subnanomolar concentration. The Wnt and Hh signaling pathways interact and coordinate during embryonic development and are implicated in tumorigenesis and metastasis. Thus, small molecules targeting both pathways simultaneously is a novel approach, which was realized by synthesizing a small library of compounds. New compounds were designed by replacing one of biphenyl system (region-A) of LGK974 **4v** (Figure 4E) with a tricyclic system. Importantly, NH in the tricyclic ring system was essential for Hh inhibitory activity as its bioisosteric replacement with oxygen or methylene group resulted in complete activity loss. However, these compounds exhibited potent Wnt inhibition. Further, optimization of region-B indicated the essential role of pyridine nitrogen for potent Wnt pathway inhibition, whereas the methyl group next to the pyridine nitrogen seemed essential to mitigate liver CYP450 inhibition. For region-C, replacement of fluorine atom with other small electron-withdrawing or electron-donating groups, such as cyano or methoxy was well tolerated for Wnt pathway inhibition, however, Hh inhibition was significantly reduced. A single oral administration of compound **4u** in mice at the dose of 10 mg/kg resulted in quick absorption with T_{max} of 0.5 hours, C_{max} of 5136 ng/mL, Cl of 6.1 mL/min/kg, $V_{\text{d, ss}}$ of 0.4 L/kg, $t_{1/2}$ of 2.1 hour, AUC of 13 534 ng. h/mL and bioavailability ($F\%$) of 49%.

Recently, Li et al⁶⁶ reported rational computer-based drug design approach to synthesize a series of unsymmetrical diaryl ureas **4w** and **4x** (Figure 4E) as dual PI3K/Akt/mTOR and Hh signaling inhibitors by combining the structural features of buparlisib and vismodegib. The SAR study around this series revealed that addition of a morpholine or a pyridine-3-yl group as R^2 resulted in potent cytotoxicity in MCF-7, MDA-MB-231, and PC-3 cells. Further, presence of a morpholine group or a pyridine-3-yl (as R^2) along with a pyridine-2-yl moiety at R^1 seemed essential for inhibition of mTOR signaling. Among all compounds, compound **4y** (Figure 4E) exhibited the most antiproliferative activity against MDA-MB-231, T74D, and MCF-7 cells and exhibited potent enzymatic activity against mTOR signaling.

Compound **4y** also suppressed the expression of p-S6K, p-Akt, and SAG elevated Gli1, induce tumor apoptosis and inhibited the migration of TNBC MDA-MB-231 cells.

Pyridine derivatives discussed in this section have the potential to be used in the clinic. The SMO inhibitor, GDC-0449 **4c**, is the first Hh pathway inhibitor approved by the FDA for treatment of metastatic BCC, or patients with recurrent, locally advanced BCC. Currently, GDC-0449 is under clinical trials as a monotherapy or in combination with other therapeutic drugs for various cancers, including MB, metastatic pancreatic, and prostate cancer, intracranial meningioma, advanced head/neck BCC, recurrent glioblastoma, and acute myeloid leukemia.

To further explore the beneficial effects of GDC-0449 in various cancers and liver fibrosis, it has been evaluated in combination with other therapeutic agents by Kumar et al.⁶⁷ Hh signaling is involved in initiation and progression of pancreatic ductal adenocarcinoma (PDAC) and contributes to desmoplasia. Expression levels of Hh ligands are increased in pancreatic cancer cells, whereas the level of tumor suppressor miR-let7b is downregulated. Codelivery of miR-let7b and GDC-0449 using micelles prepared using methoxy poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxylpropylene carbonate-*graft*-dodecanol-*graft*-tetraethylenepentamine) (mPEG-*b*-PCC-*g*-DC-*g*-TEPA) proved beneficial. This combination therapy effectively inhibited tumor growth in athymic nude mice bearing an ectopic pancreatic tumor, compared to micelles carrying GDC-0449 or miR-let7b alone. Gemcitabine (GEM) is a first-line chemotherapeutic agent for pancreatic cancer. Coadministration of GEM and IPI-926 enhanced GEM efficacy by increasing tumor perfusion. IPI-926 resulted in stromal depletion and increased the intracellular concentration of GEM resulting in an enhanced anticancer effect.⁶⁸ The effective concentration of GEM can be further enhanced by decreasing metabolism and enhanced permeability and retention (EPR) effects using delivery approach. Therefore, Karaca et al⁶⁹ have formulated the micelle mixtures grafted GEM (mPEG-*b*-PCC-*g*-GEM-*g*-DC) micelles and GDC-0449 loaded (mPEG-*b*-PCC-*g*-DC) micelles. The combined micelles resulted in a synergistic tumor growth inhibition in mice bearing subcutaneously pancreatic tumor.

The Hh pathway is upregulated in liver fibrosis, which is a liver disease characterized by excess extracellular matrix (ECM) upon chronic injury. Cyclopamine, a Hh inhibitor, has proven beneficial for the treatment of liver fibrosis. Pratap et al⁷⁰ evaluated the beneficial effects of GDC-0449 and cyclopamine to attenuate early liver fibrosis in common bile duct ligated rats. Results of the study suggest the improved liver functions, decreased hepatic stellate cell (HSC) activation, and suppression of epithelial-to-mesenchymal transition (EMT) by GDC-0449 compared with cyclopamine. Polymeric nanoparticles prepared with mPEG-*b*-p(CB-*co*-LA) encapsulating GDC-0449 and peroxisome proliferator-activated receptor gamma (PPAR- γ) agonist, rosiglitazone has the potential to treat liver fibrosis by intervening the complex fibrotic cascade.⁷¹

Further, miR-29b1 which targets several profibrotic genes like collagen types I and IV, c-MYC, PDGF- β , and PI3K/AKT upregulated in liver fibrosis was co-formulated into micelles with GDC-0449. Micelles prepared using mPEG-*b*-PCC-*g*-DC-*g*-TEPA copolymer efficiently delivered high concentrations of GDC-0449 and miR-29b1 to liver cells and

decreased liver fibrosis in mice.⁷² Further, drug delivery to HSCs within a fibrotic liver is a major challenge. Mannose-6-phosphate (M6P)/insulin-like growth factor II receptor (M6P/IGF-IIR) is overexpressed by HSCs of fibrotic livers and are serving as potential targets to treat liver fibrosis. To improve GDC-0449 delivery to HSCs, Dutta et al⁷³ have reported the mannose-6-phosphate decorated mixed micelles to target M6P/IGF-IIR. M6P conjugated mixed micelles carrying GDC-0449 were efficiently accumulated in the liver after systemic administration. This study concluded that M6P targeted mixed micelle formulation of GDC-0449 has the potential to treat liver fibrosis.

2.2.1 | Pyrimidines—Pyrimidine is a basic, six-membered, heterocyclic compound bearing two nitrogen atoms at 1 and 3 positions and is an important constituent of nucleic acids. This section describes some important reports where pyrimidines were identified as inhibitors of Hh pathway.

Xin et al⁷⁴ described a novel series of *N*-(2-pyrimidinylamino)benzamides **5a** as potent inhibitors of the Hh pathway inspired from a potent compound containing pyrimidine amino unit named ALLO-2 which possesses good Hh inhibition activity described by Tao et al.⁷⁵ The pyrimidine amino core is considered as a privileged structure commonly found in many drugs such as imatinib, and nilotinib. In the initial studies, *N*-(2-pyrimidinylamino)benzamide **5a** (Figure 5A) was demonstrated as a leading Hh pathway inhibitor; better than GDC-049, and LDE-225. To optimize the rudimentary structure, **5a** was explored systematically in four regions. For region-A, the nature and position of the substituents on phenyl ring played an important role in regulating Hh signaling inhibitory activity. Introduction of the strong electron-withdrawing group (-OCF₃, F, and CN) at either 3-position or 4-position of phenyl showed good Hh inhibition, while substitution with these groups at 2-position led to significant loss of activity. However, substitution with the electron-withdrawing group at both 4-position and 3-position led to substantial loss of activity. Although an unsubstituted phenyl ring or saturated ring such as morpholino was well tolerated, replacement of phenyl with *N*-heterocyclic ring (ie, 4-pyridinyl) was also tolerated. Interestingly, the position of nitrogen in the *N*-heterocyclic ring played a critical role in Hh inhibitory activity. Other heteroaryls such as pyrazolyl and furyl, proved less potent. Replacement of the phenyl ring with an alkyl was unacceptable for activity. Optimization of the pyrimidinyl ring (region-B) revealed that small hydrophobic groups were favored at the 5-position or 6-position of pyrimidinyl. Further, an *N*-1 nitrogen in the pyrimidinyl ring improved the Hh signaling inhibitory activity relative to an *N*-3 nitrogen. SAR studies on region-C revealed that small alkyl groups (eg, methyl) on the amide nitrogen was well tolerated and gave potent compounds. Exploration of region-D demonstrated that 3-position of phenyl bearing some *N*-functionality was well tolerated. Exchange of phenyl with a small alkyl group, such as isopropyl, resulted in the loss of potency, whereas bulky alkyl group, such as cyclohexyl, retained the potency. From this SAR study, *N*-(2-pyrimidinylamino)benzamide **5b** was selected for in vivo evaluation in SD rats and found to have a low bioavailability (*F* = 36%) with half-life of *t*_{1/2} = 7.0 hours, but large volume of distribution (*V*_{ss} = 27.74 L/kg), and low oral exposure (1705 ng. h/mL) (Figure 5A).⁷⁴

In an attempt to improve its in vivo PK profile, Xin et al⁷⁶ focused their attention on modifying region-A and region-B of compound **5b**, more specifically the C-5 position of

region-B. Analogs incorporating cyclic aliphatic amines as a side chain were more potent, whereas more basic cyclic amines, such as piperazine, significantly reduced the activity, because of strong basicity of second basic center. However, substituted piperazine and piperidine gave access to potent compounds confirming the fact that extra basicity was not favorable for activity. The pyrrolidine and amine-containing bicyclic systems also provided potent Hh inhibitors. A variety of acyclic amines with suitable length (1–3 carbon) gave potent analogs. However, long aliphatic chain bearing basic centers or bulky groups bearing analogs were moderately active, probably because of the extra basicity and steric hindrance. Besides this, analogs containing secondary amines were also suitable for potency. In addition, the inhibitory activity was retained with an amine substituted with aromatic groups. Further, attempts were made to decrease the log *P* value. Exchange of trifluoromethoxyphenyl of region-A with 1-methylpyrazolyl proved beneficial to maintain comparable potency with **5b** and much lower log *P*. This compound had significantly improved oral bioavailability by almost 64%. Therefore, based on the SAR study, compound **5c** was selected as a lead candidate for in vivo study (Figure 5A).

To further optimize the 4-(2-pyrimidinylamino)benzamide skeleton, Xin et al⁷⁷ synthesized a series of new trifluoromethyl 4-(2-pyrimidinylamino)benzamides **5d** (Figure 5B). Fluoro substitution at 5-position of pyrimidine ring resulted in potent Hh inhibitory activity and with better PK profiles than the corresponding 5-methyl substituted analogs. Based on these observations, a rational fluorine scan strategy was applied to assess new analogs by introducing a trifluoromethyl group at 5-position of the 4-(2-pyrimidinylamino) benzamide skeleton. A systematic SAR study revealed that the aromatic rings (region-B) were essential for maintaining the Hh signaling pathway inhibitory activity. Several *N*-heterocycles substituted at 5-position of the aromatic rings gave potent compounds, especially morpholine yielded the most potent compound in this class, which was 5-fold more potent than GDC-0449. Also, exploration of substituents at R¹ (region-A) revealed that strong electron-withdrawing groups on the phenyl ring gave the potent compounds, whereas unsubstituted phenyl ring demonstrated reduced potency. To further increase the hydrophilicity, several heteroaryl groups were used to replace the aryl ring. However, they all proved less active than GDC-0449. Based on this optimization study, morpholine containing trifluoromethyl 4-(2-pyrimidinylamino)benzamide **5e** was the most potent compound. Further, PK studies in SD rats, suggest this compound had high plasma exposure (AUC = 1957 ng. h/L), low clearance (Cl; 0.47 L/h/kg) and long half-life (*t*_{1/2} = 8.63 hours) (Figure 5B).

Xin et al⁷⁸ reported a new series from 4-(2-pyrimidinylamino)benzamides **5f** (Figure 5C) as potent Hh signaling pathway inhibitors. Based on their previous SAR study, the substituent at R³ position (region-A) revealed that *N*-heterocyclic systems gave potent compounds when R¹ is either hydrogen or fluorine. Among all the *N*-heterocyclic system, morpholine provided the most potent compound. Phenyl ring (region-C) replacement by pyridine gave a compound with better potency. Furthermore, removal of the methyl group from region-B (R² = H) afforded even more potent compounds. However, introduction of a cyclopropyl group in region-D reduced inhibitory activity. In this series, compounds **5g** and **5h** (Figure 5C) were selected for antiproliferative effects in tumor cell lines. Both compounds exhibited high

potency in the cancer cell lines. These compounds were further evaluated for antitumor activity in the LS-174T nude mice xenograft model. However, by tumor weight growth inhibitory measure, both compounds did not show strong tumor regression (only <25%) and lack the dose-dependent correlation. Nevertheless, these compounds were well tolerated and prevented any significant loss of mouse body weight after treatment (Figure 5C).

Based on a clinically used Hh inhibitor BMS-833923 **5i** (also known as XL-139) (Figure 5D) which contains a quinazoline core, Xin et al⁷⁹ designed a new series of compounds in which cyclization of 5-position and 6-position of pyrimidine on B-ring gave access to new fused-pyrimidine scaffold and screened them for Hh signaling pathway inhibitory activity. To realize this cyclization, pyrrolo[2,1-*f*][1,2,4]triazine skeleton **5j** (Figure 5D) was used as a privileged scaffold for B-ring surrogate based on a combination of conceptual cyclization and bioisosterism strategies. The systematic modification of this skeleton at the A-region, C-region, and D-region was performed. It has been found that the 6-membered aryl ring (region-A) was preferred over the 5-membered aromatic ring for efficacy, whereas 2-pyridinyl and 3-pyridinyl containing analogs provided potent compounds. The incorporation of saturated cyclic or aliphatic amines at the C-5 position of region-D gave potent compounds, particularly with morpholine and piperidine. Removal of the 2-methyl group from this region resulted in decreased activity. For region-C, inclusion of an electron-deficient pyridine was well tolerated. Additionally, introduction of an electron-withdrawing (ie, F) group at phenyl gave the potent compound. Based on this optimization study, compound **5k**, was selected for PK studies in SD rats and found to have the AUC of 2.23 µg h/L, clearance (Cl) of 0.41 L/h/kg and was eliminated at a much slower rate than GDC-0449 (*t*_{1/2} of 8.86 vs 1.64 hours). Based on PK properties, it was further considered for in vivo antitumor efficacy in Hh signaling–derived solid tumor models (Figure 5D).

To further explore the cyclized pyrimidines, Zhang et al⁸⁰ have reported a series of five-membered heterocyclic fused-pyrimidine, such as purines **5l**, pyrrolo[2,3-*d*]pyrimidines **5m**, pyrrolo[3,2-*d*]pyrimidines **5n**, thieno[2,3-*d*] pyrimidines **5o**, thieno[3,2-*d*]pyrimidines **5p**, furo[3,2-*d*]pyrimidines **5q** (Figure 5E), and evaluated for Hh signaling pathway inhibition. In the purine series, the analogs bearing basic amine side chains exhibited significant potency. Additionally, substitution of a methyl group at the 9-position in purines appeared help improve Hh inhibitory activity. Pyrrolo[2,3-*d*]pyrimidines have shown to potency satisfactorily compared with GDC-0449, whereas *N*-methyl pyrrolo derivatives were superior to GDC-0449 by 3-fold. Similarly, in pyrrolo[3,2-*d*]pyrimidine series, the methyl substituted derivatives showed better potency than the unsubstituted derivatives. Isosteric replacement of nitrogen with S or O atom in the five-membered ring adjacent to the pyrimidine ring junction seemed to be well tolerated and showed good Hh signaling inhibitory activity. Among the thienopyrimidine series, thieno[2,3-*d*] pyrimidines were more potent than thieno[3,2-*d*]pyrimidines. Furo[3,2-*d*]pyrimidines had better potency than thieno[3,2-*d*]pyrimidines and even better than GDC-0449. These observations suggested that five-membered heteroaromatic ring fused-pyrimidines appeared good alternatives for surrogate of pyrrolo[2,1-*f*][1,2,4]triazine and pyrimidine scaffolds. Among all the newly synthesized analogs, compounds **5r**, **5s**, **5t**, and **5u** were potent Hh inhibitors with IC₅₀ values below 1.0 nM (Figure 5E).

Recently, Xin et al⁸¹ developed a potent series of fluorine-containing 4-(2-pyrimidinylamino)benzamides **5v** (Figure 5f) inspired by the fact that fluorine-containing compounds have improved metabolic stability and membrane permeability. The newly synthesized compounds exhibited high potency for Hh signaling pathway inhibition (in a luciferase reporter assay in NIH3T3 cells). SAR investigation around region-A revealed that aliphatic tertiary amine side chain containing substituents (such as dimethylamino and diethylamino) or cyclic amines (such as pyrrolidinyl, piperidyl, *N*-methyl piperazinyl, and morpholinyl) afforded a more potent compound than vismodegib. The alkyl-bridged morpholinyl derivatives with ethoxy and propoxy spacer were also well tolerated. Further, to increase the drug-like properties of the molecules by reducing the molecular weight and lipophilicity, various groups such as (un)substituted phenyl or heterocyclic systems (pyridines and 1-methylpyrazole-3-yl) were explored in region-B, and the phenyl-bearing compound **5w** (Figure 5F) was approximately 150-fold more potent than vismodegib. Further modification of substituents at R¹ (CH₃, F, or H) and R³ (CH₃ or H) did not improve the potency. Compound **5x** bearing an *N*-methyl piperazinyl group showed impressive PK profile in SD rats by intravenous (iv) dosing (1.0 mg/kg) and PO (5 mg/kg) administration and have long half-life ($t_{1/2}$ = 14.9 hours), large volume of distribution (V_z = 71.7 L/kg), plasma exposure ($AUC_{(0-t)}$) of 0.96 μ g h/L, and clearance (Cl) rate of 3.3 L/h/kg (Figure 5F).

Despite the fact that some compounds from the pyrimidine/quinazoline series have good Hh inhibitory potential, none of these compounds has advanced into the clinical studies.

2.3 | Quinolones

Quinoline, also known as 1-aza-naphthalene or benzo[*b*]pyridine is a nitrogen-containing fused heterocyclic system found widely in nature and possessing a broad range of biological activity. Some of the quinoline analogs possess potent Hh pathway inhibitory potential and are discussed in this section.

Ontoria et al⁸² have screened a library of quinolone-based small molecules for SMO-binding potential and Hh pathway inhibition. From the screening results, 4-[3-(quinoline-2-yl)-1,2,4-oxadiazol-5-yl]piperidinyl urea **6a** (Figure 6A) was identified as a lead molecule and based on its PK profiles; it was selected for further SAR optimization. Four regions of the lead scaffold were extensively explored namely quinoline ring, 1,2,4-oxadiazole, piperidine ring, and capping group (regions A-D, respectively) (Figure 6A). Replacement of the 2-quinolyl ring (region-A) with simple phenyl, heterocycles (pyridine, pyrimidine, or pyrazine) or 2-naphthyl significantly decreased the activity. The position of the nitrogen in the quinoline ring was important as other isomeric quinoline such as 1-isoquinoline, or 3-isoquinoline analogs were inferior inhibitors of the Hh pathway. Further, the additional nitrogen into the bicyclic scaffold (such as in quinazoline, quinoxaline, and naphthyridine) proved unsuccessful to enhance the activity. In contrast, replacement of 2-quinolyl with *N*-methyl benzimidazole displayed slightly weaker affinity than the parent compound. However, such compounds have increased human *ether-a-go-go*-related gene (hERG) profile and reduced CYP450 (CYP 2C9, CYP 3A4) inhibition.⁸³ The ERG gene encodes the alpha subunit of a potassium ion channel protein. hERG inhibition can result in a potentially fatal long QT

syndrome and must be screened during drug development. The 1,2,4-oxadiazole ring (region-B) was optimal to bind with SMO protein, whereas isomeric 1,3,4-oxadiazole was less active than the parent compound. Similarly, the piperidine ring (region-C) was explored by contraction of the ring to four-membered azetidines or five-membered pyrrolidine ring which resulted in the loss of affinity for SMO protein. Efforts to further improve the metabolic stability by changing the electronics of the piperidine ring proved unsuccessful. Replacement of the piperidine with piperazine resulted in improvement in both SMO-binding affinity and activity in the Light 2 cell assay; however, this led to increased inhibition of CYP and hERG profile. For capping group (region-D), aromatic ureas were best suited at this position, as simple amides, in general, were inactive, whereas alkyl groups, benzyl carbamate, and phenolic carbamates were not stable in physiological pH and displayed moderate activity. Primary amine-based ureas were better than secondary amine-based ureas. The inclusion of the bicyclic system in this position resulted in substantially reduced activity. A variety of substituents, including electron-withdrawing and electron-donating groups, were well tolerated at the 2-position and 3-position of the aniline ring without significant impact on the affinity for SMO protein. However, replacement of aniline group by bicyclic or heterocyclic system led to many folds decrease in activity. Replacement of phenyl ring with lipophilic alkyl urea and cycloalkyl urea (like cyclopentyl and cyclohexyl) exhibited submicromolar activity. The corresponding 4,4'-difluorocyclohexyl analogs exhibited improved activity in the Light 2 and SMO-binding assays. Addition of the fluorine atoms on the cyclohexyl ring improved the stability of the molecule and displayed enhanced stability in microsomes. Based on the initial SAR and PK profiles, compounds **6b** and **6c** were identified as advanced and potent leads from this series (Figure 6A).

Further, Muraglia et al⁸⁴ have reported new structural modifications for lead compounds **6b** and **6c** (Figure 6A) to improve the potency and lower serum binding. For this, various substitutions on the urea moiety were screened to identify potent SMO antagonists with improved physicochemical properties. From this screening, morpholine containing derivative **6d** (Figure 6B) was identified as a lead compound for further optimization. The basic amine of morpholine (region-A) was found essential for activity as its removal or replacement with other groups like hydroxymethyl or insertion of carbonyl between the cyclopentyl and the amine group resulted in the loss of activity. Similarly, replacement of the morpholine group of compounds **6d** with either an *N*-acetamide or *N*-Boc gave compounds with weaker affinity for SMO. The exchange of morpholine ring with similarly lipophilic amines, such as pyrrolidine and piperidine was allowed. Acyclic amines of similar size (such as diethylamine) were also tolerated, whereas the corresponding dimethylamine or primary amine led to a drastic loss of activity. Secondary amines (ie, ethylamine or isopropylamine) were well tolerated, although the addition of large lipophilic groups (ie, cyclohexylamine) resulted in a drop of potency. Further, it was established that cyclopentyl group (region-B) was essential for optimum lipophilicity of the compound as its removal, reduction in size, or replacement with large cycloalkyl, phenyl, or pyridine resulted in a drastic loss in potency. There appears to be optimal basicity of the amine while utilizing morpholine or piperidine as they display good activity in the SMO-binding assay. Further optimization to modulate the acid dissociation constant at logarithmic scale (pKa) of these compounds by introducing one or two fluorine atoms on morpholine or piperidine ring resulted in a drop in SMO-binding

affinity. Substitution of morpholine with methoxy piperidine or piperazine resulted in the loss of potency. Attempts to replace the morpholino and cyclopentyl group with a pyrrolidine led to a significant reduction in potency. Addition of carbonyl oxygen into the ring system or ring fusion with a phenyl further led to drop in potency. Replacement of quinoline (region-C) with *N*-methyl benzimidazole proved beneficial and showed high potency in both Light 2 and SMO-binding assays. The most potent compound from this series was found from the unification of piperazine and benzimidazole to give compound **6e** showing single-digit nanomolar activity in both Light 2 cells and SMO-binding assays with 20% normal human serum (Figure 6B).

Recently, Trinh et al⁸⁵ identified a new scaffold quinolone-1-(2*H*)-ones **6f** (Figure 6C) as an inhibitor of Hh signaling pathway. These quinolone-2-(1*H*)-one analogs inhibit Ptch1 and Gli2 at mRNA levels in Shh-Light 2 cells stimulated with 100 nM SAG. Moreover, these analogs suppress Gli2 in nonactivated Shh-Light 2 cells, and also support a downstream of SMO inhibition. SAR study revealed that the C3-tethered indole group at R² (region-A) was essential for activity as replacement of indole by 4-methoxyphenyl or phenyl moiety resulted in significant decrease in activity. The presence of bulky aliphatic groups at R³ (region-B) was required to endow cytotoxicity against the TCAM-2 cell line. Based on the SAR studies, compounds **6g**, **6h**, and **6i** were identified as promising leads for future development (Figure 6C).

Ohashi et al⁸⁶ have identified thieno[3,2-*c*]quinoline-4-one **6j** (Figure 6D), as a potent Hh inhibitor by high throughput screening (HTS) using a Gli-luciferase reporter assay. To improve the metabolic stability, chemical modifications of compound **6j** were undertaken through the systematic SAR study. The furo-analog (replacing S with O) (region-A) led to decrease in the Hh inhibitory activity, whereas *N*-methyl pyrrolo analog had a significant increase in Hh inhibitory activity. Subsequently, *N*-methyl on the 5-membered ring was found essential for potency. Further, substituents at the 2, 3, and 5-positions of the pyrrolo[3,2-*c*]quinoline-4-one were explored. The methoxy group at the 3-position was optimal as ethoxy analog reduced the activity and led to instability in mouse hepatic microsomes. For optimization of position-2 (region-B), derivatives where the piperidine or morpholine linked through ethyl-bridge to the amide nitrogen were synthesized, however, this modification led to decreased Gli-luciferase reporter activity without improvement in metabolic stability. Direct attachment of the cyclic amine, such as 4-piperidiny, to the amide linker led to reduced lipophilicity and improved metabolic stability. Further extension of the side chain length by the introduction of a hydroxyethyl group onto the piperidine nitrogen confirmed potent Gli-luciferase reporter activity and retained metabolic stability in mouse and human microsomes. Introduction of hydroxy acetyl piperidine moiety provided potent activity as well as good metabolic stability without hERG inhibition. At the position 5 (region-C), phenacyl group was essential for tight binding with SMO protein as removal of the phenyl or even carbonyl from the side chain led to a drastic drop in the activity. Compound **6k** (Figure 6D) was identified as a potential lead with significant suppression of Gli1 mRNA in the mouse MB allogeneic tumor model created by transplanting Ptch1 (+/–) and p53 (–/–) tumor subcutaneously into nude mice. Oral administration of compound **6k**

for 2 weeks at the dose of 6.25 mg/kg resulted in virtually complete suppression of tumor growth (T/C ratio = 3%) without notable loss of body weight (Figure 6D).

The PK profiles of compound **6k** at the high dose in mice showed poor oral absorption ($AUC = 12.1 \mu\text{g h. /mL}$ after an oral dose of 10 mg/kg), due to its low solubility. Ohashi et al⁸⁷ have designed a new series of pyrrolo[2,3-*c*]quinoline-4-one to improve the solubility while maintaining its Hh pathway inhibitory activity. For this, the tricyclic core of the molecule was selected for further modifications to decrease the planarity and improve the aqueous solubility. First, replacement of the benzenoid (region-A) with cyclohexane in the core structure led to reduced activity, indicating the requirement of specific size and shape to maintain the potency. However, substitution of methyl or ethyl group as R^1 in compound **6l** (Figure 6E) effectively preserved activity and improved solubility compared with the parent compound, whereas ethyl derivative showed poor efficacy in vivo even at the higher dose because of low solubility. Further to improve its solubility, position-3 (region-B) was explored. Exchange of methoxy to an ethyl group significantly decreased the activity indicating the important contribution of oxygen atom for activity. Further, mono, di, or trifluoroethoxy analogs were found with significantly increased solubility and potency, particularly trifluoroethoxy analog **6m** (Figure 6E). In in vivo efficacy study of compound **6m** using a Ptc1+/-p53-/MB allograft model in mice indicated good tumor permeability and dose linearity. Compound **6m** (TAK-441) showed favorable oral bioavailability ($F[\%] = 90.3 \pm 8.8\%$) with low drug clearance; $161.3 \pm 35.6 \text{ mL/hour/kg}$ and high $AUC_{(0-24\text{hours})}$, PO ($45.40 \pm 5.81 \mu\text{g. hour/L}$) in dogs (Figure 6E).

To investigate the exact role of core skeleton of pyrrolo[3,2-*c*]pyridine-4-one analog TAK-441 including substituents at 4-position and 5-position of pyrrole (region-A), Ohashi et al⁸⁸ have modified the central core in search of new inhibitors. Since the pyrrole ring of compound **6m** was essential for inhibitory activity, modifications were focused to the region-B. For this, pyrrolo[2,3-*d*]pyrimidine analog maintained the potent inhibitory activity compared to compound **6m**, whereas the pyrrolo[2,3-*b*]pyridine analog proved much weaker Hh inhibitor. Further, the introduction of 5-benzamide instead of phenacyl group gave indole analog **6n** (Figure 6F), which has significantly increased Gli reporter inhibitory activity compared with the parent pyrrolo[2,3-*d*]pyrimidine analog. It has been revealed that the core structure like pyrrolo[2,3-*d*]pyrimidine, pyrrolo[3,2-*b*]pyridine, and pyrrolo[2,3-*b*] pyrazine retained the activity even without a 4-carbonyl group. An amide linker acquired suitable conformation with a dihedral angle of about 90° and well accommodated into the pocket of SMO protein. In human pancreatic xenograft tumor model to measure the reduction of Gli1 mRNA expression levels, all the new analogs were weaker than TAK-441 despite their potent in vitro Hh inhibitory activity. From this study, TAK-441 was considered well-balanced in PK profile ($AUC = 28.35 \mu\text{g h. /mL}$ after a dose of 10 mg/kg, PO in mice) and potent Hh inhibitory activity ($IC_{50} = 4.4 \text{ nM}$, Gli-luciferase reporter assay) (Figure 6F).

Quinolone compounds need further extensive studies to optimize their PK/PD properties and fine-tuned to be developed as more advance drug-like molecules.

2.4 | Quinazolinones

Quinazolinones are nitrogen-containing fused heterocycles that have attracted the considerable interest of medicinal chemist because of the diverse range of their biological properties. Some important leads from this class as Hh inhibitors are discussed in this section.

Brunton et al⁸⁹ have screened a small-molecule library to find potential hits to inhibit the Hh pathway. This screening resulted in the identification of quinazolinone-based lead molecule **7a** (Figure 7A) was a weak antagonist activity ($IC_{50} \sim 1.4$ mM). To improve its potency, three distinct regions of the molecules were explored. Initially, removal of both methyl groups in region-A of compound **7a** (bis-desmethyl analog) led to increased activity compared with the parent compound, whereas monomethyl analog proved less active than the parent compound. Next, the 4-fluorophenyl group on quinazolinone ring (region-B) proved essential for activity as removal of 4-fluorophenyl or substitution with an alkyl group (*N*-isopropyl) gave analogs with much less potency than the parent compound. Finally, the requirement of the quinazolinone ring (region-C) was explored by preparing the truncated pyrimidinone analogs. However, such analogs proved much less active than the parent compound suggesting the critical role of the quinazolinone system for activity. Compound **7b** was identified as the most potent analog from this series, with a 20-fold increase in potency (Gli-Luc IC_{50} 70 nM) over the parent compound **7a** (Figure 7A).

Bhattarai et al⁹⁰ have utilized the ring-closing structure-hopping approach to design a cyclized version of sonidegib from its comparatively flexible amide linkage by addition of a heteroatom. The newly synthesized quinazolinones **7c** (Figure 7B) were potent Hh pathway inhibitors in Gli-luciferase reporter assay. Further, quinazolinone **7c** was explored starting with simple 8-chloro-3-(4-fluorophenyl) quinazoline-4(3*H*)-one, which exhibited an IC_{50} value of 269 nM in Hh inhibitory assay. Various substituents were tried as R^1 on the structure **7c** (region-A) such as Cl, phenyl, and phenyl ring bearing electron-withdrawing (F, CF_3 O, etc.) as well as electron-donating (OCH_3 and CH_3) groups. Among these, an unsubstituted phenyl ring or fluorine-bearing phenyl ring was best for Hh inhibitory activity. For optimization of R^2 position (region-B), a phenyl ring appropriately substituted at the 4-position was essential for good Hh inhibitory activity, whereas aliphatic chain bearing analogs were significantly less active. Replacement of the phenyl ring with heterocyclic systems, such as pyridines or pyrimidines were well tolerated without substantial loss of activity. Further, substitution at the 4-position of the phenyl ring was allowed. The analog bearing dimethyl morpholine was the most potent. From this study, quinazolinone analogs **7d** and **7e** (Figure 7B) were the most potent lead compounds to inhibit the Hh pathway. These new scaffolds inhibited the Gli transcription binding activity in Gli-luciferase reporter cells and *Ptch1*^{-/-} mouse embryonic fibroblast cell line. This novel quinazolinone scaffold may be utilized for the development of new and improved Hh pathway inhibitors for drug resistance cancer (Figure 7B).

2.5 | Phthalazines and piprazic acids

Phthalazine is a fused two nitrogen-containing heterocyclic compound also known as benzo-orthodiazine or benzopyridazine and a positional isomer of quinoxaline, cinnoline, and

quinazoline, whereas piprazic acid is a saturated, monocyclic, two nitrogen-containing heterocyclic system also known as piperidine-3-carboxylic acid. Some important compounds from this class as Hh inhibitors are discussed in this section.

Miller-Moslin et al⁹¹ had screened a small-molecule library and identified 1-amino-4-benzylphthalazine **8a** (Figure 8A), as potent inhibitors of the Hh pathway in Gli shift assay. To enhance the SMO-binding affinity, SAR around each unit of the system was studied (regions A-D) (Figure 8a). Changing region-A from a phenyl to a 2-pyridyl resulted in a significant improvement in SMO-binding affinity and Hh pathway inhibition and relative to lead structure **8a**. The introduction of electron-withdrawing (-CF₃, -CN, primary amide, sulfonamide) or electron-donating substituents (alkyl amines, alkyl-linked amides, and tertiary amine) at the 4-position resulted in excellent improvement in activity. Primary, secondary, and bulky tertiary alcohols were also well tolerated. However, a carboxylic acid at this position decreased the activity. For region-B, a piperazine was essential as homo piperazine or piperidine derivatives displayed low activity. Substitution on the piperazine ring with a methyl group did not improve the potency. For region-C, phthalazine ring was essential for potency as either isoquinoline regioisomer or similar scaffolds such as furo[2,3-*d*]- or 1*H*-imidazo[4,5-*d*]pyridazines resulted in a drastic loss of activity. Further, the introduction of a halogen, such as a chloro, on the phthalazine led to significant improvement in activity, whereas a strong electron-withdrawing group, such as cyano, decreased activity. For region-D, a phenyl ring was essential for activity as pyridyl analogs significantly decreased activity. Electron-withdrawing groups on the phenyl ring (such as -CF₃, -CO₂Me, and -CO₂H) decreased activity, whereas 4-chloro or 4-cyano substitutions increased activity. Compound **8b** (Figure 8A) showed excellent in vitro activity (IC₅₀ = 5.0 and 8.0 nM in mouse and human SMO membrane filter binding assay, respectively) and selected for further profiling in vivo studies. The degree of Gli1 mRNA inhibition and the tumor growth inhibition correlated well in the efficacy study in Ptch^{+/-}p53^{-/-} mouse MB allograft model when dosed with 40.0 mg/kg QD (Figure 8A).

Lucas et al⁹² have performed an HTS and identified phthalazine **8c** (Figure 8B) as potent SMO antagonist; however, with low stability in rat and human microsomes, and elevated levels of CYP 2D6 inhibition. Replacement of thiophene in region-A with phenyl ring provides a compound with good potency, reduced turnover in microsomes, and low inhibition of CYP 3A4 and 2D6. Thus, the benzoyl piperazine moiety was selected for further optimization. Further, analogs with 2-*S*-methyl piperazine and its regioisomeric 3-*R*-methyl piperazine (region-B) were potent SMO antagonist. However, for 3-*R*-methyl piperazine analog, microsomal clearance was increased significantly. Further, exploration of pendent aryl group (region-C) revealed that its replacement with heteroaryl system resulted in compounds with low potency. Compounds bearing electron-withdrawing groups (4-Cl or 4-CF₃) at the 4-position of pendent phenyl ring afforded good potency without compromising the stability in microsomes. For 3-*R* methyl piperazine core, small electron-donating groups at the 4-position of the pendant aryl ring provided promising compounds with SMO antagonist activity in the subnanomolar range. From all the screened analogs, compound **8d** was potent in both mSMO and hSMO assays. The in vivo efficacy of **8d** was

demonstrated in a $Ptch^{+/-}$ MB tumor allograft model where it significantly reduced the tumor volume (Figure 8B).

Peukert et al⁹³ optimized the 1-amino-4-benzylphthalazine **8e** and identified a new molecule, NVP-LEQ506 **8f** (Figure 8C) with improved potency, low hERG activity, and enhanced aqueous solubility. Initial attempts were focused to further increase the aqueous solubility and to decrease the hERG activity by exploring the substituents at R¹ on the phenyl ring (region-A). Various groups such as ionizable basic and acidic substitutions as well as polar groups inhibited hERG with low potency. Truncation of phthalazine core (region-B) was attempted to improve its aqueous solubility. Replacement of the annealed benzene ring either with a cyclopentyl ring or with two methyl groups, afforded compounds with improved potency. However, removal of both or either of methyl group decreased activity. The dimethyl substituted compound was further optimized at R¹, and installation of polar groups like 2-hydroxypropyl increased potency (Gli shift = 5 at 1.0 nM dose) but with low aqueous solubility (>5 μ M at pH 6.7), and potent hERG activity (hERG IC₅₀ = 1.3 mM). Subsequently, replacement of the 2-pyridyl (region-A) with a 2-pyrazine resulted in excellent improvement in potency and solubility without insignificant binding to the hERG channel. The addition of a methyl group on the piperazine linker further increased the aqueous solubility. The *R*-enantiomer was approximately 2-fold more potent than the *S*-enantiomer. Notably, replacement of the benzylic methylene linker (region-D) with an oxygen atom was well tolerated, whereas substitution with an NH resulted in decreased Hh inhibitory activity. Overall, compound **8f** provided the best combination of substituents with excellent potency in the Gli-luciferase assay, low hERG channel binding, and acceptable aqueous solubility. A separate study conducted on $Ptch^{+/-}$ Hic^{-/-} (hypermethylated in cancer) MB bearing mice and rats using compound **8f** revealed that it required 2-fold higher dose than sonidegib to achieve similar tumor regression in both rodent models. However, compound **8f** achieved similar efficacy with 3-fold to 8-fold lower exposure in plasma and tumor, because of its higher intrinsic potency. The higher dose required for compound **8f** was due to its shorter half-life in rodents ($t_{1/2}$ = 1.2–1.4 hours) in comparison with sonidegib ($t_{1/2}$ = 3.9–4.2 hours). Inhibition of the Hh pathway in tumors upon treatment with compound **8f** in $Ptch^{+/-}$ Hic^{-/-} MB bearing mice demonstrated almost complete (>90%) and sustained inhibition of Gli1 mRNA over 24 hours (Figure 8C).

Zhao et al⁹⁴ have further explored benzylphthalazine containing analog Anta XV **8g** (Figure 8D). The new modifications were based on the finding from the cocrystal structure of Anta XV and SMO protein that shows the hydrogen-bond interaction between the R400 and the phthalazine core, N219 with the pyridine *N* atom and K395 with the hydroxyl O atom. The piperazine ring of Anta XV play an important role as a linker and can adjust the orientation of pharmacophores phthalazine and pyridine-3-propanol. Exchange of region-B of Anta XV (Figure 8D) from piperazine to various four-membered, five-membered, or six-membered heterocyclic systems led to significant alteration of Hh pathway inhibition. A change from piperazine to pyrrolidine-3-amine was tolerable, and further replacement of piperazine with piperidine-4-amine significantly enhanced activity, whereas piperidine-3-amine analog completely lost activity. Meanwhile, a compound with the tertiary amine in the linker (*-N*-CH₃) was equipotent with Anta XV. For region-A, the bulky 3-pentanol substituent at the 4-

position of pyridine in pyrrolidine-3-amine analogs was tolerated, whereas its replacement with piperidine-4-amine resulted in a drastic drop in activity. In this study, compound **8h** (Figure 8D) was most active to antagonize the Hh pathway in in vitro study and significantly suppressed the MB cells proliferation. This compound exhibited acceptable PK profile of in SD rat, with both iv and oral dosing, moderate systemic plasma clearance ($CL = 55 \text{ ml/min/kg}$), the volume of distribution was moderate relative to total body volume and half-life ($t_{1/2}$) of about 1.0 hours after iv injection. Following a single oral dose of 10 mg/kg, compound **8h** showed bioavailability ($F\%$) 40.1 ± 2.5 and $T_{\max}$ around 2.0 hours (Figure 8D).

Khatra et al⁹⁵ reported a new series of piperazic acid **8i** (Figure 8E) as potent Hh pathway inhibitors. Piperazic acid (hexahydropyridazine-3-carboxylic acid) is a nonproteinogenic, cyclic α -hydrazino acid with remarkable biological activity. Analogs of piperazic acid including piperazic acid methyl ester, azepane acid, and azepane acid methyl ester were prepared, however, only piperazic acid showed downregulation of Gli-dependent signaling. Both nitrogens were required for the activity as *DL*-pipecolic acid (lacking 1-nitrogen) was inactive in firefly luciferase activity up to 50 μM . Further, various analogs of piperazic acid were prepared such as di-Boc-piperazic acid, δ -*N*-Cbz-piperazic acid, dimethyl-piperazic acid, δ -*N*-methyl-piperazic acid, and piperazic acid amides. However, none of the analogs proved activity except the amides. Interestingly, primary and secondary amides exhibited higher activity than the parent piperazic acid. Further, amide **8j** reduced the Gli1 protein expression levels with better potency as confirmed by the Western blot analysis. Ptch1 protein expression was unaltered by these compounds, confirming the likely target of these compounds was either upstream of Ptch1 or the receptor itself (Figure 8E).

In another report, Hipskind et al⁹⁶ identified a novel molecule **8k** (LY2940680) (Figure 8F) that bound to the SMO receptor and potently inhibited Hh signaling in human MB tumor cell line Daoy, and in mouse mesenchymal progenitor cell line C3H10T1/2. Importantly, **8k** was active in resistant SMO mutant (D473H) produced by treatment with GDC-0449. Compound **8k** also had excellent PK properties in rodent and nonrodent species. Oral administration of **8k** in MB (Ptch^{+/-} p53^{-/-} transgenic) bearing mice produced significant efficacy and improved their survival. Further, **8k** treatment induced caspase-3 activity and reduced proliferation as confirmed by immunohistochemical analysis of MB tumors. Compound **8k** inhibited Hh-regulated gene expression in xenograft tumor model and resulted in significant antitumor activity. Currently, **8k** (LY2940680) is being tested in Phase I and Phase II clinical trials for advanced solid tumors and esophageal cancers.

Lu et al⁹⁷ have further optimized the lead compound Taladegib by replacing the 4-methylamino-piperidine moiety (region-A) in **8l** (Figure 8F) with various four-membered, five-membered, or six-membered azacyclic, or azaspirocyclic building blocks. Replacement of piperidin-4-amine with 2,6-diazaspiro[3.3]heptane, azetidin-3-amine, or piperidin-3-amine significantly decreased Hh signaling. However, pyrrolidin-3-amine was well tolerated. Surprisingly, use of a reversed pyrrolidin-3-amine linker as connector resulted in the loss of activity. Further optimization of 1-methyl-1*H*-pyrazole (region-B) was conducted within the pyrrolidin-3-amine derivative. Replacement of 1-methyl-1*H*-pyrazole by a phenyl ring was well tolerated. Introduction of electron-withdrawing groups, such as cyano-position, chloro-position, or acetyl-position, at the para-position of the phenyl ring resulted drop in potency.

However, an electron-donating substituent, such as paramethyl or parahydroxymethyl substitution, showed a distinct improvement in activity. Further, replacement of phenyl ring by pyridine was also well tolerated with a slight drop in activity. However, replacement with an amino aryl group, amino benzyl, or 1-methyl-1*H*-pyrazole-4-yl resulted in loss of potency. Chiral resolution of pyrrolidin-3-amine derivatives proved beneficial as the *R*-enantiomer **8n** provided a 5-fold improvement in activity compared with the racemate whereas the *S*-enantiomer exhibited significantly low activity. Compound **8m** and **8n** (Figure 8F) were found most potent in inhibition of the proliferation of MB cells isolated from Ptch^{+/-} p53^{-/-} mice (IC₅₀ = 20.1 and 24.6 nM, respectively). In Ptch^{+/-} p53^{-/-} mouse MB allograft model (daily dose at 50 mg/kg *bid* or 100 mg/kg *qd* by oral), compound **8n** inhibited the growth of allografted MB. However, in vivo efficacy of **8n** was inferior to Taladegib due to poor PK properties. Following a single 2.0 mg/kg *iv* dose to SD rat, compound **8n** showed high plasma clearance (CL = 74.7 ± 6.3 ml/min/kg), short half-life (*t*_{1/2} = 0.32 ± 0.012 hours) and relatively low oral bioavailability (F% = 7.4 ± 0.67) after PO dose (10 mg/kg) (Figure 8F).

Recently, Wang et al⁹⁸ have further optimized the structural units of taladegib by replacing the phthalazine core with dimethylpyridazine to give a scaffold **8o** (Figure 8G). For region-A, exchange of 1-methyl-1*H*-pyrazole by a phenyl ring bearing either electron-withdrawing or electron-donating groups exhibited reduced inhibition potential compared with the parent compound. However, 4-cyano or 2-chloro substituted phenyl rings resulted in a slight deduction in activity, whereas replacement with pyridine-4-yl resulted in a significant potential loss. SAR study for region-B revealed that exchange of 4-methylamino-piperidine with other five-membered or six-membered heterocycles such as pyrrolidin-3-amine or piperazine resulted in significant potency loss. For region-C, exchange of 4-fluoro-2-trifluoromethylbenzoyl group with other heterocycles such as pyridine, pyrazine, or pyridazine resulted in drastic potency loss. Interexchange of amide linker between region-B and region-C with urea and carbamate was not tolerated. Further, the trifluoromethyl substituent on the aromatic ring seemed essential for activity due to enhanced π - π interaction with Phe484. Further, introduction of a phenyl ether or phenyl thioether onto the phenyl group of region-C were tolerated with slight reduction in Hh inhibition activity. Among these compounds, compound **8p** (Figure 8G) was further tested in Ptch^{+/-} p53^{-/-} MB mouse model (10 or 20 mg/kg by oral gavage twice a day) where it inhibited the growth of allografted MB slightly more than vismodegib without significant loss of body weight.

2.6 | Vitamin D3 analogs

Vitamin D₃ (VD3) **9a** (Figure 9A) is a metabolic precursor to 1 α ,25-dihydroxy vitamin D₃ [1 α ,25(OH)₂D₃, calcitriol], which inhibits the Hh signaling pathway by binding to SMO protein. Vitamin D binds to its nuclear vitamin D receptor (VDR) and acts as a transcription factor to regulate apoptosis, proliferation, and differentiation. However, the clinical usefulness of 1 α , 25 (OH)₂ D₃ and its analogs have been limited by its tendency to promote hypercalcemia.

Considering these facts, Banerjee et al⁹⁹ have prepared several analogs of VD3 by modifying 3-(*S*)-hydroxyl group of VD3. First, oxidation of alcohol to ketone (region-A, **9a**)

decreased the Hh inhibitory activity whereas amine analogs were less active than VD3. Inversion of the stereochemistry at C-3 was allowed suggesting that the *S*-configuration of natural VD3 was not essential for activity. Interestingly, the amine epimer 3-(*R*)-**9b** and 3-(*S*)-**9c** were most active for SMO protein binding and Hh pathway inhibition (Figure 9A).

VD3 scaffold, particularly in region-A, was not sufficient to prevent its cellular activation of VDR. Thus, to determine the regions of VD3 essential for Hh inhibition, DeBerardinis et al¹⁰⁰ synthesized two new series of compounds; the first series was based on semisynthetic analogs of VD3 and second series comprises of truncated VD3 molecules. The first series where the side chain of VD3 (region-B) was altered revealed that the modifications were allowed in the side chain as modified analog VD2 **9d** (Figure 9A) equipotent in Hh pathway inhibition compared to VD3. By contrast, truncation of the side chain led to the loss of activity. This suggested that this region was available for minor modifications while retaining the chemical and hydrophobic nature of the natural alkyl side chain. The second series of the compound consisting of truncated CD-ring with a modified side chain. Compound **9e** (Figure 9A) retained the activity, whereas modification or removal of the C-8 hydroxyl group significantly reduced the Hh inhibition in the CD-ring analog suggesting its presence and orientation were important for activity. However, truncated A-ring analog **9f** (Figure 9A) was inactive in Hh inhibitory assay. Most of the new VD3 analogs did not activate VDR. In addition, all compounds induced CYP24A1 significantly less than that observed with VD3. This indicates that truncated analogs would not activate canonical VDR signaling and hold potential for selective Hh pathway inhibition (Figure 9A).

With the understanding that CD-ring alcohol derived VD3 (Grundmann's [GA] alcohol) are equipotent to VD3, and its inactivity for purified VDR, DeBerardinis et al¹⁰¹ have designed a new series of compounds **9g** (Figure 9B) in which the carbocyclic E-ring of VD3 was isosterically replaced with an aromatic ring to maintain the steric and hydrophobicity as that of natural aliphatic E-ring. The aromatic E-ring and the CD-ring were connected through the ester linkage. The new analogs bearing 3-hydroxy/aminobenzoic acid (region-A) demonstrated potent Hh inhibitory activity. However, masking of hydroxyl or amine groups by methyl or benzyl ether resulted in decreased activity. Interestingly, an analog containing 3-COOH group was a weak Hh inhibitor, like VD3. This reduced activity resulted from the extension of hydrogen-bond forming unit away from the aromatic ring, preventing key interaction at the binding site. Inhibitory activity of these analogs was attributed to the intact structures and not due to the metabolic product after cleavage of the weak ester linkage. The most active compound **9h** inhibited the Hh signaling in a dose-dependent fashion in C3H10T1/2 cells. This compound inhibited the pathway signaling in Hh-dependent mouse fibroblasts and cultured cancer cells (Figure 9B).

Further, DeBerardinis et al¹⁰² have explored the aromatic ring substituents (region-A) of the lead compound **9i** (Figure 9C). Interestingly, incorporation of the hydroxyl group in the 4-position of the aryl group resulted in potent Hh signaling inhibition, whereas shifting the hydroxyl group to 2-position resulted in the loss of activity. This might be because the 2-hydroxyl group may prevent the key interaction(s) available with the 3-hydroxy or 4-hydroxy due to increased distance or orientation. Similarly, incorporation of an amine in the 4-position decreased activity when compared with the 3-position amine. The 3-substituted

analog incorporating chlorine or masking the phenol or amine resulted in inactive analogs. Further, hetero and biaryl analog of the aromatic ring indicated that the 2-pyridyl substituted analog retained modest inhibition (Gli1 expression = 14.1%) whereas shifting the nitrogen to the 3-position or 4-position resulted in decreased potency. Five-membered heterocycles, as well as bicyclic aryl analogs, did not inhibit the Hh signaling and did not activate the VDR indicating that a six-membered ring was an optimal size for the aromatic ring mimic in region-A. Further, analogs containing multiple hydroxyl groups on the aryl or pyridine moiety, such as 3,5-dihydroxy and 3,4,5-trihydroxy benzene, demonstrated inhibition compared with the monosubstituted analogs, whereas the hydroxy-substituted pyridines displayed decreased activity. Analog **9j** (Figure 9C) was identified as the most potent Hh pathway inhibitor. Extensive in vitro (C3H10T1/2 and ASZ001; murine BCC, and DAOY; MB cells) and in vivo studies suggested that VD3 exerts its anti-Hh activity via vitamin D receptor, whereas **9j** inhibits the Hh pathway through a separate mechanism.

Using analog **9j** as a lead scaffold, further modifications in the ester bond (region-A) was explored by DeBerardinis et al.¹⁰³ Analog incorporating additional methylene in the linker region, demonstrated good potency and selectivity compared to **9j**, whereas inclusion of any further additional atom in the linker chain proved detrimental for activity. In addition, saturated linker analogs proved more active than the corresponding unsaturated analogs suggesting the requirement of flexibility in hydroxyl moiety for key binding interactions. Further, extended ester analogs significantly reduced VDR activation. Interestingly, epimeric analogs at C-8 position (region-B) of the VD3 scaffold were less active for Hh inhibition and led to significant activation of VDR. The linkerless analogs were also designed by appending the A-ring directly to the CD-ring region. However, such analogs proved moderately active against the Hh signaling pathway and were completely inactive for VDR. The related one carbon linker analog bearing a ketone-based linker and inverted stereochemistry (compared to lead compound **9j**), proved potent downregulator of Gli1 mRNA. Further, nitrogen-based linker represented a new series of compounds incorporating a hydrogen-bond forming unit into the linker region and demonstrated potent inhibition of Hh signaling. In addition, amine-linked analogs upregulated CYP24A1 at a modest level. The amide equivalent of the lead compound also maintained potent Hh inhibition, whereas its potency for upregulation of CYP24A1 was 3-fold less than the lead compound. Further in amide-linked analogs, modest Hh signaling inhibition suggested that extending the linker length in this region may help to make key interactions within the analog binding site. Compounds **9k** and **9l** were identified as potent leads bearing an amine linker and 4-hydroxyphenyl ring (Figure 9C).

Hadden and coworkers¹⁰⁴ were inspired by the observations that incorporation of extended side chain onto the piperidine ring in the steroidal natural product cyclopamine gave 10 times more potent analog KAAD-Cyc. A new series of VD3-based analogs has been reported bearing modified side chain as potent Hh pathway inhibitors. Various analogs with linear side chains were synthesized with the varied length of side chain compared to the natural VD3 chain. However, these changes were less active than lead analog **9j**. Analog retaining six-carbon chain length were still active. Introduction of a terminal short branched alkyl chain in new analogs (isopropyl or *t*-butyl) decreased activity. However, medium

length terminally branched chains (isobutyl or $-\text{CH}(\text{C}_2\text{H}_5)_2$) were equipotent to the corresponding unbranched analogs with similar side chain length. Interestingly, induction of rigidity in the side chain by the addition of an unsaturated olefin resulted in a 2-fold increase in the activity compared with the saturated analog. Further incorporation of a terminal aromatic benzyl or a primary hydroxyl group in the side chain decreased the activity. Linear or branched alkyl chains of five or six carbons were optimal for selective and potent Hh pathway inhibition. The most potent analog, **9m** revealed potent inhibition of Hh signaling in MEFs and BCC cells, compared to the lead compound **9j** (Figure 9D).

Recently, Maschinot et al¹⁰⁵ have reported the C-ring modifications at the C-11 position of **9l** and **9m** for probing the binding pocket for additional interactions. In the new analogs, the substituents varied in size from small ethyl to benzyl to propargyl groups. There was a sharp loss in activity as the size of the functional group changed from ethyl to cyclohexyl and suggested a narrow binding pocket. However, the introduction of hydrophilic groups at this position led to the restoration of the inhibitory activity. Interestingly, for C-11 analogs of **9m**, only small hydrophobic substitutions, such as an ethyl group in the α -orientation (as in compound **9n**) retained the anti-Hh activity. By contrast, the analogs with a hydrophilic β -substituent retained the activity. Overall this study concluded no significant potency gains. However, the addition of ethyl group at C-11 position improved the selectivity for the receptor (Figure 9D) pathway.

To gain in-depth understanding of GA's alcohol and its analogues as Hh inhibitors, Wen et al¹⁰⁶ recently reported the synthesis and Hh inhibitory potential of GA linked to a substituted phenyl or benzyl ring via an ether or thioether linker. For phenyl-substituted GA isomers and corresponding ether-linked 4-aniline analogues demonstrated potent anti-Hh activity in C3H10T1/2 cells, whereas precursor 4-nitro derivatives were inactive. The most potent analogue, **9o**, has 3a*R*,4*R* hexahydroindane scaffold and showed slight upregulation of Cyp24A1 relative to VD3 suggesting the selectivity of ether-linked analogues. Further, optimization of 3a*R*,4*R* hexahydroindane scaffold was made by altering the substitutions on the aromatic ring attached via either linkage. Compounds bearing phenol or anilines at *meta*-position or *para*-position showed potent anti-Hh activity, whereas masking of these groups demolished the activity. Among phenols, 3-hydroxy-benzyl or 4-hydroxy-benzyl, both compounds showed comparable potencies. Similar trend in activity was observed for amine-containing analogues suggested that *para*-substituents and *meta*-substituents were well tolerated in the phenyl ether class of VD3 analogues. However, more potent anti-Hh activity was observed in the 4-aniline derivative relative to 4-hydroxy analogue. Among the *meta*-phenols and *para*-phenols with the 3a*R*,4*R* orientation, both compounds were equipotent but less active than the corresponding aniline analogue. Further, GA's thiols were found completely inactive, whereas its thioether derivative was almost 10 times less potent than the corresponding ether analogues signifying the importance of C4 hydroxy group of GA for the anti-Hh activity. Preliminary in vitro PK profiling of few potent compounds suggested that the ether-linked analogues were stable and had enhanced solubilities in phosphate buffered saline (PBS) at pH 7.4 and 4.0.

The cellular mechanisms through which the *seco*-steroid class of compounds exhibited potent anti-Hh signaling pathway was recently investigated by Thompson et al.¹⁰⁷ The new

investigations confirmed the major role of canonical Hh and VDR signaling to inhibit the Hh signaling pathway. The synthetic VD3 analogues (*seco*-steroids), but not VD3 or calcitriol, also regulated target genes associated with TGF- β signaling in Hh-dependent mode. Additionally, these activities were not correlative with the ability of the *seco*-steroids to bind TGF- β R1 or TGF- β R2 thus, suggesting the involvement of some unidentified binding factor in the mechanism.

Vitamine D3 analogs are in their primitive stage of development and need much more concerted efforts to be developed as a potent Hh pathway inhibitor. Many of the compounds have shown promising activities in cell culture-based assays. However, it would be interesting to evaluate these compounds in *in vivo* models.

2.7 | Piperazine analogs (itraconazole analogs)

Piperazine is a six-membered, heterocyclic, two nitrogen-containing saturated basic system. One of its derivatives, itraconazole is a clinically used broad-spectrum antifungal drug. Changing its structure for enhancing Hh inhibition potency and reducing the possible side effects are now being explored extensively. This section deals with some important analogs of itraconazole described as potent Hh inhibitors.

Itraconazole **10a** (Figure 10) is a clinically used broad-spectrum antifungal drug. Alteration in the structure of clinically used drugs to explore their side effects is a well-known practice. This strategy may reduce the overall period to appear any such drug in the clinic for its alternate use. Itraconazole possesses potent anti-Hh pathway activity acting on SMO by different mechanism from other known SMO antagonists.¹⁰⁸ Itraconazole resembles other azole antifungals, such as terconazole and ketoconazole. Although their equipotent antifungal activity compared to itraconazole, these drugs do not exhibit antiproliferative activity. The only structural differences between them are with respect to the triazolone ring and its *sec*-butyl side chain. Based on these observations, Shi et al¹⁰⁹ have designed and synthesized a library of itraconazole analogs, compound **10b** (Figure 10) in which the *sec*-butyl side chain (region-A) was modified and evaluated for Hh signaling inhibition as measured by Gli1 transcript levels. The modified analogs revealed that a side chain with more than three carbons (up to *n*-octane) without polar groups or branching at the β -carbon provided strong inhibition of the Hh pathway. Further, small bulky groups were allowed at the distal end of these extended side chains, but not large groups such as benzophenone. Branching in the side chain on the α -carbon or γ -carbon was also allowed. Hh pathway inhibition was also favored in analogs containing a terminal alkyne and alkene or alkyne linkages between γ and δ carbons. From this series, many compounds were potent inhibitors of Hh pathway, particularly analog **10c** (Figure 10).

In itraconazole **10a**, the triazole moiety can interact with the heme of CYP 3A4 leading to multiple drug-drug interactions and adverse side effects. Pace et al¹¹⁰ have reported a new series of itraconazole analogs to identify the functionalities required for anti-Hh and anti-angiogenic properties. The new compounds revealed that absolute stereochemistry at the 2'-position (region-A) of the *sec*-butyl side chain did not significantly affect the activity, whereas removal of the methyl group from the side chain reduced the activity. Interestingly, removal of the triazolone/side chain did not affect the Hh inhibitory activity. Regarding

modifications to the region-B, removal of the chlorine atoms from the phenyl ring did not affect the activity, whereas removal of the phenyl ring decreased activity. Elimination of the triazole did not affect the Hh inhibitor activity. Removal of both the triazole and phenyl rings significantly reduced the activity. In the stereochemically defined analogs, the *trans* orientation of the dioxolane region gave potent compound, specifically the *trans* 2*R*,4*R* analogs. Based on the ability to down-regulate endogenous Gli1 mRNA expression in MERP MB cell line, analog **10d** exhibited most potent activity (Figure 10).

2.8 | Cyclopamine and related steroidal analogs

2.8.1 | Cyclopamine analogs—Steroids are fused carbocyclic systems with four rings arranged in a specific molecular configuration. Among these four fused rings, three are six-member cyclohexane rings, and one is five-member cyclopentane ring. Steroids represent one of the most potent biologically active compounds found widely in nature. This section is dedicated to the remarkable potency of steroids as Hh inhibitor.

Cyclopamine **11a** (Figure 11A) is a natural product isolated from *Veratrum californicum* (Melanthiaceae), which acts directly on the SMO protein and has well-known antitumor activity in several xenograft models. However, its use is limited because of poor aqueous solubility (~5 µg/mL), low metabolic stability and moderate potency.¹¹¹ Cyclopamine readily converted to inactive veratramine **11b** (Figure 11A), because of an acid-catalyzed opening of the spirotetrahydrofuran *e* ring followed by rapid aromatization of the *d* ring. To overcome this limitation, Tremblay et al¹¹² have reported modified cyclopamine analogs with improved water solubility and chemical stability. To improve the acid stability of cyclopamine, the influence of the *d*-ring allylic ether on the hydrolysis of the spirotetrahydrofuran *e*-ring was analyzed, while minimal change to the *d*-ring geometry. For this, synthesis of new ring-expanded (region-A), *d*-homocyclopamine analog were synthesized. However, these were weaker than cyclopamine in Hh inhibition assay. Further, Oppenauer oxidation of enol gave enone analog **11c** (Figure 11A) which was equipotent to cyclopamine and was further selected for side chain modification at region-B. The study around enone **11c** revealed that basicity of the *f*-ring nitrogen was a key element in determining the binding to cyclopamine target. However, the *N*-acetamide analog displayed good potency. Relatively bulky alkyl groups were detrimental to activity, whereas elongated appendages, such as the glycolate analogs had potency similar to that of cyclopamine and analog **11c**. Interestingly, the desmethyl analogs had significantly reduced biological activity. More importantly, analog with side chain, such as CH₂CH₂NHCO(CH₂)₅NHCOCH₂CH₂Ph (KAAD-cyclopamine **11d**) (Figure 11A), had the hydrophobic chain and was many folds more potent. A truncated version of this side chain was also proved beneficial and provided a 10-fold increase in potency. However, hydroxylamine as side chain displayed many folds decrease in potency compared to enone **11c**. The new *d*-homocyclopamine analog **11c** possessed enhanced stability in simulated gastric fluid compared to cyclopamine. Compound **11c** showed excellent exposure (80% oral bioavailability) after oral administration in mice. It was cleared from plasma with an elimination half-life (*t*_{1/2}) of 3.2 hours and had a large *V*_d of about 18 L/kg (Figure 11A).

Tremblay et al¹¹³ have further optimized the core structure **11c** which led to the discovery of more potent cis-decalone analog **11e** (Figure 11B). The presence of polar functionalities at the C3' position (in *R* configuration) provided analogs with best Hh inhibitory activity, whereas more hydrophobic deoxygenated analogs proved less potent than ketone **11e**. Methyl oxime analog was less active than oxime, which suggests the preference for a hydrogen-bond donor. The oxime was as active as ketone **11e** in the differentiation assay, but with less metabolic stability. Compounds with primary amines at C3' position were weak inhibitors, whereas the amide and sulfonamide analogs (with *R* configuration) were potent inhibition of Hh pathway. Particularly, sulfonamides were superior to the amides, because of lower pKa of the *N*-H and larger steric volume of the sulfonamide group. Importantly, the C3'-(*R*)-sulfonamide was equipotent to the C3'-(*R*)-alcohol and with improved metabolic stability in human liver microsome assay. To further improve the metabolic stability of the C3' ketone in compound **11e**, the introduction of heterocycles, such as pyrazole and isoxazole fused onto the A-ring (region-A) was envisaged. The position of the pyrazole on the A ring has a significant effect on the potency and metabolic fate, as C2'-C3' fused analogs are superior than C3'-C4' fused analogs. The nature of the heterocyclic ring influenced the biological activity, as pyrazole being favored over thiazole, oxadiazole, and isoxazole. In another approach to modifying A-ring, a seven-membered ring lactam analog and a *N*-methyl lactam analog were prepared. However, the lactam was only slightly less potent than the pyrazole and sulfonamide analog whereas an *N*-methyl lactam showed low potency compared with the lactam. After extensive SAR around the A-ring of reduced *d*-homocyclopamine, compound **11f** (IPI-926) (Figure 11B) was identified as a novel Hh antagonist with improved pharmaceutical properties and potency, as well as a better plasma PK profile [$F(\%) = 50\%$, $Cl = 0.66$ L/h/kg, $V_d = 15.3$ L/kg, $t_{1/2} = 15.5$ hours in Beagle dog (4.0 mg/kg, PO)].

2.8.2 | Steroidal analogs—The difference in the orientation of the nitrogen atom (region-A) relative to the steroid plane in cyclopamine and tomatidine **12a** (Figure 12A) has been considered a major factor contributing in teratogenicity of cyclopamine while tomatidine being a nonteratogenic.^{114,115} Based on these observations, Winkler et al¹¹⁶ have designed a series of new compounds where the *C*-nor-*D*-homo steroidal ring system of cyclopamine functions as a scaffold for the orientation of the heterobicyclic framework of the *e,f* rings relative to the *C*-3 β oxygen functionality in cyclopamine. Replacement of the *C*-nor-*D*-homo steroidal system with the androstane ring system gave compound **12b** (Figure 12A). This compound lacks the *C*-nor-*D*-homo ring system of cyclopamine and exhibited significant homology with cyclopamine. Further, stereochemical simplification of compound **12b** was achieved by aromatization of ring *a* and *f* to give compound **12c** (Figure 12A). However, the three-dimensional (3D) overlap of compound **12c** was not as good as that of compound **12b** with cyclopamine, but the ease of synthesis of **12c** led to the synthesis and biological activity of bis-aromatic analog **12c**. In ligand-induced Shh signaling inhibitory activity in a luciferase-based assay, steroidal analog **12c** (10.0 mM), was equipotent to cyclopamine in inhibiting Shh-induced granule neuron precursors (GNP) proliferation.

Further, Zhang et al¹¹⁷ have reported the synthesis of *f*-ring saturated analogs of **12c**, which has the same stereochemistry at C-22 and C-23 as cyclopamine. Biological evaluation of new compounds **12d** and **12e** (Figure 12A) revealed that both compounds are potent inhibitors of Hh signaling and compared to cyclopamine in potency in Shh-Light 2 cells assay.

Efforts have focused on identifying the pharmacophore responsible for high activity and metabolic stability of cyclopamine analog **12c** and related structures. The energy-minimized structures of cyclopamine and its analogs suggested an important role of C-17 stereochemistry. The C-17 oxygen atom of compound **12f** (the C-17 epimer of **12c**) (Figure 12A) was oriented on the α -face of the steroid plane, which led to the orientation of the *f*-ring nitrogen atom of **12f** on the β -face of the steroid plane. This is the same orientation as found in tomatidine. Further, the role of C-3 oxygen, C-17 oxygen, and C-21 nitrogen atoms in analog **12f** was evaluated for Shh activity in Light 2 cells at 5.0 mM concentration, which inhibited signaling (approximately 3-fold increase in Hh signaling inhibition compared with cyclopamine). This result contradicted the three-point recognition hypothesis, where the C-3 oxygen and each of the other *e* and *f*-ring heteroatom functionalities were required for potency. Structures with either orientation at C-17, as in compounds **12c** and **12f**, were potent inhibitors of Shh signaling. The orientations of the oxygen (in tetrahydrofuran) and nitrogen (in pyridine) relative to the steroid plane did not contribute in activity. The role of C-3 oxygen functionality in analog **12c** and **12f**, the C-3 deoxy compound **12g** (Figure 12A) was synthesized. Biological evaluation of **12g** using a luciferase assay revealed a potent inhibition of Shh signaling (80% inhibition at 5.0 mM; compared to 70% inhibition with **12c**). Analog **12g** acts on the Shh pathway and significantly decreased the Gli1 expression in human MB cells. The remarkable potency of the analog **12g** indicated by the two-point interaction (of the C-3 β hydroxyl and the *e,f* heterobicyclic ring) concluded that the 3D structures of cyclopamine was not a critical feature for activity, thereby offering scope to further simplify the structure of the cyclopamine (Figure 12A).¹¹⁸

Further, Winkler et al¹¹⁹ have simplified the structure of analog **12g** by systematic truncation of the steroidal framework. For this, compound **12h** (Figure 12A) was synthesized, which lacks the *a, b* ring system present in analog **12g**. However, biological evaluation of compound **12h** using the Shh-Light 2 cells luciferase-based assay revealed that the analog **12h** failed to inhibit Hh signaling, suggesting the *a, b* ring system is essential for activity. New analogs **12i** and **12j** (Figure 12A), in which aromatic *a*-ring was present but lack the *b*-ring of the analogs **12g** were synthesized. The C-3 hydroxy containing analog **12j** was more potent compared to compound **12i**, which contrasts with the previous observation where C-3 hydroxyl containing analog **12f** was more potent. Further investigation revealed that analog **12d** was a potent Hh signaling inhibitor as demonstrated in the GNP proliferation assay (approximately three times more potent than cyclopamine). In the DAOY MB cell viability assay, it was equipotent to cyclopamine (Figure 12A).

Several steroidal alkaloids, such as solanidine and veramiline, were known for their teratogenic activity. However, only a few reports are available for their potential as Shh signaling modulator. Guerlet et al¹²⁰ have described aza-steroid analog of cyclopamine

bearing a piperidine ring at C22 as in compound **12k** (Figure 12A). The biological evaluation of compound **12k** for the Shh pathway inhibition using the Gli-dependent luciferase reporter assay in Shh-Light 2 cells revealed that desmethylveramiline **12k** is a potent inhibitor of Shh signaling pathway. These results demonstrate that desmethylveramiline, without much structural similarity to cyclopamine, can inhibit the Shh signaling.

Chatzopoulou et al¹²¹ have taken the lead from a natural product taepeenin D **12l** (Figure 12A), a cassane-type diterpenoid isolated from *Caesalpinia crista* (also isolated from *Acacia pennata*) capable of inhibiting Hh-mediated Gli transcription activity ($IC_{50} = 1.6 \mu M$) Hh reporter cells and displayed selective cytotoxicity against cancer cells with increased Hh signaling in PANC1, DU145, and C3H10T1/2 cells ($IC_{50} = 3.2 - 3.4 \mu M$).¹²² The compound lack C-14 methyl substitution but bearing, respectively, a hydroxy or an acetoxy substituent at C-6, which inhibited the Gli transcription. However, no inhibition was observed with a compound that lacks C-6 substitution. Interestingly, dihydrobenzofuran bearing analog was found inactive despite the presence of 6-acetoxy moiety. These results highlight the importance of benzofuran moiety and demand further study of taepeenin D analogs with different electronic/steric on the aromatic region. The removal of the C-14 methyl substitution lead to structural simplification of taepeenin D, since it allowed an expedient entry to related analogs exploiting the chemistry of abietic and podocarpic acid. From these series, compounds **12m** and **12n** were most potent inhibitors compared with cyclopamine (Figure 12A).

Besides these, some oxyterol (OHC) base molecules also demonstrated Hh modulation. Recently, Dash et al¹²³ have reported a molecular dynamics-based approach to identify OHC-based Hh pathway inhibitors. In this study, various computational approaches, such as molecular dynamic simulations and MM/GBSA energy calculations, were applied and identified compound **12o** (Figure 12B) as a potent inhibitor of the Hh signaling pathway. These compounds stabilize the SMO by interacting with its *N*-terminal cysteine-rich domain (CRD). Another oxysterol, compound **12p** (Figure 12B), was identified by Nachtergaele et al¹²⁴ that maintained its ability to inhibit Hh pathway activity even in the presence of Hh activator, SAG in vitro. More importantly, compound **12p** demonstrated potent inhibition of Hh signaling in a clinically relevant mutant form of SMO resistant to the currently FDA approved vismodegib (Figure 12B).

Extensive preclinical studies revealed that cyclopamine has the potency to inhibit the growth of various tumors, such as human glioma, melanoma, colon, pancreatic, prostate cancers, small cell lung cancer, and MB. Topical cream of cyclopamine was also found effective in inhibiting human BCC. Cyclopamine derivatives including KAAD-cyclopamine and IPI-926 (Saridegib) showed improved metabolic stability, PK profile, and potency over cyclopamine. IPI-926 in a single oral dose (130.0 mg) in BCC patients showed a half-life of 19.3 hours and $AUC_{(0-24)}$ of 823.0 ng h/mL.¹²⁵ In in vivo studies, IPI-926 effectively attenuated tumor growth in MB, chondrosarcoma, and pancreatic cancer. However, clinical trial (Phase II) of IPI-926 for pancreatic cancer or myelofibrosis were terminated due to its safety-related issues and poor clinical benefits.⁵³

Hh signaling modulates I/R injury in cholestatic rat liver as described in a study by Pratap et al,¹²⁶ inhibition of Hh signaling with cyclopamine before ischemia-reperfusion (I/R) prevented liver injury. Seven days after CBDL, rats were exposed to either cyclopamine or vehicle for 7 days daily before being subjected to 30 minutes of ischemia and 4.0 hours of reperfusion. Preconditioning with cyclopamine reduced the histological damage and serum liver injury markers and reduced cholangiocyte proliferation, and apoptosis. Cyclopamine suffers from poor water solubility and stability thus needs formulation for clinical translation. Cyclopamine and Gefitinib play a key role in treating tumors with aberrant activation of Hh and EGFR signaling. Chitkara et al⁶ have reported that the micelles formed by mPEG-*b*-P(CB-*co*-LA) copolymer efficiently deliver cyclopamine and gefitinib to tumors by EPR effect. Combination therapy showed a synergistic effect on L3.6pl cells and increased caspase 3/7 activity and increased apoptotic cell death. Combination of these two drugs decreased tumor growth rate significantly in L3.6pl-derived xenograft mouse tumors.

Chemoresistance may arise due to repeated treatments of cancer stem cells (CSCs) and results in therapy failure. Combination of cyclopamine and paclitaxel (PTX) has been shown to efficiently inhibit PTX-resistant CSCs cells.¹²⁷ In another study, Yang et al¹²⁸ have utilized the mPEG-*b*-PCC-*g*-PTX-*g*-DC (P-PTX) and mPEG-*b*-PCC-*g*-CYP-*g*-DC (P-CYP) polymer-drug conjugates for codelivery, which were self-assembled into micelles. The combination of P-PTX and P-CYP alleviated PTX resistance and suppressed tumor colony formation, and upregulated tumor suppressor miRNAs. Further, P-PTX and P-CYP combined micelles significantly inhibited chemoresistant prostate cancer growth compared to monotherapy. Additionally, P-CYP and microtubule stabilizer docetaxel (DTX) as polymer-drug conjugates, P-DTX could synergistically inhibit orthotopic pancreatic tumor growth in NSG mice.⁸³

In another study, Olive et al⁶⁸ found that coadministration of IPI-926 and GEM resulted in enhanced GEM efficacy by increasing tumor perfusion. IPI-926 resulted in stromal depletion and increased intracellular concentration of GEM resulting enhanced anticancer efficacy.

2.9 | Benzimidazoles

Benzimidazole is a nitrogen-containing heterocyclic compound composed of a fusion of benzene and imidazole ring. Many benzimidazoles containing drugs are clinically used specifically for their anthelmintic properties. Here in this section, we have discussed some important benzimidazole analogs as potential Hh inhibitors.

SANT-2 **13a** (Figure 13A) is a benzimidazole bearing compound with high potency to inhibit the Hh signaling pathway in nanomolar concentration in both wild-type and oncogenic SMO.²⁹ To establish the synthetic route to SANT-2, and to search for potential new Hh inhibitors, Büttner et al¹²⁹ have altered the pattern of substitution particularly at the phenyl ring (region-A). This SAR study revealed that the benzimidazole core is essential for Hh inhibitory activity. Substituents on the phenyl ring in region-A were of minor relevance. This study concluded that the modifications of aryl moieties, particularly the benzimidazole core in SANT-2 reduces the ability of analogs to inhibit the Shh signaling pathway. Compound TC132 **13b** was identified as the most potent compound in this study (Figure 13A).

Munchhof et al¹³⁰ have reported optimization study of benzimidazoles **13c** (Figure 13B), which possess significant SMO inhibition potential. To address the limitations such as poor metabolic stability, low aqueous solubility, and high plasma protein binding, compound **13c** was segregated in three fragments: benzimidazole (region-A), phenyl (region-B), and aminoacylaryl (region-C) groups for exploration. A phenyl group contributes to a significant amount of lipophilicity, an alternative option, such as cyclohexyl, was explored and among many analogs, (1*R*,3*S*)-isomer **13d** (Figure 13B) emerged as promising analog in the Gli-luciferase reporter assay. Other heterocycles, such as pyrrolidines and piperidines, as phenyl ring replacements resulted in piperidine analog **13e** (Figure 13B) about 2-fold more potent than analog **13d**, whereas the other diastereomers are many folds less potent than **13d**. Compound **13e** was stable in rat and human microsomes and was cleared rapidly (>70 ml/min/kg) after oral dose in rats. To further improve the potency and in vivo PK properties of compound **13e**, a replacement for the 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxy group on the 4-amino piperidine proved most effective. Replacement of the amide with the substituted urea with small alkyl, cyclic alkyl, and aryl groups was also tried. Among them, phenyl-substituted analog was identified as an attractive lead for further investigation. Further, substitution on phenyl ring at the 2-position was not tolerated whereas substitution at 3-position and 4-position led to improved activity. Substitution of the electron-withdrawing group at the 4th position of the phenyl was preferred whereas replacement of phenyl with heterocyclic systems such as pyridyl diminished the potency. Interestingly, methylation of either of the urea nitrogen gave inactive compounds. Among all the newly synthesized compounds, compound Glasdegib **13f** (Figure 13B) was most potent in Gli-luciferase reporter assay. Additionally, it did not interact with CYP450 isoforms and did not show affinity for glutathione. A permeability study in Caco-2 cells indicated that the compound **13f** is a *p*-glycoprotein substrate. Further, PK studies demonstrated that the in vivo clearance was in line with in vitro microsomal data, and volumes of distribution were moderate, with oral bioavailabilities of 33% and 68%, in rat and dog, respectively. The preclinical in vitro and PK data suggest that compound **13f** had low plasma clearance (1.03 ml/min/kg), a moderate volume of distribution ($V_d = 2.7$ L/kg), the half-life ($t_{1/2}$) of 30 hours, and oral bioavailability ($F\%$) of 55% in humans (Figure 13B).

Compound MRT-10 **13g** (Figure 13C) was identified as a potent Hh signaling inhibitor by virtual screening of a commercial library. Inspired by the structural features of compound **13g** and glasdegib **13f**, Sun et al¹³¹ have designed a new series of compounds with general structure **13h** (Figure 13C) with the aim to improve its Hh inhibition potential. The newly designed compounds may be divided into region-A, region-B, and a connector (region-C) for optimization. The connector (acylthiourea or acylurea) between region-A and region-B was retained. In region-B, the addition of 2-chloro at the phenyl ring (at R²) slightly increases the activity. The addition of fluorine at 2-position or 4-position had a significant impact on the activity. Further, oxidation of acylthiourea (region-C) led to significant enhancement of the antiproliferative activity. For region-A, the addition of a chlorine atom or a methyl group at R¹ was well tolerated at the 4-position of the phenyl ring. The study identified compound **13i** as a potential lead. However, it is inferior in potency to GDC-0449 suggesting that compound **13i** was a promising template to develop new Hh inhibitor (Figure 13C).

Further, Chen et al¹³² have reported a tetrahydrothieno[3,2-*c*]pyridine-based new potential Hh signaling pathway inhibitors **13j** (Figure 13D). They initially focused their attention to the compounds bearing various basic substitutions at the carbonyl group (region-A). It was found that cyclic amines like pyrrole, methylpiperidine, morpholine, 4-hydroxypiperidine, and piperazine showed better activity compared with aliphatic amines. Among all these amines, piperazine, and anilines had the most potent activity. Exploration of the 4-position of the piperazine revealed that addition of small alkyl groups like ethyl or methyl gave moderately active compounds. However, 4-(methylsulfonyl)-piperazine showed promising potency. Surprisingly, the introduction of an amide group at 4-position of the piperazine decreased activity. Further, bulky amides like Boc analogs showed many folds low activity indicating unacceptability for a carbonyl or bulky groups at this position. Also, *N*-phenyl piperazine displayed potent activity. Introduction of halogen atoms onto the phenyl ring had low potency. Interestingly, 3,4-dichlorophenyl piperazine displayed improved activity. Other electron-donating substituents, such as the methoxy at the 2-position or 4-position, reduced the potency. Replacement of the phenyl ring by a pyridinyl resulted in moderate activity, whereas addition of second nitrogen to the heteroaryl improved the activity. For a simple aniline analog, introduction of methyl or ethyl on aryl ring reduced the activity. The influence of aromatic ring (region-B) substitution in the core region was also explored, revealing that the chlorine atom was most suitable at this position and afforded good potency in the Gli-luciferase reporter assay. The result of the present study showed that compounds based on the tetrahydrothieno[3,2-*c*]pyridine template produced good Hh inhibition by antagonizing SMO particularly compound **13k** (Figure 13D).

In phase I study of glasdegib, it was found to be safe, tolerable, and with potential efficacy in acute myeloid leukemia, myelodysplastic syndrome, myelofibrosis, chronic myelomonocytic leukemia, and advanced solid tumors.⁵³

2.10 | Medium size/macrocyclic rings

Medium size rings are composed of seven to eight-membered cyclic systems (including heteroatoms), whereas cyclic systems with nine or more members are considered as macrocyclic rings. Some important medium size/macrocyclic ring containing compounds are discussed in this section as potential Hh inhibitors.

Polyketide synthase-derived macrolactones, such as puromycin, erythromycin, and enterobactin epothilones, have rich structural and biological diversity. Peng et al¹³³ have reported the synthesis of macrolactones leading to the development of new and potent small-molecule probe for the Hh signaling pathway. Previously, they have reported the screening results of macrocycle **14a** (Figure 14A) that displayed concentration-dependent binding to Shh N-terminal peptide (ShhN). They have exploited 1,2-amino alcohols as simple templates upon which 12-membered, 13-membered, and 14-membered macrocycles were synthesized. Compound **14a** showed low ability to inhibit Gli expression. Modifications in the substituents of the macrocyclic core of compound **14a** resulted in improved activity. The synthesis of 12-membered ring analogues led to the identification of robotnikinin **14b** (Figure 14A), the most active compound of the series. Robotnikinin acts upstream of SMO, binding directly to Shh. Further, by inserting a methylene unit into the scaffold of

robotnikinin and reversing the orientation of the two stereogenic centers, Gli suppression was completely abolished. Next, stereochemically and skeletally related compounds were tested in the Shh-Light 2 Gli reporter assay. The stereochemically inverse of robotnikinin (*2S,6R*) had reduced the activity, whereas the corresponding 14-membered compound (*2S,6R*) had complete loss of activity in the same assay. Another 14-membered analog of robotnikinin (*2R,6S*), was found without detectable Gli repression. Alteration in the stereochemistry to the (*2R,6R*) configuration with the identical 12-membered ring size provided a significant drop in activity. Addition of another methylene unit to the macrolactone scaffold with (*2R,6R*) did not change the activity appreciably, whereas the 14-membered macrolactone analog (*2R,6R*) had significantly decreased potency. The robotnikinin diastereomer (*2S,6S*) was moderately an active inhibitor. The related 13-membered macrolactone (*2S,6S*) resulted in decreased activity relative to robotnikinin. The 14-membered analog (*2S,6S*) was cytotoxic at low concentration and had poor solubility in surface plasmon resonance (SPR) studies. Many compounds in this study bound with purified ShhN and verified by SPR experiments. Furthermore, the apparent off rate of robotnikinin was significantly longer than that of its (*2S,6R*) 13-membered homolog explaining the fact that both compounds bind to ShhN, but one lacks significant activity (Figure 14A).

To further improve the potency of robotnikinin **14b**, Dockendorff et al¹³⁴ have reported a new series of macrocyclic structure **14c** (Figure 14A). For the preparation of macrocyclic analogs of robotnikinin, a build/couple/pair strategy was used that afforded rapid access to diverse analogs. Methylation of macrocyclic nitrogen (region-A) gave a slight improvement in activity as compared to unsubstituted analog whereas inversion of stereochemistry at the 2-position decreased the potency. Substitution of methyl or benzyl was well tolerated at the 11-position and gave compounds with good activity whereas 11-isopropylamino-substituted analog was weakly active. Modifications of the olefin (region-B) are also tolerated, and the *Z*-olefin (minor product of a metathesis reaction) proved more potent than the *E*-olefin analog. Saturated analogs were as active as unsaturated analogs. The presence of macrocyclic ring was essential for activity as noncyclic dienes were inactive. At position 6 (region-C), a lipophilic chain was necessary for potency as truncated analogs were inactive or were only partial inhibitors. Analogs possessing a trifluorobutyl or amides were active, suggesting that the amide moiety was not critical for activity. Analogs with 4-chlorobenzylamide were potent inhibitors, whereas those with 3-chlorobenzylamide were even better in potency. The macrocyclic carbamate was also prepared to eliminate the chiral center at the 6-position. However, such analog showed poor activity. Analog **14d** (Figure 14A) emerged as a potential lead compound. After re-examining the SAR for 2-position (region-D) of the new scaffold, the potency was maintained with cyclohexyl or benzyl groups. However, aliphatic alkyl groups such as *n*-butyl or isopropyl analog proved very weak in activity compared with the parent compound **14d**. Further, compounds bearing electron-withdrawing groups at 4-position of aryl ring gave most active compounds of the series particularly the 4-chloro bearing analog. Replacement of aromatic ring with heteroarene proved that unsuccessful indicating important role of the hydrophobic aromatic ring at the 2-position of the scaffold. The most potent compound from this study was the 4-chlorophenyl analog **14e** and in competition studies with the SAG and purmorphamine, it

was active in a *Ptch*^{-/-} cell line. Compound **14e** (BRD-6851) seemed to act predominantly as an SMO antagonist. It contrasted with the mechanism of action of robotnikinin (Figure 14A).

Tropane, an 8-azabicyclo[3.2.1]octane is the structural core of more than 600 biologically active alkaloids. Narayan et al¹³⁵ have developed a first catalytic enantioselective approach to assess tropane scaffold **14f** (Figure 14B) through a CuI-catalyzed [3 + 2] cycloaddition of 1,3-fused cyclic azomethine ylides and nitroalkenes. Members of this series represent the first example to modulate the Hh signaling pathway by tropane alkaloids and their analogs. The Hh pathway inhibition in mouse embryonic mesoderm fibroblast C3H10T1/2 cells, most of the compounds from this series were potent inhibitors with IC₅₀ values in the low μ m range for several compounds particularly, compounds **14g** and **14h** (Figure 14b).

Schaefer et al¹³⁶ have screened a compound library in a cell-based assay using Shh-Light 2 cells. From a library of 6700 compounds, primary screening data resulted in the identification of eight-membered ring skeleton as active lead. From this screening, compound BRD50837 **14i** (Figure 14C) was found with high potency with good solubility in PBS (64.3 μ M). New analogs of compound **14i** were synthesized by varying the attachments on the aniline and the extra-annular amine as well as removing the extra-annular alcohol. Changes on the aniline moiety (region-A) of the compound were well tolerated. However, activity was optimal with saturated ring systems (cyclohexyl or cyclopropyl). The cyclopropyl analog, such as in BRD50837, has better solubility in PBS than the cyclohexyl analog, whereas complete removal of the aniline moiety resulted in the loss of activity. Analogs with urea instead of amides were active but displayed low solubility. Changing the sulfonamide group (region-B) from 4-chlorobenzene sulfonyl chloride to 2-chlorobenzene sulfonyl chloride or 3-chlorobenzene sulfonyl chloride reduced activity, whereas analogs without a 4-chloro atom or with a methyl group had reduced activity. Replacement of sulfonamide with a tertiary amine led to decreased activity. Removal of extra-annular alcohol (region-C) resulted in the loss of activity. Other related analogs, having eight-membered or nine-membered rings with similar structural elements to BRD50837 were inactive. In this study, compound BRD50837 **14i** and BRD9526 **14j** (Figure 14C) were selected for further study. The extensive study suggests that BRD50837 and BRD9526 may function by mechanisms distinct from cyclopamine and not easily described by traditional linear models of the pathway.

Recently, Liu et al¹³⁷ optimized the core skeleton of the lead compound **14j** through cyclization of two side substituents of the bicyclic skeleton combined with Natom. The new tricyclic compounds were evaluated for the Hh signaling pathway inhibition by dual luciferase reporter assays with Shh-Light 2 cells. A SAR study around the benzo [*b*]pyrido[4,3-*g*][1,5]oxazocin-7(12*aH*)-one **14k** (Figure 14D) skeleton bearing 4-chlorophenyl sulfonyl (region-A) and cyclopropanecarboxamido moiety in region-B revealed that *N*-methylpiperidin-4-yl or 4-methoxybenzyl as R¹ in region-C was optimal and showed comparable Hh inhibition with the lead compound **14j**. However, a small hydroxyalkane as side chain proved detrimental for the activity. Screening of various sulfonyl or aryl acyl groups in region-A, revealed that aliphatic, cyclic, or heterocyclic sulfonyl (such as 4-methyl piperazine-1-sulfonyl) were unsuitable, whereas aryl sulfonyl as

substituent provided potent compounds. Further, aryl sulfonyl bearing electron-donating substituents provided weak Hh pathway inhibitors, whereas electron-withdrawing groups gave access to potent compounds. Further, heterocyclic sulfonamides also proved beneficial, whereas benzoyl-substituted analog was inactive. Interestingly, a strong electron-withdrawing group bearing carbamate also provided potent compounds.

The screening of 2,3,4,4a,5,6-hexahydro-1*H*-benzo[*b*]pyrido[3,4-*g*][1,5]oxazocin-(12*aH*)-ones **14k** (where N2-atom is walking to N3 position) revealed that 4-chlorosulfonyl group was favorable in region-A providing the most potent compound **14n** (Figure 14D), whereas other *N*-substituted analogs had low potency. Interestingly, chiral resolution of racemic **14n** proved beneficial as the (+)-enantiomer **14n** with 4*aR*,12*aS*-configuration retained the high potency, whereas, the (–)-enantiomer was nearly 17-fold less potent. Further, ring-expanded analogs bearing a nine-membered 1,5-oxazonan-6-ones **14l** (Figure 14D) or ring contracted analogs 3,4-dihydrobenzo[*f*] [1,4]-oxazepin-5(2*H*)-ones **14m** (region-B) (Figure 14D) were weak inhibitors of Hh pathway despite bearing similar substitutes as of the most active compound. Further, compound **14n** was a selective inhibitor of the Hh signaling pathway in both SMO wild-type and its two major mutants (SMO D473H and SMO W535L). Compound **14n** inhibited the proliferation of MB cells isolated from *ptch*^{+/-}*p53*^{-/-} mice and showed significant tumor growth inhibition after intraperitoneal injection into the *ptch*^{+/-}*p53*^{-/-} MB allograft mouse model at the dose of 25 or 50 mg/kg twice a day for 15 consecutive days (Figure 14D).

None of the medium size ring containing compounds has advanced into the preclinical studies.

2.11 | Biphenyl derivatives

The cross-coupled product of two aromatic rings is generally known as biphenyl systems. The biphenyl system plays a key role as a synthetic intermediate for many clinically used drugs. This section deals with some of the biphenyl systems possessing potent Hh inhibitory potential.

Peukert et al¹³⁸ have screened a small compound collection and identified *ortho*-biphenyl carboxamides particularly LAB687 **15a** (Figure 15A) as a micromolar inhibitor of Hh pathway. Additionally, in binding assays, this compound displayed weak affinity for the mouse and human SMO receptors. Thus, an optimization study was undertaken by synthesizing new series of compounds **15b** (Figure 15A) to identify potent compounds. The benzylamines containing analogs were preferred to sulfonamides or carboxamides analogs (region-A) in Hh inhibitory activity. Further, Hh inhibitory potency was stereospecific at the aminoindane moiety (region-B) as the *N*-benzylamines in the *S*-enantiomer being mostly the potent. Shifting of the methyl group (region-C) on the phenyl ring drastically decreased activity. Other substituents, such as an electron-donating group, such as a methoxy, at the R³-position resulted in the loss of activity. Removal of the *ortho*-methyl group led to a slight drop in activity. Substituents at R¹ (region-D) played a crucial role in the activity and trifluoromethyl was optimal for this position. Further, exploration of *S*-enantiomers with *N*-benzyl amine analogs revealed that substitution on the phenyl ring or replacement of the phenyl ring with heterocyclic systems, such as pyridyl or quinoliny, resulted in a drastic

drop in Hh inhibitory activity. Substitution on the methylene unit (between NH and X in region-A) either as methyl or trifluoromethyl, resulted in the slight loss of potency. Removal of methylene unit to attach the phenyl ring directly with the nitrogen atom also reduced activity. Interestingly, a 5-membered heterocycle at the benzylic position gave the most potent compound. An additional methyl group on the heterocycle slightly improved the activity. Based on this optimization study, compounds **15c** and **15d** were the most potent Hh signaling pathway inhibitors capable of binding with human and mouse SMO receptors (Figure 15A).

Pan et al¹³⁹ screened a solid phase synthesis-based compound library using a reporter gene assay in a mouse cell line. This screening identified biphenyl carboxamide **15e** (Figure 15B) as potent Hh signaling inhibitors. Optimization of structure **15f** (Figure 15B) at region-A revealed that replacement of electron-rich 1,4-diaminophenyl moiety could lead to toxicity by forming a quinone-like reactive intermediate upon metabolic activation. To reduce the electron density from this moiety, various approaches, such as the introduction of electron-withdrawing groups (F or CF₃) at the R² position, were tried, which proved beneficial to increase the activity. In addition, replacement of phenyl with an electron-deficient 2-pyridyl was allowed, however, regioisomeric, 3-pyridinyl analog, led to significant loss of activity. Further, when morpholine nitrogen was moved one carbon atom away from its direct attachment to the phenyl ring, a significant loss in activity was observed. At position R³, a piperidinyl substituent possesses good activity equivalent to morpholino analog. An enhancement in activity was observed when a *cis*-dimethyl group was introduced onto the morpholine ring. In addition, introduction of a small electron-donating group at the 2-position of the phenyl ring (region-B) led to a remarkable increase in the potency. Substitution with chlorine atom at this position also gave comparable activity. Addition of cyano, methoxy, trifluoromethyl, and trifluoromethoxy groups as R⁴ in region-C was well tolerated that led to highly potent analogs. Based on these findings, compound **15g** LDE-225 (Erismodegib/Sonidegib) (Figure 15B) was found suitable for further study. Treatment with **15g** in a subcutaneous Ptch^{+/-} p53^{-/-} MB allograft mice model led to tumor growth inhibition. The established tumors showed slower tumor growth relative to control animals (124 ± 37% as compared to baseline). Further, compound **15g** successfully penetrated the blood-brain barrier in tumor-bearing animals and results in significant tumor growth inhibition (Figure 15B).

Malancona et al¹⁴⁰ have screened a series of bicyclic hydantoin **15h** (Figure 15C) as potent SMO antagonist and inhibitor of Hh pathway.¹⁴⁰ Most of the hits in this series has the *trans* 2-phenylcyclopropyl portion in the region-A. Retaining the key [6,5]-bicyclic hydantoin and the *trans* 2-phenylcyclopropyl portion (region-B) revealed that simple methyl carbamates were slightly more active than simple amides or cyclohexyl amides. Further, the addition of lipophilic groups, such as *t*-butyl or cyclohexyl methylamine groups, led to weak inhibitory activity. By contrast, the urea fragments, such as dimethyl urea, or cyclohexyl urea, proved beneficial, whereas corresponding acyclic analogs were less active, which highlights the significance of the N-H in the urea for activity. Further, in the phenyl urea series, substitution with two lipophilic groups, such as chlorine atom on the phenyl ring, improved the potency. Interestingly, 2-biphenyl urea analog displayed high potency in both SMO-

binding and Light 2 assay. The position of the phenyl ring was also important for activity, as revealed from the isomeric 4-biphenyl analog which has significantly less activity. Optimizing the region-A revealed that the stereochemistry of cyclopropyl ring was critical for activity as *trans*-isomer displayed high activity while the *cis*-isomer was modest in SMO-binding affinity. Replacement of *trans* 2-phenylcyclopropyl with other groups like 2-phenethyl, benzyl, naphthyl, 3-biphenyl, spiro-fused ring analog or butyl group is not tolerated and led to significant drop in activity. However, 3-phenyl cyclobutane or indane led to significant loss of activity. From this, 2-biphenyl analog **15i** (Figure 15C) was selected for further optimization. Replacement of the 2-phenyl ring with substituents like trifluoromethyl, cyano, or methoxy groups proved detrimental for affinity whereas larger groups like isopropyl substituent retained some activity. Replacement of 2-phenyl with five-membered heterocycles, such as thiophene, retained the activity, whereas polar heterocycles, such as 1,3-oxazol-5-yl, resulted in an almost 20-fold loss in activity. Further, introduction of heteroatoms in the phenyl ring (ring-d) was well tolerated, as the 3-pyridyl analog proved highly active in both SMO-binding and Light 2 assay. However, the regioisomers, such as 4-pyridyl, proved less active than the 3-pyridyl analog. Finally, exchange of biphenyl fragment and 2-arylpyrid-3-yl motif with the *N*-phenylpyrazole moiety provided the most potent compound **15j** (Figure 15C) of this series. In addition, exchange of core scaffolds (region-E) with the corresponding phthalimide, and the removal of one carbonyl of the hydantoin, as well as manipulation of the hydantoin ring by ring expansion to six-membered rings, were also tried, which led to a substantial loss in affinity.

Solinas et al¹⁴¹ have screened a commercial compound library and identified an acylthiourea derivative MRT-10 **15k** (Figure 15D) as a potent Hh inhibitor.¹⁴¹ Structure optimization of the lead compound was undertaken by retaining aromatic ring with alkoxy residues in region-A. Variations in the position of the alkoxy substituents on the phenyl ring affected the activity significantly. Removal of one methoxy group from the 4-position or from the 3-position resulted in the loss of activity. Also, the introduction of ethoxy groups resulted in limited enhancement in inhibition. Further, exchange of two methoxy groups with a dioxolane ring or introduction of a dioxane cyclic system at this position was not allowed and led to decrease in activity. Modifications in the region-B revealed that phenyl ring was essential for activity, as cyclohexyl or isopropyl analogs were weak inhibitors. Replacement of phenyl ring with heterocycles, such as thiophenes, pyridine, or 1-naphthalenyl and 2-naphthalenyl, decreased activity. Introduction of an electron-donating group, such as methoxy, on this phenyl ring led to a slight decrease in activity, whereas a chlorine atom in the 4-position retained the activity. A significant improvement of 10-folds in activity was observed with the introduction of a second phenyl ring at 4-position. Further, while maintaining the biphenyl structure, replacement of the amide bond (region-C) with urea led to decreased inhibition. Replacement of both (region-B and region-C) components with isoelectronic or isosteric fragments, such as indole, led to 2 to 3-fold increased inhibitory activity compared to the lead compound **15k**. For central aromatic ring (region-D), introduction of a chloro, fluoro, or a methyl group at the C-4 carbon retained the activity, whereas a methoxy group in this position decreased the inhibition. Further, the introduction of substituents at C-4 or C-6 failed to improve the activity. The improved inhibitory potency may be attributed to the presence of a chloro or a methyl substituent at the C-4 position in

region-D, that helps in partial disruption in the conjugation of the central phenyl, and the amide bond exploration of acylthiourea (region-E) revealed that conversion from thiourea to urea or guanidine substantially increased potency in the Shh-Light 2 cells assay. The SMO receptor could accommodate (thio)urea and guanidine moieties despite their differences in the electronic. In this SAR study, analogs **15l**, **15m**, and **15n** (Figure 15D) were found as potent inhibitors as potent as GDC-0449 in several Hh assays and in inhibiting BODIPY-cyclopamine (BC) binding to human SMO. Interestingly, compound **15n** did not block the Wnt signaling, but inhibited the Hh pathway in mouse brain, suggesting this compound can block SMO in vivo (Figure 15D).

Further, Hoch et al¹⁴² have optimized the structure of compound **15n** and reported a highly potent SMO inhibitor, MRT-92, **15o** (Figure 15D) which is insensitive to the D473H mutation and uniquely bound to the entire transmembrane cavity of the SMO receptor.

Recently, Vesci et al¹⁴³ reported the ADME properties of acylguanidine **15o** and acylthiourea derivative **15p** (Figure 15D) in mouse. Both compounds were moderately metabolized by hepatic system. In PK profiles in nude mice after single oral (200 mg/kg), compound **15p** showed low bioavailability due to extensive extra-hepatic metabolism, whereas compound **15o** showed a half-life of about 2.5 hours. Compound **15o** effectively inhibited the tumor growth of colorectal cancer, showing high distribution of compound **15o** beyond systemic circulation resulting in low systemic exposure. From this study, compound **15o** was found suitable for further development owing to its good PK profiles: $C_{max} = 11.0 \pm 2.2$ hours, $t_{1/2} = 2.5$ hours, $AUC = 137 \pm 23$ $\mu\text{g} \cdot \text{h/mL}$, $V_d = 5280$ mL/kg .

This series of compounds has presented very potent analogs as active as GDC-0449 that can block the Hh signaling at the subnanomolar range. Fine tuning of these molecules may deliver some leading compounds like **15m** or **15n**.

2.12 | Miscellaneous compounds

There are many small molecules that cannot be discussed in the above-mentioned chemical classes, but still present some viable leads in Hh pathway inhibition. Therefore, we are discussing such molecules separately under this section.

Mahindroo et al¹⁴⁴ reported a new series of benzophenone analogs based on their previously designed ketoprofen amide analogs **16a** and **16b** (Figure 16A), which inhibits transcription by Gli1. These compounds were unstable in liver microsomes due to the presence of phenol in their structure. Taking inspiration from the observation that metabolic stability and bioavailability of phenolic compounds may be improved by replacing with indole or other NH-containing heterocyclic rings, they have synthesized various indole analogs (region-A) and were connected to amid nitrogen through two carbon linkers, methylene linker or without a linker. Compounds with only one or no methylene spacer were found with high activity. Indole attached through 6-position was most suitable for the activity. Further, reversing the amide group of compounds bearing two carbon spacer linkers was weak in activity whereas compound with methylene spacer was good in activity. Next, exploration of an alkyl groups substitution on the amide (region-B) revealed that a methyl substitution led to improved activity. Further, increasing the hydrophilicity of the alkyl group led to decrease

in the potency. Compounds substituted with indole at 4-position and 5-position were potent inhibitors. From this study, compounds **16c** and **16d** more weakly inhibited the Gli1-transfected C3H10T1/2 cells compared with the compound **16a** and **16b**, however, with significantly enhanced stability in mouse liver microsomes (Figure 16A).

Samanta et al¹⁴⁵ developed a novel series of chromone-based compound library **16e** (Figure 16B) as a potent inhibitor of the Hh signaling pathway. Substitution at C2-position of chromone (region-A) was essential for inhibiting Hh pathway. The potent inhibitor had a heteroaromatic group at this position, whereas heterocyclic or acyclic bearing analogues were less active or completely inactive. The most potent inhibition was observed with compound **16f** (Figure 16B), bearing a 6-fluoroindole at C2-position of chromone. Introduction of aryl group at C3-position also led to improved activity. However, analogs bearing 4-methoxyphenyl and 4-(trifluoromethyl) phenyl at C3-position displayed reduced cell viability. Further, substituents at C6 to C8 position of chromone (region-B) were insufficient to improve the inhibitory activity. This report presents the first example in which modulation of the Hh signaling pathway by chromone was observed (Figure 16B).

2*H*-pyrroles and 3*H*-pyrroles are core structures of many biologically active compounds including natural products, pharmaceutical agents, such as Precorrin-6B and alkaloids, such as Calycophylline G and Chamobtusin A. Kötzner et al¹⁴⁶ have developed new therapeutic strategies to access 2*H*-pyrroles and 3*H*-pyrroles in high yields and screened this library for Hh pathway inhibitory activity. From the various synthesized analogs, some selected 3*H*-pyrroles and 2*H*-pyrroles, particularly *rac*-2*H*-pyrrole **16g** (Figure 16C) showed potent inhibition of Hh signaling pathway without being cytotoxic to the cells. Compound **16g** bound with SMO as observed by competition experiments with BODIPY-cyclopamine in HEK293T cells ectopically expressing SMO (Figure 16C).

Yang et al¹⁴⁷ have reported a novel series of 6-(pyridine-3-yl) benzo[*d*]thiazole **16h** (Figure 16C) as a lead to develop dual Hh and PI3K inhibitors. The simplest benzamide (region-A) was important for potent Hh inhibition and was less active than the FDA-approved GDC-0449. Addition of a chlorine atom on the phenyl ring led to different results depending on the position. 3-Chloro substitution improved the activity, while 2-chloro and 4-chloro substitutions were detrimental to activity. Substitution with a fluorine atom at 4-position was most favored. Replacement of phenyl with the small aliphatic group, such as cyclopropane, diminished the activity. Further, the inclusion of methylene or reduction of amide bond reduced activity. Replacement of acetamide (region-B) with secondary amines was also well tolerated with reduced activity. The most potent compound **16i** was tested for SMO-binding ability in BODIPY-cyclopamine displacement experiment in the U2OS cells to confirm its Hh inhibition potential (Figure 16C).

A cell-based screening for small-molecule antagonists for GLI-mediated transcription led to the identification of two potent molecules, GANT58 **16j** and GANT61 **16k** (Figure 16C) (Shh-Light 2 cells assay IC₅₀ $\cong$ 5 μ M for each compound) that could selectively inhibit GLI-mediated gene transactivation (*N*-myc, D-cyclins, BMI1, GLI1, Bcl-2, and IRS-1).¹⁴⁸ These molecules block GLI function, and one of them interfered with GLI1-DNA binding in the nucleus. Importantly, these new compounds efficiently inhibited in vitro tumor cell

proliferation in a GLI-dependent manner, and completely blocked cell growth in a human prostate cancer cell-derived xenograft mouse model. Further studies also demonstrated that GANT61 robustly suppressed proliferation of colon, ovarian cancer cells, and canine osteosarcoma cells.

Beside these molecules, an inorganic compound arsenic trioxide (ATO) **16l** (Figure 16C) is potent GLI inhibitor that directly binds to GLI1 and GLI2 and decreasing the expression of endogenous GLI target genes.¹⁴⁹ ATO has been approved for treatment of acute promyelocytic leukemia, and currently, a few of clinical trials of ATO are underway for solid tumors and hematological malignancies^{53,150} (Figure 16C).

Chen et al²⁹ have described SANT-1 **16m** (Figure 16C) as potent SMO inhibitor, which was identified through small-molecule screen. SANT-1 inhibits the SMO with high potency in the Shh-Light 2 assay (Figure 16C).

Recently, Berardozzi et al¹⁵¹ reported the design, synthesis, and anti-Hh pathway potential of isoflavone-based scaffold **16n** (Figure 16D). The design of new compounds was based on the computational analysis of isoflavones where ring B was variedly substituted. The interaction pattern of these compounds with SMO and Gli protein showed interesting phenomenon where presence of a bulky substitution on the *para*-position of ring B was preferred for interaction with SMO, whereas when the same substitutions were present at *meta*-position, interaction preference for Gli1 was observed. Among various flavonoids, compounds **16o** and **16p** (Figure 16D) bearing bulky substitutions at meta-position on the B-ring were found most active in SMO^{-/-} mouse embryonic fibroblasts (MEFs) transfected with ectopic Gli. The SuFu^{-/-} MEFs model proved that compounds **16o** and **16p** act downstream of SMO. Compound **16q** (Figure 16D) bearing bulky groups at the *para*-position of the ring B, fail in inhibiting Hh-targeted gene expression because pathway activation occurs downstream of SMO receptors. However, compound **16r** (Figure 16D) bearing two bulky groups on the ring B acts as specific SMO antagonist. Molecular docking study using compounds **16o** and **16p** with Gli protein (1ZF) revealed that these compounds share comparable binding sites. In SMO-binding study, compounds **16q** and **16r** were bound to the narrow lipophilic site located within the heptahelical bundle of the receptor. Further, combination of these two different isoflavones showed the synergistic effects in primary MB cells at submicromolar concentration.

Lu et al¹⁵² recently screened a virtual compound library based on the crystal structure of SMO complexed with antagonists. From this screening, few structurally diverse compounds were identified as potent inhibitors of Hh pathway in a NIH3T3-GRE-Luc reporter gene assay. The most potent compound **16s** (Figure 16E) was equipotent to vismodegib. Compound **16s** was a potent SMO antagonist in a fluorescence-based competitive binding assay. However, further optimization of compound **16s** by various substituents in regions A and B as shown in structure **16t** (Figure 16E) employing aliphatic or cyclic amines or pyridines, respectively, did not improve the activity. These findings resulted in the identification of compound **16s** that can be used as templet for further optimization.

In a recent study, He et al¹⁵³ studied Hh inhibitory potential of dehydroeffusol **16u** (Figure 16E) isolated from *Juncus effusus*. This perennial herb traditionally used in Chinese medicinal system for the treatment of insomnia and akathisia. The dehydroeffusol **16u** inhibits Hh signaling pathway by suppressing the expression of SMO and Gli1 at 10 and 20 μ M at mRNA and protein levels in SHSY-5Y and SK-N-SH cells. However, its exact mechanism of actions is still unknown.

2.13 | Active compounds from plants

Many natural compounds have been isolated and identified as potent Hh inhibitors, some important leads are discussed in this section.

Arai et al¹⁵⁴ identified and isolated pentacyclic triterpenes from the methanolic extract of *Zizyphus cambodiana* (Rhamnaceae) namely colubrinic acid **17a**, betulinic acid **17b**, and alphitolic acid **17c** (Figure 17). These compounds inhibited Gli1-mediated transcriptional activity with little effect on cell viability. Further, these compounds decreased the expression of antiapoptotic protein BCL-2 and to show cytotoxicity against cancer cells. These compounds might become good tools and/or led to new agents in the investigation of Hh/Gli signaling pathway inhibitors.

Hosoya et al³² screened a natural product library in a newly developed cell-based assay system, which includes terpenoids, flavonoids, phenylpropanoids, their glycosides, and bisindole alkaloids. Sesquiterpenes zerumbone **17d** and zerumbone epoxide **17e**, bisindole alkaloids staurosporinone **17f**, 6-hydroxystaurosporinone **17g**, arcryaflavin C **17h**, and 5,6-dihydroxy arcryaflavin A **17i** (Figure 17) inhibited Gli1 transcription dose-dependently and had little effect on cell viability at the same concentrations. Further, they screened the methanolic extract of *Physalis minima* and isolated most active compounds, which were identified as physalin F **17j** and physalin B **17k** (Figure 17). Physalins F and B dose-dependently inhibited Gli1-mediated transcriptional activity with little or no effect on cell viability. Further, five of these compounds namely **17d-17f**, **17j**, and **17k** (Figure 17) inhibited Gli2-mediated transcription in a dose-dependent manner. Compounds **17d**, **17f**, **17j**, and **17k** also decreased the expression of Gli1 and PTCH. For Zerumbone **17d**, SAR analysis revealed that an α,β -unsaturated carbonyl group might play a pivotal role in the inhibition of Gli-mediated transcription. Similarly, for bisindole alkaloids that inhibited Gli-mediated expression, the C2 and C2' bonds in the structure seemed important for the inhibition of Gli-mediated transcription. From this study, compounds **17d**, **17f**, **17j**, and **17k** had a different mechanism of action from cyclopamine, because cyclopamine did not decrease the reporter activity in this assay system (Figure 17).

In another report, Rifai et al¹²² reported the methanolic extract of *Acacia pennata* as potent Hh/Gli signaling inhibitor. From this extract, taepeenin D **17l**, (+)-drim-8-ene **17m**, and quercetin 3-O- β -D-glucopyranosyl-4-O- β -D-glucopyranoside **17n** were identified as a potent inhibitor of Hh/Gli-mediated transcriptional activity. Further, the inhibition was caused by downregulation of Ptch-1 and BCL-2 in HaCaT cells expressing exogenous Gli1 and PANC1 cells. Therefore, compounds **17l**, **17m**, and **17n** may serve as potent leads (Figure 17).

Further, Shintani et al¹⁵⁵ isolated new bisindole alkaloid, 6-hydroxy-9'-methoxystaurosporinone **17o** from the methanolic extract of field-collected fruiting bodies of the myxomycete *Perichaenachrysosperma*. Compound **17o** dose-dependent inhibition of Gli-mediated transcriptional activity. Further, compound **17o** showed cytotoxicity against PANC1 and DU145 without affecting the normal cell line. The cytotoxicity of **17o** against PANC1 and DU145 cells may be related to its inhibition of Hh/Gli transcriptional activity (Figure 17).

From the methanolic extract of dried leaves of *Excoecaria agallocha*, two new flavonoid glycosides, **18a** and **18b** (Figure 18) were isolated by Rifai et al.¹⁵⁶ Among these compounds, compound **18a** inhibited the translocation of Gli1 into the nucleus of PANC1 cells. Quantitative reverse transcription polymerase chain reaction (RT-PCR) of compound **18a** exhibited that the reduction of Hh signaling is not caused by SMO interference, but rather by other unrevealed mechanisms related to Gli1 inhibitory function (Figure 18).

Methanolic extract of tropical plant *Adenium obesum* was screened by Arai et al¹⁵⁷ for its potential to inhibit Hh/Gli signaling in their newly developed screening protocol. This extract led to the isolation of 17 cardiac glycosides including three new compounds that inhibited Gli-related protein expression, particularly with compounds **18c-18g** (Figure 18) in human pancreatic cancer cells (PANC1). Introduction of 16 β -Hydroxy, acetyloxy, and formyloxy unit decreased the activity. A modification of the aglycone moiety also reduced the activity. Interestingly, there is no significant structural similarity in the reported Hh inhibitors.

Arai et al¹⁵⁸ reported Hh inhibitors luteolin **18h**, vitetrifolin D **18i**, compound **18j** and nishindanol **18k** (Figure 18) extracted from the methanolic extract of areal parts of *Vitex negundo* (Verbenaceae). Although compound **18j** seemed to have Hh inhibition activity, decrease in the cell number was because of the cytotoxicity of the compound. Vitetrifolin D **18i** and nishindanol **18k** showed cytotoxicity in Hh-activated cancer cells. Vitetrifolin D **18i** showed inhibition of Gli1-DNA complex formation.

Yoneyama et al¹⁵⁹ screened the methanolic extract of leaves of *Withania somnifera* to assess its potential to inhibit Gli1-mediated transcription. After purification, withaferin **18l**, 27-deoxywithaferin **18m**, 5,6-deoxywithaferin **18n**, 2, 3-dihydrowithaferin **18o**, 2,3-dihydro-3-methoxywithaferin **18p**, and 2,3-dihydro-3- β -O-sulfate withaferin **18q** (Figure 18) were isolated, which show potent inhibition of Hh/Gli1-mediated transcriptional activity particularly withaferin **18l** and 27-deoxywithaferin **18m**. Furthermore, **18l** also inhibited Gli1-DNA complex formation in electrophoretic mobility shift assay (EMSA).

The methanolic extract of leaves of *Artocarpus communis* (Moraceae) and methanolic extract of flowers of *Hyptis suaveolens* (Lamiaceae) were screened for possible Hh inhibitory potential by Arai et al.¹⁶⁰ Among the isolated compounds, 4 α -hydroperoxy-5-enovatodiolids **19a**, 4-methylene-5 β -hydroperoxyovatodiolide **19b**, and ovatodiolide **19c** (Figure 19) possessed potent Hh signal inhibitory activity. These compounds also showed high cytotoxicity against PANC1 and DU145 cancer cells, which have active Hh signal. Compounds **19a-19c** decreased the expression of antiapoptotic protein Bcl2 in DU145 cells.

Disruption of the Gli1-DNA complex by compounds **19a** and **19b** suggested that the mechanism of action of these compounds might include inhibition of Gli1-DNA complex formation.

Gao et al¹⁶¹ isolated some potent Hh inhibitors from the ethanolic extract of an entire plant of *Veratrum Grandiflorum* Loes. They isolated three new steroidal alkaloids together with some known compounds. The inhibitory activity of Hh pathway using a cell-based bioassay system (Shh-Light 2 cells) revealed that compounds **19d-19g** (Figure 19) possessed potent inhibitory activities particularly compound **19g**, which showed the most prominent inhibition activity. Overall, this study may provide an opportunity to develop potent Hh inhibitors from natural products for the treatment of various cancers.

Despite large number of lead compounds from the natural sources, only a few compounds, such as cyclopamine (targeting SMO), genistein (targeting Gli1), curcumin (targeting Shh, Gli1), epigallocatechin-3-gallate (targeting Ihh, Gli1), and resveratrol (targeting Gli1) had shown promising inhibition of Hh signaling pathway even downstream of SMO.¹⁶² Modification of natural products has proven a valuable tool to assess potent clinically used drugs. Many natural products discussed in this section may serve a source of inspiration for the development of new semisynthetic Hh inhibitors.^{32,154–157,159–162}

3 | STRUCTURES OF SMO RECEPTOR BOUND TO DIFFERENT LIGANDS AND THE LIGAND BINDING POCKETS

Structure and binding sites within the SMO receptor have been studied extensively.^{53,124,163–166} The SMO receptor contains an extracellular domain (ECD) consist of an extracellular CRD and an ECD linker domain, a seven-transmembrane helical (7TM) domain and an intracellular carboxy-terminal domain.¹⁶⁷ SMO receptors are classified as a Frizzled-class GPCR because unlike the “classical” Class A G protein-coupled receptors (GPCRs), the SMO contains stereotypical seven-pass, α -helical transmembrane bundle (TMD), and extracellular and intracellular domains.¹⁶⁸ The SMO receptor structure has a canonical GPCR 7TM bundle fold with a short helix VIII packed parallel to the membrane bilayer. The ECD linker domain and long extracellular loops (ECLs) form intricate structures stabilized by four disulfide bonds: C193-C213, C217-C295, C314-C390, and C490-C507.¹⁶³

Crystal structures of SMO with various antagonists (such as SANT-1, Anta XV, LY2940680, and cyclopamine) and agonist (such as SAG1.5) has revealed distinct binding sites in the long and narrow cavity defined by the 7TM helices, ECD and ECLs of SMO.

The binding sites in the 7TM domain of SMO may be classified into two distinct sites, site-1 present near to extracellular loops (binding site for LY2940680, cyclopamine,¹⁶⁶ GDC-0449)¹⁶⁹ and site-2, buried deep in the 7TM cavity (binding site for SANT-1).¹⁷⁰ The extracellular entrance can accommodate the attachment of reporter moieties or other bulky groups to the ligands through a long-chain flexible linker, such as that of KAAD-cyclopamine (Figure 20).

Presence of long and deep binding pocket in SMO receptor offers distinct binding sites to structurally diverse small molecules. Different binding sites in the SMO receptor provides opportunity to bind different SMO inhibitors even if it develops resistance against few of the antagonist due to point mutation. For an example, LY2940680 showed high potency and bound to the D473^{6.54f}H mutant SMO, which is resistant to GDC-0449. This can be explained by structural analysis where the LY2940680 forms weak interactions with D473^{6.54f}. In contrast, Anta XV requires a more substantial involvement of D473 in its binding with SMO^{6.54f}, regardless of its similar scaffold with LY2940680.¹⁷⁰ SMO-cyclopamine interactions in cocrystal structure revealed that cyclopamine interacts predominantly with the extracellular loops of SMO.¹⁷¹ Some molecules, such as MRT-92, exposed a third type of SMO antagonists where the molecules entirely fill the SMO-binding cavity from the upper extracellular part to the lower cytoplasmic-proximal subpocket.¹⁴² Itraconazole did not compete to the BODIPY-cyclopamine binding site, suggesting indirect action on SMO. Binding sites for various SMO antagonist with SMO are shown in Figure 20 along with site of point mutation that results in chemoresistance.¹⁰⁸

The SMO activity could also be regulated by distinct binding sites in the CRD segment. Oxysterols and its derivatives, such as compounds **12o** and **12p**, regulate the SMO activity through the CRD site, known as oxysterol binding site. The Hh inhibitors targeting the SMO CRD provides an orthogonal approach to modulate the Hh signaling in regeneration and cancer.^{123,124}

4 | CONCLUDING REMARKS AND FUTURE DIRECTION

Promises and challenges of Hh inhibitors as cancer therapeutics are associated with the development of potent compounds. Although Hh inhibitors that act as downstream targets of SMO have the potential to be quite effective in treating Hh pathway-dependent cancers, most of the known Hh pathway antagonists directly target SMO, which is susceptible to mutation and develops chemoresistance. Therefore, the development of compounds that can target the cascade downstream of SMO represents a more promising strategy. There are already some compounds identified to target Gli1/2 transcription factor. One of the promising compounds in this context is GANT61, which is highly effective against various cancer cells. However, GANT61 is not stable at physiological pH and hydrolyzes into an inactive benzaldehyde species.¹⁷² Further, PDE4 inhibitor EGM1 that acts downstream of SuFu is also being explored for drug development.

We have discussed many synthetic as well as few natural products, which inhibit the Hh pathway at multiple targets. We have discussed the SAR of various molecules and highlighted the most potent compounds. Interestingly, large variation in the molecular structures can be seen that can inhibit the Hh pathway including thiophenes, pyridines, pyrimidines, quinolones, phthalazines, vitamin D analogs, benzimidazoles, medium size rings, and biphenyl-derived molecules. Besides, chemical leads from the natural products were also fine-tuned to optimize the PK properties to assess whether they can be used as potent Hh inhibitors. Strategies to further develop lead compounds as potent Hh inhibitors have also been discussed. A selection of most potent and currently known Hh inhibitors

from synthetic and natural origin are shown in Figure 21, along with their corresponding molecular targets.

We have also included some reports that describe compounds that were first time identified as potent Hh inhibitors. For examples, chromes, benzothiazoles, and pyrroles. Itraconazole represents a clinically used antifungal drug that is now being fine-tuned as a potent Hh pathway inhibitor. Similarly, we had witnessed some examples in which complex structures (chemically and stereochemically) were simplified to assess potent leads such as in steroidal and vitamin D analogs. Table 1 highlights the most potent compounds reported in the recent literature from each chemical class discussed in this review. Some of which are already approved and some may be recognized as potential drugs in the future.

We hope that this review will stimulate discussion on the development of new Hh pathway inhibitors for treating cancer, fibrosis, and several other diseases. While it is important to develop potent Hh inhibitors, several studies suggest that oncogenic pathways, such as PI3K, MAPK, KRAS/BRAF, and TGF- β cooperate in Hh signaling. Therefore, it is advantageous to use Hh inhibitors as a combination or adjuvant therapies in cancers. Furthermore, new molecules targeting novel components of the pathway hold great promise.

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Abbreviations:

AUC

area under the curve

BCC

basal cell carcinoma

Cl

clearance

CREB

cAMP-response element binding

CSC

cancer stem cell

CYP

cytochrome

Dhh

dessert Hedgehog

DTX

docetaxel

Dyrk1

dual-specificity tyrosine phosphorylation-regulated kinase-1

ECM

extracellular matrix

EGM1

Eggmanone

EMT

epithelial-to-mesenchymal transition

EPR

enhanced permeability and retention

F%

fraction absorbed (oral bioavailability)

FGF

fibroblast growth factor

hERG

human *ether-a-go-go*-related gene

HSC

hepatic stellate cell

HTS

high throughput screening

GEM

gemcitabine

Gli

glioma

HEPM

human embryonic palatal mesenchyme

Hh

Hedgehog

Hhat

Hh acyltransferase

IGF-IIR

insulin-like growth factor II receptor

I/R

ischemia reperfusion

Ihh

Indian Hedgehog

LipE

lipophilicity efficiency

M6P

mannose-6-phosphate

MAPK

mitogen-activated protein kinase

MB

medulloblastoma

mPEG-*b*-P(CB-co-LA)methoxy-polyethylene-glycol-*b*-poly(carbonate-*co*-lactide)**mPEG-*b*-PCC-*g*-DC-*g*-TEPA**methoxy poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxylpropylene carbonate-*graft*-dodecanol-*graft*-tetraethylenepentamine)**mPEG-*b*-PCC-*g*-GEM-*g*-DC**methoxy poly (ethylene glycol)-*block*-poly(2-methyl-2-carboxylpropylene carbonate-*graft*-gemcitabine-*graft*-dodecanol**NCEs**

new chemical entities

PDE4

phosphodiesterase

PI3K

phosphatidylinositol 3-kinase

PD

pharmacodynamic

PDGFR α platelet-derived growth factor receptor- α **PK**

pharmacokinetics

PKA

protein kinase A

PKC

protein kinase C

PO

per oral

PPAR- γ

peroxisome proliferator-activated receptor gamma

PTCH1

patched1

PTX

paclitaxel

SAR

structure-activity relationship

Shh

sonic Hedgehog

ShhN

Shh *N*-terminal peptide

SMO

Smoothened

SPR

surface plasmon resonance

SuFu

suppressor of fused

TGF β

transforming growth factor- β

VD3

vitamin D₃

VDR

vitamin D receptor

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FIGURE 1. Overview of the Hedgehog signaling pathway. Hh, Hedgehog; Shh, Sonic Hedgehog; SMO, Smoothened; SuFu, suppressor of fused; PTCH1, patched1 [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 2.

Various classes of compounds capable of inhibiting Hh pathway. Hh, Hedgehog [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3.

A and B, Development of thiophenes as inhibitors of Hh signaling pathway. EGM1, Eggenone; Hh, Hedgehog [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 4.

A-E, Development of pyridine inhibitors of the Hh signaling pathway. Hh, Hedgehog; HEPM, human embryonic palatal mesenchyme [Color figure can be viewed at wileyonlinelibrary.com]

(A)

5a

For A

- # Strong EWGs at 3- or 4-position improve the activity
- # Substitution of EWGs at 2-position significantly reduce the activity
- # Unsubstituted phenyl or *N*-heterocycles are well tolerated
- # Alkyl groups as replacement are unacceptable

For B

- # *N*-1 nitrogen in the pyrimidinyl ring is important than the *N*-3 nitrogen

For C

- # Small alkyl groups on amide nitrogen is well tolerated

For D

- # Replacement of phenyl ring with small alkyl group is not tolerated whereas bulky alkyl group retained the potency
- # *N*-functionality at 3-position of phenyl ring is well tolerated

5b

Gli-luciferase rep IC₅₀ = 1.3 nM
AlogP = 7.8

For A

- # Replacement with 1-methylpyrazolyl proved beneficial

For B

- # At C-5 position, cyclic aliphatic amines found best
- # More basic cyclic amines significantly reduced the activity
- # Analogs with secondary amines are well tolerated
- # Amines substituted with aromatic groups are well tolerated

5c

Gli-luciferase rep IC₅₀ = 1.3 nM
AlogP = 3.6

(B)

5d

For A

- # Strong EWG on the phenyl is essential for potency
- # Heteroaryl replacing the aryl ring reduce potency

For B

- # Hydrophilic substituent on the aromatic ring is beneficial
- # *N*-heterocycles at 5-position of the aromatic rings is best

5e

Gli-luciferase rep IC₅₀ = 1.44 nM

FIGURE 5.

A-F, Development of pyrimidine inhibitors of the Hh signaling pathway. Hh, Hedgehog
[Color figure can be viewed at wileyonlinelibrary.com]

For A
2-Quinolyl ring is essential for activity
Addition of second nitrogen into the bicyclic scaffold is ineffective to improve activity
For B
The 1,2,4-oxadiazole ring is optimal for activity
For C
Ring contraction to four- or five-membered ring is not allowed
Piperazine is better than piperidine
For D
Aromatic ureas are best choice
Primary amine are better than secondary amine based ureas
Replacement of aniline with a bicyclic or heterocyclic system decrease potency
Replacement of phenyl ring with lipophilic alkyl urea or cycloalkyl urea improve activity

For A
Morpholine like groups such as pyrrolidine or piperidine and acyclic amines of comparable size are tolerated
Large lipophilic groups such as cyclohexylamine are disfavored
For B
Cyclopentyl group is essential for optimum lipophilicity and activity
For C
Replacement of quinoline with methyl benzimidazole improve the activity

FIGURE 6.

A-F, Development of quinolone inhibitors of the Hh signaling pathway. Hh, Hedgehog
[Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 7.

A and B, Development of quinazolinone inhibitors of the Hh signaling pathway. Hh, Hedgehog [Color figure can be viewed at wileyonlinelibrary.com]

(A)

8a

Gli shift 1 nM (IC_{50}) = 414 ± 4
 Gli shift 25 nM (IC_{50}) = 2246 ± 1514

For A

Replacement with 2-pyridyl improves the activity
 # Substitution at 4th position of pyridine is allowed
 # Tertiary alcohol is best

For B

Piperazine is essential for activity
 # Substitution on piperazine do not improve the activity

For C

Phthalazine ring is essential for potency
 # Addition of halogen on phthalazine ring improves the activity
 # Addition of EWGs reduces the activity

For D

Phenyl ring is essential for activity
 # Substitution with 4-chloro or 4-cyano group improves the activity

8b

Gli shift 1 nM (IC_{50}) = 2.7 ± 0.9
 Gli shift 25 nM (IC_{50}) = 35.0 ± 3

(B)

8c

hSMO (IC_{50}) = 7.0 nM
 CYP3A4 inhib. = 10%
 CYP2D6 inhib. = 47%

For A

Replacement of thiophene with phenyl ring improves the potency

For B

2-(S) or 3-(R)-methyl substitution on piperazine improves the activity

For C

Phenyl ring is essential for activity
 # Substitution of EWG at 4th position of phenyl ring improves the activity

8d

hSMO (IC_{50}) = 2.8 nM
 CYP3A4 inhib. = <10%
 CYP2D6 inhib. = 15%

(C)

8e

For A

Various ionizable groups did not improve the aqueous solubility and potency
 # 2-Hydroxypropyl is best for potency
 # Replacement of 2-pyridyl with a 2-pyrazine improve the potency

For B

Truncation of phthalazine core is allowed

For C

R-Me improves the potency

For D

Replacement of benzylic methylene linker with an oxygen atom is tolerated

8f NVP-LEQ 50665

Gli shift 1 nM (IC_{50}) = 1.0 nM
 Gli shift 25 nM (IC_{50}) = 10.0 nM
 hERG (IC_{50}) = 30000 nM

(D)

8g Anta XV
Gli-luc reporter
 $IC_{50} = 7.1 \pm 1.8$ nM

For A

Bulky 3-pentanol substituent at the 4-position of pyridine is essential

For B

Replacement of piperazine to a pyrrolidine-3-amine or piperidine-4-amine significantly improve the activity
Piperidine-3-amine lead to complete potency loss

8h
Gli-luc reporter
 $IC_{50} = 0.58 \pm 0.15$ nM

(E)

8i **8j**

Gli-mediated transcription (IC_{50}) = 10.0 μ M

(F)

8k Taladegib (LY2940680)
 $IC_{50} = 6.3 \pm 1.2$ nM

8l

8m
Gli-luc reporter
 $IC_{50} = 1.16 \pm 0.18$ nM

8n
Gli-luc reporter
 $IC_{50} = 0.17 \pm 0.01$ nM

For A

Replacement of piperidine-4-amine is not tolerated except pyrrolidine-3-amine
R-enantiomer is more potent than racemate
Reversed pyrrolidine-3-amine linker is not allowed

For B

Pyrazole can be replaced with aryl or heterocycles
EDG at 4th position of phenyl improve the activity

FIGURE 8.

A-G, Development of phthalazine and piprazoic acid inhibitors of the Hh signaling pathway.

Hh, Hedgehog; hERG, human ether-a-go-go-related gene [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 9.

A-D, Development of vitamin D₃ analogs as inhibitors of the Hh signaling pathway. Hh, Hedgehog; MB, medulloblastoma; mRNA, messenger RNA; VDR, vitamin D receptor [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 10.

Development of itraconazole analogs as inhibitors of the Hh signaling pathway. Hh, Hedgehog; MB, medulloblastoma [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 11.

A and B, Development of cyclopamine analogs as inhibitors of the Hh signaling pathway.

Hh, Hedgehog; SMO, Smoothed [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 12.

A and B, Development of steroids as inhibitors of the Hh signaling pathway. Hh, Hedgehog

FIGURE 13.

A-D, Development of benzimidazoles as inhibitors of the Hh signaling pathway. Hh, Hedgehog [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 14.

A-D, Development of medium size/macrocyclic rings as inhibitors of the Hh signaling pathway. Hh, Hedgehog [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 15.

A-D, Development of biphenyl derivatives as inhibitors of the Hh signaling pathway. Hh, Hedgehog; Shh, Sonic Hedgehog [Color figure can be viewed at wileyonlinelibrary.com]

For A
 # Replacement of phenol with indole-6-yl with one carbon space or without spacer give most potent compound
For B
 # Reverse amides are week inhibitors
 # Alkyl substitution on amide nitrogen improve activity

For A
 # Substitution at C2-position of chromone with heteroaromatics is essential
 # Substitution with heterocyclic or acyclic substituents at C2 give inactive compound
 # Aryl group at C3-position of chromone improve activity
For B
 # Substitution on aryl ring of chromone did not improve activity

For A
 # 4-F-Benzamide is best for activity
 # Alkyl amides are inactive
 # Reduction of amide give inactive compounds
For B
 Replacement of acetamide with *sec*-amide is allowed

FIGURE 16.

A-E, Miscellaneous compounds as inhibitors of the Hh signaling pathway. Hh, Hedgehog
[Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 17.

Some naturally occurring compounds identified as inhibitors of Hh signaling pathway. Hh, Hedgehog

FIGURE 18.

Some naturally occurring compounds identified as inhibitors of Hh signaling pathway. Hh, Hedgehog

FIGURE 19.

Some naturally occurring compounds identified as inhibitors of Hh signaling pathway. Hh, Hedgehog

FIGURE 20.

Map of the structural basis for the modulation of SMO by small molecules. The N-terminal CRD is shown using the CRD of zebrafish SMO (PDB id: 4C79). The boundaries of the membrane bilayer are shown as dashed lines (printed with permission from the publisher).

¹⁷⁰ SMO, Smoothened; CRD, cysteine-rich domain [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 21.

Most potent small molecules capable of inhibiting Hh pathway with their mode of action.

Hh, Hedgehog; PKA, protein kinase; SMO, smoothened; Shh, Sonic Hedgehog; SuFu, suppressor of fused [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Most potent synthetic and natural inhibitors of Hh signaling from various chemical classes with their mode of action/target

Compounds	Category/compound	Target	Efficacy	Refs
Thiophenes	3c	PDE4	EC ₅₀ = 82 ± 10 nM	49
	3j RU-SKI 201	PDE4	IC ₅₀ = 200 ± 90 nM	52
Pyridines	4c GDC-0449	SMO	Gli-LUC S12 IC ₅₀ = 13.0 nM	56
	4h	SMO	Firefly IC ₅₀ = 12 nM Shh EC ₅₀ = 13 nM BST p38a IC ₅₀ = 2700 nM	58
Pyrimidines	5t	SMO	IC ₅₀ = 0.35 nM	80
	5w	SMO	Gli-luciferase rep IC ₅₀ = 0.05 nM	81
Quinolones	6m TAK-441	SMO	IC ₅₀ = 4.4 nM	87
	6n	SMO	Gli-luciferase rep IC ₅₀ = 2.6 nM	88
Quinazolinones	7d	SMO and Gli2	IC ₅₀ = 38 nM	90
Phthalazines and piprazoic acids	8h	SMO	IC ₅₀ = 0.58 ± 0.15 nM	94
	8k Taladegib (LY2940680)	SMO	Gli-luciferase report IC ₅₀ = 6.3 ± 1.2 nM	96
	8n	SMO	Gli-luciferase report IC ₅₀ = 0.17 ± 0.01 nM	97
Vitamin D3 analogs	9k	SMO	Gli1 mRNA = 0.7 ± 0.2% CYP24A1 mRNA = 45.3 ± 19	103
	9l	SMO	Gli1 mRNA = 1.3 ± 0.6% CYP24A1 mRNA = 20.6 ± 3.6	103
Itraconazole analogs	10d	SMO	Gli1 mRNA in MERP MB cells IC ₅₀ = 190 ± 70 nM	110
Cyclopamine and related steroidal analogs	11a Cyclopamine	SMO	EC ₅₀ = 170 ± 70 nM	111
	11f	SMO	IC ₅₀ = 7.0 ± 2.0 nM	113
	12k	SMO	IC ₅₀ = 1100 nM	120
Benzimidazoles	13i	SMO	Gli-luciferase rep IC ₅₀ = 25 ± 20 nM	131
	13k	SMO	Gli-luciferase rep IC ₅₀ = 25 ± 20 nM	132
Medium size/macrocyclic rings	14b Robotnikinin	Shh	Gli EC ₅₀ = 4000 nM	133
	14j BRD9526	Shh	IC ₅₀ = 60 nM	136
	14n	SMO	Gli-luciferase rep IC ₅₀ = 29 ± 9 nM	137
Biphenyl derivatives	15g Sonidegib (LDE-225)	SMO	IC ₅₀ = 1.3 nM	139
	15m	SMO	Shh-Light 2 IC ₅₀ = 1 ± 2 nM C3H10T1/2 IC ₅₀ = 25 ± 3 nM	141
Miscellaneous compounds	16f	...	IC ₅₀ = 990 ± 120 nM	145
	16j GANT58	Gli1	IC ₅₀ = 5000 nM	148
	16i Arsenic trioxide	Gli1/2	NIH3T3 cells IC ₅₀ = 830 nM	149
Natural products	17k Physalin B	Gli1	Gli1 tran inhib. IC ₅₀ = 620 nM Gli2 tran inhib. IC ₅₀ = 1500 nM	32
	18g	Gli1	Gli1 tran inhib. IC ₅₀ = 110 nM	157

Compounds	Category/compound	Target	Efficacy	Refs
	18I	Gli1	Gli1 tran inhib. IC ₅₀ = 500 nM	159
	19g	Gli1	Shh-Light 2 cells IC ₅₀ = 630 ± 20 nM	161

Abbreviations: Hh, Hedgehog; SMO, smoothened; mRNA, messenger RNA; MB, medulloblastoma.