

Targeting GLI factors to inhibit the Hedgehog pathway

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Hedgehog (Hh) signaling has emerged in recent years as an attractive target for anticancer therapy because its aberrant activation is implicated in several cancers. Major progress has been made in the development of SMOOTHENED (SMO) antagonists, although they have shown several limitations due to downstream SMO pathway activation or the occurrence of drug-resistant SMO mutations. Recently, particular interest has been elicited by the identification of molecules able to hit glioma-associated oncogene (GLI) factors, the final effectors of the Hh pathway, which provide a valid tool to overcome anti-SMO resistance. Here, we review results achieved in developing GLI antagonists, explaining their mechanisms of action and highlighting their therapeutic potential. We also underline the relevance of structural details in their discovery and optimization.

Hh pathway and cancer

The evolutionarily conserved Hh pathway plays a crucial role in patterning and organogenesis during early development, in adult tissue maintenance and repairing functions [1]. The Hh signaling represents a complex transduction pathway orchestrated by several regulatory components and post-translational events. A simplified model of Hh signaling describes that in the absence of Hh ligand (Sonic, Indian and Desert Hh), the PATCHED receptor (PTCH) inhibits the class F G-protein-coupled receptor (GPCR) SMO. When PTCH is engaged by Hh, it relieves the inhibition of SMO and the signal is transduced to the downstream transcription factors GLI1, GLI2, and GLI3, which in turn regulate the expression of Hh target genes involved in key cellular processes, such as cell cycle, survival, migration, and metabolism [2]. Given the significant involvement of Hh signaling in the development of several districts including pancreas, kidney, lung, nervous system, and limb [3–5], its misregulation results in multiple birth and developmental defects [6,7]. Aberrant Hh pathway activation is responsible for the tumorigenesis of several disparate human cancers including

medulloblastoma (MB), rhabdomyosarcoma, melanoma, basal cell carcinoma (BCC), and breast, lung, liver, stomach, prostate, and pancreas tumors [8–11]. Hh-dependent carcinogenesis may result from abnormal upregulation of Hh ligands or deregulation of the expression or function of downstream components such as loss of *PTCH* [12] or suppressor of fused *SUFU* (the main negative regulator of Hh signaling) [13], activating mutations of *SMO* [14], amplification or chromosomal translocation of *GLI1* [15], *GLI2* gene amplification, or stabilization of GLI2 protein. Moreover, alterations of phosphorylation, ubiquitylation, and acetylation post-translational processes can also contribute to Hh-dependent tumorigenesis by modulating GLI1 function [16–18]. Remarkably, Hh signaling is active in cancer stem cells (CSCs) of various tumor types [19,20], sustaining the proliferation of these niche cells that are responsible for tumor relapse and resistance to conventional anticancer therapy. Indeed, the Hh pathway controls the functional properties of CSCs, such as self-renewal, survival, metastatic spread, and neoangiogenesis by the regulation of stemness-determining genes such as *Nanog*, often overexpressed in cancer. Given the increasing evidences supporting the crucial role of the Hh pathway in cancer initiation, proliferation, metastasis, chemoresistance, and in the survival of CSCs [10,17], its components represent attractive druggable targets for anticancer therapy.

SMO antagonists: pitfalls and limitations

The first Hh inhibitor discovered was cyclopamine, a naturally occurring alkaloid isolated from *Veratrum californicum* [21], which inactivates SMO by directly binding to its heptahelical bundle. Nevertheless, cyclopamine has shown several limitations as a drug candidate, such as toxicity and teratogenicity, poor oral bioavailability, and suboptimal pharmacokinetics with relatively short elimination half-life [22].

In recent years, drug discovery efforts directed against the Hh pathway have been focused predominantly on the development of SMO antagonists and a remarkable number of small molecules of natural, semisynthetic or synthetic origin have been developed and extensively reviewed in recent reports [23–25]. Several SMO antagonists have demonstrated efficacy in mouse xenografts and, most notably, have been investigated in clinical trials against a

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large range of metastatic and advanced cancers [26,27]. Among them, vismodegib (GDC-0449/Erivedge) became the first Hh inhibitor to receive approval from the USA FDA in January 2012 for the treatment of locally advanced or metastatic BCC [28].

Despite the initial enthusiasm, clinical development of SMO antagonists has ultimately proved disappointing, due to scarce pharmacokinetics, low selectivity on CSCs, severe side effects, and the emergence of drug resistance. Indeed, after an initial clinical response to treatment with vismodegib, a patient with metastatic MB showed tumor regrowth within 3 months due to D473H point mutation that rendered SMO insensitive to the drug [29]. Furthermore, acquired resistance has been also observed in BCC patients under treatment with vismodegib and in a Phase I study of saridegib (IPI-926). In this study, nine patients with BCC, previously progressed on vismodegib, failed to respond to saridegib, suggesting the existence of overlapping mechanisms of resistance [30]. Recently, genomic analysis of SMO resistance to vismodegib in BCC patients has revealed a number of additional SMO mutations and variants that confer constitutive activity and drug resistance [31,32]. All variants have shown partial or complete resistance to vismodegib, while the aPKC- γ /GLI inhibitor PSI and the GLI2 antagonist arsenic trioxide (ATO) were both able to suppress Hh pathway activation in the presence of any SMO variants [31].

Several lines of evidence also suggest that cancer cells can acquire resistance to SMO antagonists via SMO-independent hyperactivation of the powerful downstream GLI transcription factors, or mutations at different nodal points of the Hh pathway. Indeed, preclinical and clinical trials have shown that SMO drug resistance can be the consequence of (i) *GLI2* amplification during vismodegib or sonidegib (LDE-225) treatment; (ii) upregulation of noncanonical and synergistic GLI signaling [e.g., phosphoinositide 3-kinase (PI3K) pathway, observed during sonidegib treatment]; and (iii) increase of the expression of ATP-binding cassette transporters (ABCs), such as P-glycoprotein (Pgp), which diminishes drug efficacy by increasing its cellular clearance [29,33–36].

Moreover, the onset and progression of some types of Hh-driven cancers are related to Hh-pathway-activating mutations downstream of SMO, such as loss of *SUFU* or *GLI1* amplification, thus rendering SMO antagonists ineffective in these scenarios.

Not least, studies investigating systemic treatments with SMO antagonists have revealed several side effects including dysgeusia, alopecia, fatigue, nausea, diarrhea, decreased appetite, hyponatremia, weight loss, and especially muscle cramping due to noncanonical SMO signaling (SMO–AMP-activated protein kinase axis) and Ca²⁺ influx [37,38].

Consequently, the development of Hh inhibitors that modulate targets acting downstream of SMO or independently by SMO, such as GLI, has recently emerged as a more promising therapeutic strategy for the treatment of Hh-dependent tumor. This approach would allow overcoming anti-SMO resistance and adverse effects, which are responsible for >50% dropouts rates in SMO antagonists clinical trials.

GLI factors: new attractive targets in Hh-dependent tumors

GLI transcription factors are the final effectors of the Hh pathway and share common structural features, such as five highly conserved tandem zinc fingers (ZFs), a fairly conserved N-terminal domain, several potential protein kinase A (PKA) binding sites, and additional conserved regions at the C terminus. Nevertheless, GLI proteins exert different functions *in vivo*: GLI1 acts only as a transcriptional activator, whereas GLI2 and GLI3 can act both as transcriptional activators and as repressors, depending on the specific cell context and on the activation state of Hh signaling. In absence of upstream Hh signaling, some protein kinases [PKA, glycogen synthase kinase (GSK)3 β and casein kinase (CK)1] phosphorylate GLI proteins, leading to ubiquitylation/proteasome-dependent GLI1 degradation or GLI2 and GLI3 proteolytic cleavage into repressor forms (GLI2R and GLI3R). These events are mediated by Cullin1/beta-transducin repeats-containing proteins (β -TrCP) E3 ubiquitin ligase complex [1,39]. In contrast, the activation of Hh signaling inhibits this processing, resulting in full-length GLI2 and GLI3, which have activator function (GLI3A and GLI2A) [38]. The balance between activator and repressor functions of GLI transcription factors determines the status of the Hh transcriptional program and consequently the behavior of responding cells.

The fine interplay among post-translational modifications and intersection with other pathways, such as PI3K–AKT, has a crucial role in the regulation of GLI activity and in the generation of their repressor forms [40–42]. In addition to β -TrCP, for example, other E3 ligases belonging to the really interesting new gene (RING) (Cullin3/speckle-type POZ protein (HIB-SPOP)) or homologous to E6-AP carboxy terminus (HECT) (ITCH) family have been identified to be responsible for *Drosophila* and mammalian Ci/GLI proteolysis through phosphorylation-independent mechanisms [43,44]. Furthermore, acetylation/ubiquitylation interplay, mediated by histone deacetylases (HDACs) and members of Cul3 adaptor proteins family potassium channel tetramerization domain, (KCTDs) (KCASH, KCTD containing Cullin3 Adaptor Suppressor of Hedgehog), has been recently discovered as a key transcriptional checkpoint of GLI function [45,46].

Albeit several aspects of GLI regulation remain unclear, it is evident the key role of GLI in embryogenesis and adult homeostasis. Indeed, GLI factors activate the expression of a number of genes involved in functions related to tissue development, such as cell proliferation and differentiation (e.g., *CyclinD1* and *D2*, *N-Myc*, *Wnts*, *PDGFR*, *IGF2*, *FoxM1*, *FoxA2*, *Nkx2.2*, *FoxF1*, *Myf5*, *HES1*, and *IGFBP3*), survival (*Bcl2*), self-renewal and cell fate determination (*Bmi1* and *Nanog*), angiogenesis (*VEGF*), epithelial–mesenchymal transition (*Snail1*, *Sip1*, *Elk1*, and *Msx2*) and tumor cell invasiveness (*Osteopontin*) in several tissues [19,47]. Remarkably, GLI factors control the expression of both *GLI1* and *PTCH1*, thus forming a feedback loop enhancing or repressing Hh response. Given the broad spectrum of cellular events under GLI control, dysregulation of *GLI* genes could clearly lead to unfavorable developmental and pathological consequences, such as oncogenesis. *GLI1* was indeed the first Hh pathway gene found to be amplified in several cancers, such as glioblastoma, glial tumors, prostate cancer,

and a panel of brain tumors in a study that identified *GLI1* expression as the only reliable marker of Hh pathway activity [15,48]. Moreover, the level of *GLI1* transcript can be used to discriminate BCC from certain other skin tumors [49] and *GLI* deregulations have been revealed in MB, the most common malignant pediatric brain tumor. The primary mediator of Hh signaling, *GLI2*, has been also identified as pleiotropic oncogene, whose upregulation induces genomic instability and a number of the acquired characteristics of tumor cells [50]. Interestingly, *GLI2* is upregulated in a wide variety of human tumors such as melanoma [51], prostate cancer [52], BCC [53], and hepatocellular carcinoma [54], becoming potentially an attractive therapeutic target. Noteworthy, several reports have highlighted noncanonical mechanisms of *GLI* activation, in addition or independent of upstream Hh signaling [26,47,55]. For instance, *GLI* expression, stability, and/or transcriptional activity in normal and cancer cells may be positively modulated via the persistent stimulation of different growth factor cascades, such as EGF/EGFR (epidermal growth factor receptor), Wnt/ β -catenin, and the TGF- β 1/TGF- β R (transforming growth factor β receptor) system [56]. The aberrant crosstalk among Hh signaling and these pathways can induce tumor transformation. In this regard, K-RAS and TGF- β were shown to regulate *GLI1* expression in absence of SMO and they collaborate with Hh signaling to initiate pancreatic ductal adenocarcinoma (PDAC) development [57]. In contrast, EGFR signaling modulates Hh/*GLI* target gene expression during keratinocytes transformation inducing activation of JUN/activator protein-1 (AP1), which cooperates with *GLI1* and *GLI2* [58]. Moreover, the interaction between Hh and platelet-derived growth factor (PDGF) signaling has been shown in cultured murine fibroblasts, BCC cells, and CNS tumors [59]. Activation of the Hh/*GLI* signaling was also observed in Ewing sarcoma family tumors (ESFTs), where the oncogenic transcription factor Ewing's Sarcoma/Friend leukemia integration 1 (EWS/FL1), resulting from the chromosomal translocation t(11;22), induces directly *GLI1* expression [60]. Furthermore, PI3K/AKT signaling was described to negatively regulate the degradation of *GLI2* and potentiate *GLI1* transcriptional activity [61]. Hh signaling is also differently modulated by distinct members of the PKC family: upregulation of α PKC λ potentiates Hh signaling by directly phosphorylating and activating *GLI1* and because of α PKC λ is also an Hh target gene, it sustains a positive feedback loop contributing to Hh activation [62].

It is important to consider that, whatever alteration leads to aberrant Hh pathway activation, such as genetic mutations of pathway components or other SMO-dependent or -independent mechanisms, all trigger the downstream effector *GLI1*. For this reason, *GLI* factors are emerging as attractive targets for the development of novel anticancer drugs. However only a few *GLI* antagonists have been identified, most likely due to the lack of structural details related to *GLI* activity.

Most effective *GLI* antagonists developed so far are herein reviewed and classified based on their mechanism of action. Direct *GLI* antagonists act by contacting directly *GLI* effectors and blocking their transcriptional functions, whereas indirect *GLI* antagonists inhibit *GLI* functions

through mechanisms that control *GLI* activity, such as proteolytic degradation or post-translational modifications.

Indirect *GLI* antagonists

Cyclohexyl-methyl aminopyrimidines (CMAPs)

A series of CMAPs (Figure 1) have been identified by a phenotypic screening approach, designed to classify compounds as SMO or non-SMO antagonists. Bassilana and coworkers have discovered a CMAP chemotype able to block *GLI* functions and, through a chemoproteomics strategy, they also have identified the orphan GPCR GPR39 as the specific target of this molecule [63]. Although the authors failed to demonstrate the direct binding of CMAP to GPR39, data document that CMAP is a specific agonist of this receptor. Indeed, ectopic expression or knockdown of GPR39 conferred or decreased, respectively, responsiveness to CMAP. Interestingly, CMAP analogs were able to inhibit *GLI*-activated signaling following overexpression of *GLI1* or *GLI2*, providing evidence that CMAPs affect Hh signaling (Table 1). Specifically, CMAPs-activated GPR39 leads to inositol triphosphate production and activation of mitogen-activated protein kinase (MAPK) pathway, thus repressing *GLI* signaling. However, the mechanism by which GPR39 activation impinges *GLI* function has not been elucidated (Figure 2). Since MAPK activation occurs also via G_i -coupled signaling and β arrestin recruitment, the discovery of CMAPs/GPR39 axis has identified a new mechanism for Hh inhibition downstream of SMO, triggering signaling pathways that cooperate to counteract Hh activation.

JQ1 and I-BET151

Recently, epigenetic enzymes have emerged as druggable targets for attenuating the growth of Hh-dependent tumors [64]. A new class of compounds targeting bromo and extra C-terminal (BET) bromodomain (BRD) proteins has been described to specifically affect *GLI* transcriptional activity. Members of the BET family proteins (BRD1–4) are involved in cell cycle progression, chromatin compaction, and chemoresistance. These proteins, through their bromodomains, bind to acetylated lysines in histones, and through their interaction with the positive transcription elongation factor (p-TEFb) stimulate RNA polymerase II activity and enhance gene expression [65]. Among different BET enzymes, suggestive evidences identify BRD4 as an attractive candidate target for developing therapeutics against Hh-driven tumors. Tang and colleagues have demonstrated that BRD4 activates Hh signaling in a ligand-independent manner, finely tuning *GLI1*- and *GLI2*-mediated transcription by direct occupancy of their promoters. Accordingly, they have identified the BET inhibitor JQ1 (Figures 1 and 2) as a potent *GLI* antagonist that alters the recruitment of *GLI* proteins on their target genes, determining growth arrest and cell death of tumors from patients with aberrant Hh pathway activation (Table 1). Importantly, JQ1 also impaired *GLI* signaling in a context of acquired resistance to SMO antagonism. Another BRD4 inhibitor, I-BET151 (Figures 1 and 2), has been recently described to suppress the expression of Hh target genes in a SMO-independent fashion, thus inhibiting MB cells growth,

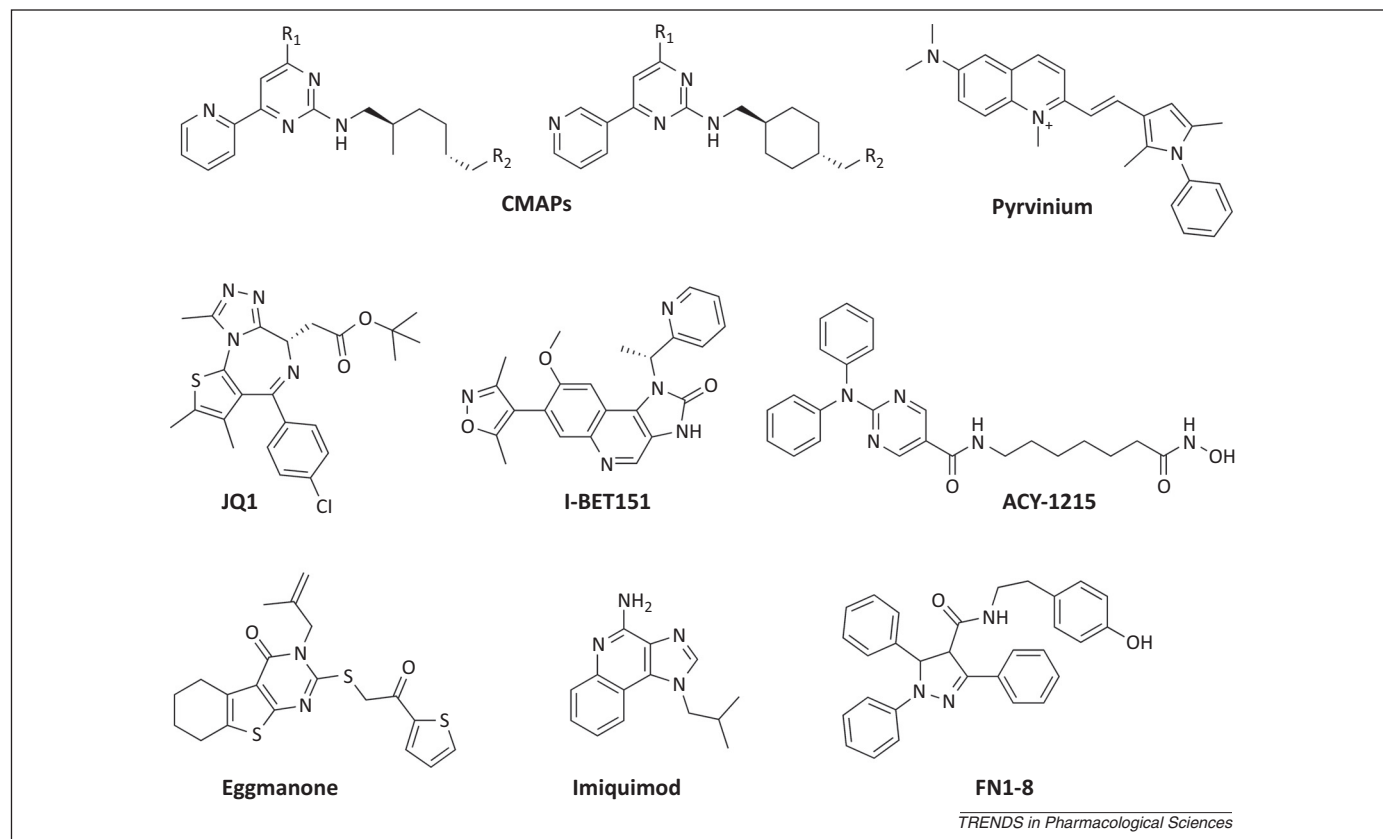


Figure 1. Indirect GLI antagonists. Chemical structure of the most effective GLI antagonists acting by an indirect mechanism of action. Abbreviations: CMAP, cyclohexyl-methyl aminopyrimidines; GLI, glioma-associated oncogenes.

in vitro and *in vivo* (Table 1) [66]. Notably, BRD4 has been found overexpressed in PDAC cell lines, being able to sustain a high proliferative growth rate and chemoresistance [67]. All these findings suggest that BET inhibitors can represent an innovative therapeutic tool for the treatment of aggressive Hh-dependent tumors besides MB and BCC.

HDAC inhibitors (HDACi)

HDACs are a class of enzymes known as epigenetic readers that play a key role in the regulation of multiple biological events. Members of HDACs family are involved in the control of Hh pathway. GLI1 and GLI2 have been reported to be acetylated proteins [45,68] and their HDAC-mediated deacetylation has been described to promote their transcriptional activation. This mechanism sustains a positive autoregulatory loop through Hh-induced upregulation of HDAC1 and plays a role in ubiquitylation-dependent control of GLI1 function. Indeed, a Cul3 E3 ubiquitin ligase complex, formed by a family of the adaptor proteins KCASH1, KCASH2, and KCASH3, triggers HDAC1 degradation [45,46]. These findings unveil an integrated HDAC- and ubiquitin-mediated circuitry, where acetylation of GLI proteins functions as an unexpected key transcriptional checkpoint of Hh signaling. These data identify HDACi as Hh antagonists able to prevent transcriptional activity by inducing GLI1 and GLI2 hyperacetylation (Figure 2) [41,45,68]. Next to HDAC1, also HDAC5 and HDAC9 were found to be upregulated in MB, and their expression was associated with poor prognosis [69]. Interestingly, Dhanyamraju and colleagues have recently observed that another deacetylase, HDAC6, implicated

in primary cilium biology, is overexpressed in a murine model of Hh-driven MB. These findings suggest that selective inhibition of HDAC6 can represent a new therapeutic approach in the treatment of Hh-dependent malignancies. Indeed, the specific HDAC6 antagonist, ACY-1215 [70] (Figures 1 and 2 and Table 1), reduced strikingly *in vivo* tumor growth. Hence, HDAC6, despite exerting a dichotomous role (it is required to achieve full pathway activity, but it has also been described to repress basal Hh target gene transcription) is a critical player in Hh pathway regulation, promoting the maximum expression of a subset of GLI target genes [70]. Since the upregulation of HDACs has been documented in various types of tumors, the development of novel potent and selective HDACi is ongoing. In this regard, several chemical classes of HDACi are currently being tested in human cancer therapy. To date, three HDACi (vorinostat [SAHA], romidepsin and belinostat) have been approved by the FDA for treating refractory cutaneous and peripheral T cell lymphoma, while other HDACi have entered clinical trials in both solid and hematological malignancies [71,72]. More importantly, it has been reported that SAHA can penetrate the blood-brain barrier and induces apoptosis in mouse models of MB [73].

Pyrvinium

Recently, the anthelmintic drug pyrvinium (Figure 1) has been described to affect on Hh signaling by inducing GLI destabilization [74,75]. In particular, pyrvinium has been described as a specific activator of CK1 α , a kinase that negatively regulates GLI transcription factors by promoting the processing of GLI3 into repressor form and GLI2

Table 1. Efficacy and *in vivo* study of GLI antagonists

Compound	Assay	Efficacy ^a	<i>In vivo</i> mouse study	Dosage	Refs
CMA-1	TM3 (1 nM Ag1.5) GliLuc-assay	1.1 μ M (EC ₅₀)	n.d.	n.d.	[63]
CMA-2	TM3 (1 nM Ag1.5) GliLuc-assay	11.3 μ M (EC ₅₀)	n.d.	n.d.	[63]
CMA-3	TM3 (1 nM Ag1.5) GliLuc-assay	0.004 μ M (EC ₅₀)	n.d.	n.d.	[63]
CMA-4	TM3 (1 nM Ag1.5) GliLuc-assay	0.12 μ M (EC ₅₀)	n.d.	n.d.	[63]
CMA-5	TM3 (1 nM Ag1.5) GliLuc-assay	0.02 μ M (EC ₅₀)	n.d.	n.d.	[63]
CMA-6	TM3 (1 nM Ag1.5) GliLuc-assay	n.d.	n.d.	n.d.	[63]
CMA-7	TM3 (1 nM Ag1.5) GliLuc-assay	0.01 μ M (EC ₅₀)	n.d.	n.d.	[63]
CMA-8	TM3 (1 nM Ag1.5) GliLuc-assay	0.05 μ M (EC ₅₀)	n.d.	n.d.	[63]
CMA-9	TM3 (1 nM Ag1.5) GliLuc-assay	0.2 μ M (EC ₅₀)	n.d.	n.d.	[63]
I-BET151	Shh-Light2 cells (SAG)/firefly luciferase	31 nM (IC ₅₀)	Allograft of spontaneous MBs from <i>Ptch</i> ^{+/-} mice	30 mg/kg	[66]
JQ-1	Cell viability in Smo WT -MB and Med1-MB cells	~50–150 nM (IC ₅₀)	Allograft of Med1-MB cells; SmoWT-MB or SmoD477G-MB cells. Allograft model of <i>Ptch</i> ^{+/-} ; <i>K14-creER2</i> ; <i>p53</i> ^{fllox/fllox} -derived mouse BCC cells	50 mg/kg	[109]
HDiA and HDiB	Shh-Light2 cells (SAG)/firefly luciferase	n.d.	n.d.	n.d.	[45]
ACY-1215	Shh-Light2 cells (SAG)/firefly luciferase	n.d.	Allograft of primary SmoA1 MB cells (MB99-1 cells)	50 mg/kg	[70]
Pyrvinium	Shh-Light2 cells (SAG or SHH)/firefly luciferase	~10 nM (IC ₅₀)	(a) Allograft of spontaneous MBs from <i>Ptch</i> ^{+/-} mice (b) APC ^{min} mice	(a) 0.8 mg/kg 5 mg/kg (b) 25 mg/kg	[74,75]
GANT58	Shh-Light2 cells (SAG)/firefly luciferase	~5 μ M (IC ₅₀)	Xenograft of 22Rv1 prostate cancer cells	50 mg/kg	[81]
GANT61	Shh-Light2 cells (SAG)/firefly luciferase	~5 μ M (IC ₅₀)	Xenograft of 22Rv1 prostate cancer cells Xenograft of Huh7 hepatocellular carcinoma cells Orthotopic xenograft of CFPAC-1 pancreatic cancer cells	50 mg/kg	[81,86,91]
HPI-1	GLI1 overexpressing NIH 3T3 cells/firefly luciferase	6 μ M (IC ₅₀)	Subcutaneous and orthotopic xenografts of Huh7 hepatocellular carcinoma cells	30 mg/kg	[100,101]
HPI-2	GLI1 overexpressing NIH 3T3 cells/firefly luciferase	>30 μ M (IC ₅₀)	–	–	[100]
HPI-3	GLI1 overexpressing NIH 3T3 cells/firefly luciferase	>30 μ M (IC ₅₀)	–	–	[100]
HPI-4	GLI1 overexpressing NIH 3T3 cells/firefly luciferase	>30 μ M (IC ₅₀)	–	–	[100]
ATO	NIH 3T3 (ShhN) GliLuc-assay	0.7 μ M (IC ₅₀)	(a) Allograft of primary MBs from <i>Ptch</i> ^{+/-} <i>p53</i> ^{-/-} mice (b) Allograft of BCC tumor derived from <i>Ptch</i> ^{+/-} ; <i>K14-creER2</i> ; <i>p53</i> ^{fllox/fllox}	(a) 2.5–10 mg/kg (b) 7.5 mg/kg	[92,94]
GlaB	GLI1-overexpressing HEK293T cells/firefly luciferase	12 μ M (IC ₅₀)	(a) Allograft of spontaneous MBs from <i>Ptch</i> ^{+/-} mice (b) Orthotopic xenograft of Daoy MB cells (c) Allograft of ASZ001 BCC cells	(a)–(b) 35 mg/kg (c) 50 mg/kg	[106]

^aEfficacy is expressed as EC₅₀ or IC₅₀ as reported in parentheses. n.d., not determined/unknown.

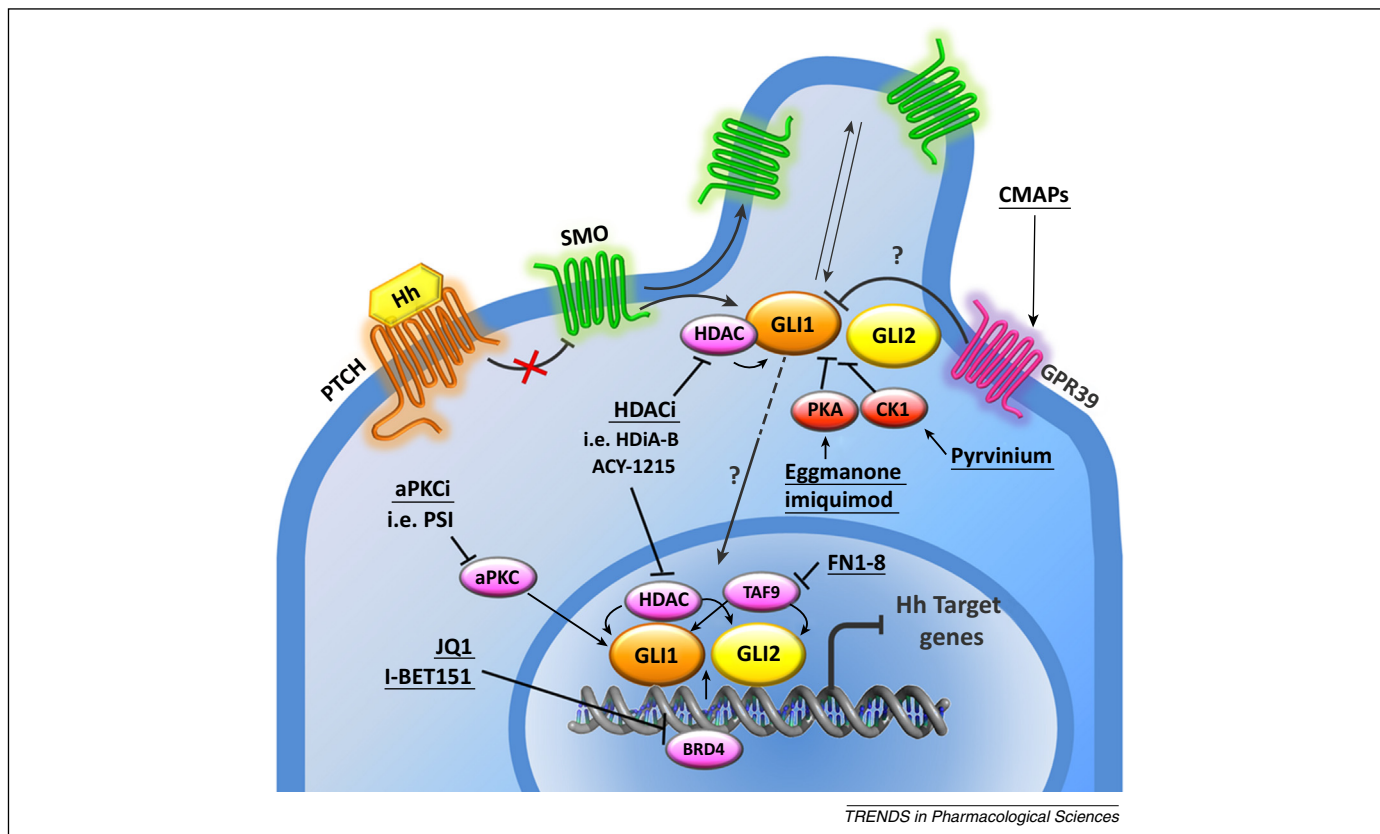


Figure 2. Mechanism of Hh inhibition by indirect GLI antagonists. CMAPs activate the orphan receptor GPR39, which represses GLI signaling through a not yet elucidated mechanism. Pyrvinium is an activator of CK1 kinase, while eggmanone and imiquimod are PKA activators; these molecules negatively regulate GLI transcription factors by promoting GLI2 repressor form and Gli1 degradation. HDACi, such as HDiA-B (HDAC1-2i) or ACY-1215 (HDAC6i) inhibit HDAC-mediated GLI deacetylation, which is required for GLI transcriptional activation. JQ1 and I-BET151 inhibit BRD4, which activates GLI1 and GLI2 transcription by direct occupancy of their promoters. PSI inhibits aPKC, which promotes GLI1 binding to DNA. FN1-8 affects GLI-mediated transcriptional activity by inhibiting GLI1 and GLI2/TAF9 interaction. Abbreviations: aPKC, activated protein kinase C; BRD4, bromodomain-containing protein 4; CK1, casein kinase 1; CMAPs, cyclohexyl-methyl aminopyrimidines; FN1-8,; GLI, glioma-associated oncogenes; GPR 39, G-protein-coupled receptor 39; HDACi, histone deacetylase inhibitor; Hh, Hedgehog; PKA, protein kinase A; PTCH, PATCHED; SMO, SMOOTHENED; TAF9, TBP (TATA-box binding protein)-associated factor 9.

ubiquitin-dependent degradation (Figure 2) [76]. Notably, pyrvinium attenuates Hh-dependent MB cell proliferation downstream of SMO, both *in vitro* and *in vivo* (Table 1). This occurs as a consequence of its potent efficacy in down-regulation of *GLI1* and *PTCH* expression also in a context of hyperactivation of the Hh pathway, resulting from the loss of the negative regulator *SUFU* or overexpression of GLI proteins. Moreover, pyrvinium strongly suppressed Hh signaling induced by the oncogenic SMO mutant, which instead showed resistance to the treatment with vismodegib. Anti-SMO resistance indicates the presence of bystander co-regulatory mechanisms of the Hh pathway and suggests that their targeting may synergize with Hh inhibitory drugs [29]. Indeed, PI3K/AKT inhibitors revert anti-SMO resistance, such as that induced by LDE225 compound [33]. Further, the ability of the mammalian target of rapamycin (mTOR)/protein-chinasi 1 p70-S6 (S6K1)-mediated phosphorylation and activation of GLI1 might explain the effect of AKT/PI3K inhibitors because of their ability to downregulate mTOR function [77].

Eggmanone, imiquimod, and other SMO-independent antagonists of GLI

The SMO antagonist vismodegib recently approved by the FDA for the treatment of BCC, causes frequently resistance and severe side effects. Thus, the discovery of novel

clinical strategies for BCC therapy is increasingly becoming important. In this regard, new compounds, eggmanone and imiquimod, which both target Hh signaling through PKA activation, are under clinical investigation. Eggmanone (Figure 1) is a small molecule identified by an *in vivo* chemical genetic screening of 30 000 others compounds for their ability to reproduce the Hh-null phenotype in zebrafish embryo. This molecule affects some typical structural features such as ventral tail curvature, small eyes, loss of pectoral fins, enlarged rounded somites, loss of neurocranial chondrogenesis and impaired slow muscle formation [78]. Specifically, *in vitro* studies show that eggmanone antagonizes Hh signaling by inhibition of phosphodiesterase (PDE)4D3, thus determining an increase of cAMP levels (Figure 2). This event leads to selective activation of PKA at the base of the primary cilium, a PKA-localization required to promote GLI processing. Imiquimod (Figure 1) is a synthetic nucleoside analog of the imidazoquinoline family, recently approved for topical treatment of small superficial BCCs. This molecule counteracts Hh signaling downstream of SMO by inducing PKA activity (Figure 2) with consequent GLI2/3 phosphorylation and their cleavage into repressor forms [79]. Other compounds have been identified to impinge the Hh pathway by targeting proteins and/or interactors that modulate GLI factors activity. For instance, rapamycin inhibits GLI1 functions

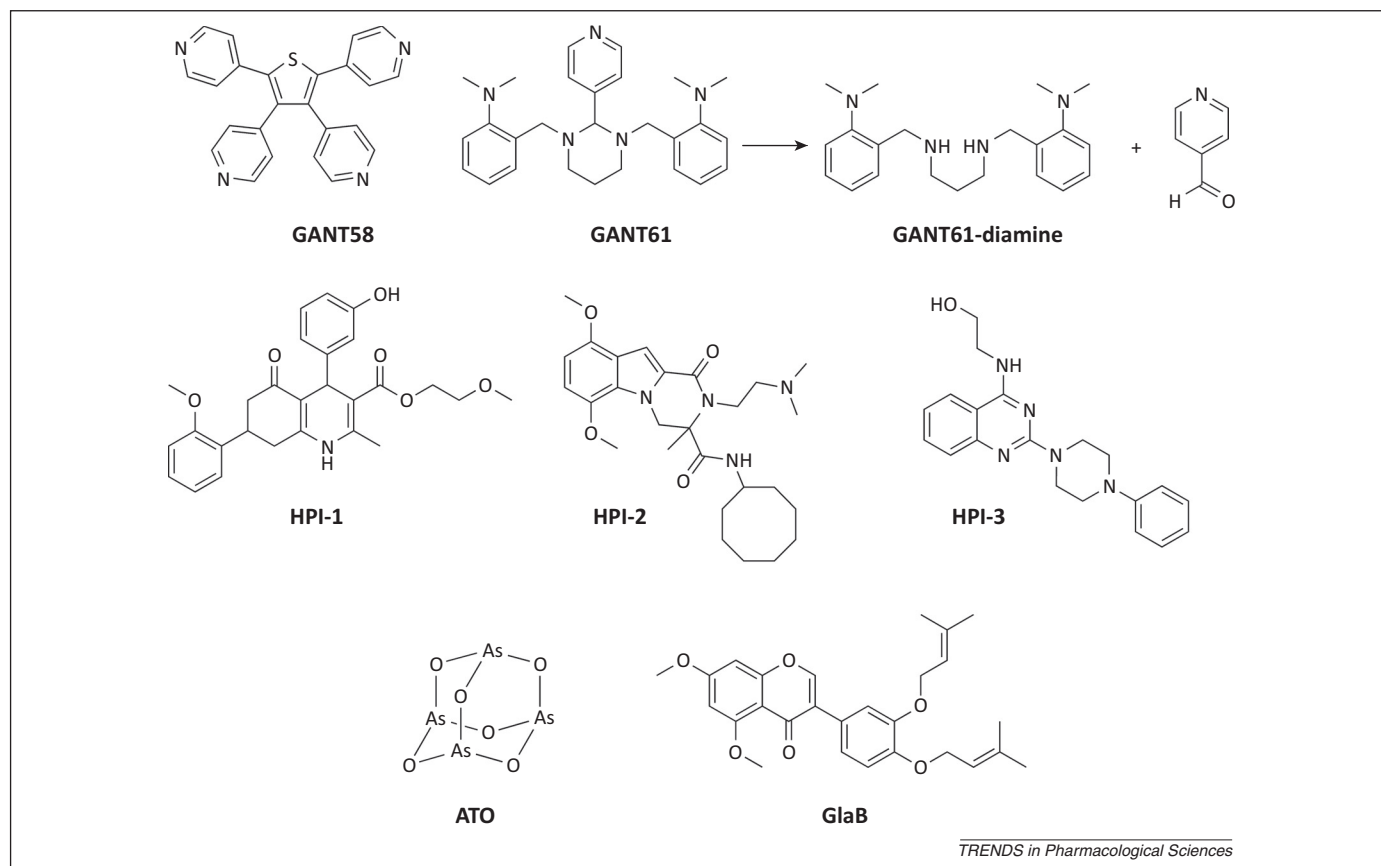


Figure 3. Direct GLI antagonists. Chemical structure of the most effective GLI antagonists acting by a direct mechanism of action. The putative mechanism of GANT61 hydrolytic degradation is also reported. Abbreviations: ATO, arsenic trioxide; GlaB, Glabrescione B; GLI, glioma-associated oncogenes; GANT, GLI antagonist; HPI, Hedgehog pathway inhibitors.

through repressing its phosphorylation and activation induced by tumor necrosis factor (TNF) α and mTOR/S6K1 in esophageal adenocarcinoma cells [77]. FN1-8, a synthetic small molecule comprising a pyrazoline structure (Figure 1), strongly reduces GLI-mediated transcriptional activity by disrupting the interaction of both GLI1 and GLI2 with TBP (TATA box-binding protein)-associated factor (TAF)9, a transcription coactivator (Figure 2). Noteworthy, FN1-8 was able to suppress the proliferation of lung cancer cells *in vitro* and *in vivo* and to inhibit the cell growth of numerous cancer cells that express high GLI levels, including prostate, pancreatic, colon cancer, and glioma [80].

Direct GLI antagonists

GLI antagonist 61 (GANT61)

In 2007, a cellular screen for small molecule inhibitors of GLI1-mediated transcription led to the identification of GANT58 and GANT61 (Figure 3) as the first GLI antagonists [81]. GANT61 proved to counteract GLI-mediated tumor growth more efficiently than GANT58, becoming therefore the lead candidate for further investigations and the most valuable tool for pharmacological Hh pathway inhibition. Although its mechanism of action has not been elucidated and no records of preclinical or clinical studies are available, GANT61 is the reference GLI1/GLI2 antagonist in many biological and drug design studies. GANT61 inhibits Hh signaling by impairing GLI1- and GLI2-mediated transcription *in vitro* and *in vivo*, affecting the GLI1/DNA interaction only in living cells probably by inducing

post-translational modifications of GLI1 (Figure 4) [81]. Moreover, GANT61 is poorly stable at physiological conditions, and quickly hydrolyzes into a benzaldehyde species (inactive against Hh signaling) and a diamine derivative (Figure 3), which has showed the same efficacy as GANT61 in inhibiting GLI-mediated transcription [82].

GANT61 has been successfully used to inhibit the Hh-GLI1 axis at the GLI1 and/or GLI2 level in several cancer cells, CSCs, and tumor animal models, such as pancreatic, [83] prostate and lung cancer [81,84], colon [85] and hepatocellular carcinoma (Table 1) [86], as well as against tumors for which the role of GLI transcription was not elucidated before, including ovarian cancer [87], esophageal adenocarcinoma [88], and melanoma CSCs [89], emphasizing the therapeutic potential of GANT61. Finally, GANT61 has been used to monitor the relationship between specific GLI targeting and autophagy. Indeed, GANT61 has been found to induce cancer suppressing autophagic processes in PDAC and hepatocellular carcinoma cells, whereas pro-survival autophagy has been observed in neuroblastoma cells [86,90,91].

Arsenic trioxide (ATO)

ATO (Figure 3) is an FDA-approved drug for second-line treatment of acute promyelocytic leukemia that functions by binding to cysteine residues of proteins involved in MAPK, nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), c-Jun N-terminal kinases (JNK) and other pathways. In 2010, Kim and collaborators observed that ATO antagonizes the Hh pathway *in vitro* and *in vivo* by

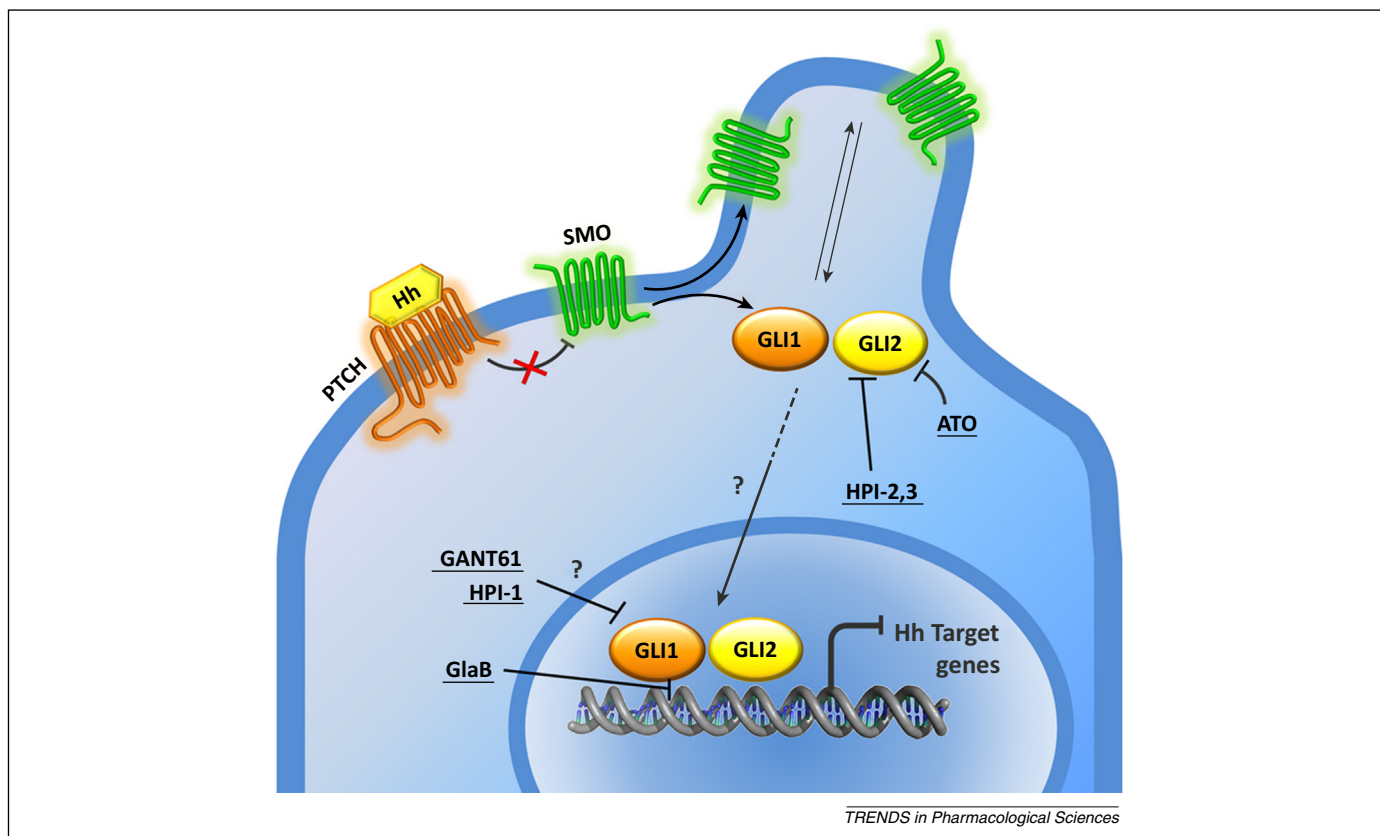


Figure 4. Mechanism of Hh inhibition by direct GLI antagonists. GANT61 inhibits GLI-mediated transcription by an unclear mechanism (most likely by inducing post-translational modifications of GLI). ATO affects ciliary accumulation of GLI2 and enhances its degradation after longer treatment regimens. HPI-1 has been observed to target post-translational events of GLI processing/activation downstream of SMO. HPI-2 and HPI-3 seem to alter the cellular trafficking of GLI1 and to increase the stability of GLI2. GlaB inhibits GLI1/DNA interaction by its ability to bind GLI1 zinc-finger domain. Abbreviations: ATO, arsenic trioxide; GANT, GLI antagonist; GlaB, Glabrescione B; GLI, glioma-associated oncogenes; Hh, Hedgehog; HPI, Hedgehog pathway inhibitors; PTCH, PATCHED; SMO, SMOOTHENED.

directly interfering with GLI transcription factors (Table 1) [92]. ATO has been observed to inhibit ciliary accumulation of GLI2 in the short term, and to enhance GLI2 degradation after a prolonged incubation time in MB cells (Figure 4). In 2011, Beauchamp *et al.* showed that ATO inhibits Hh signaling by interacting directly with GLI1, without altering GLI1/DNA interaction or modifying GLI1 cellular trafficking and stability [93]. Although these works have proposed rather different mechanisms, they have clearly indicated that ATO, used alone or in combination with other anticancer drugs, may represent a valuable therapeutic option to treat Hh-dependent tumors, particularly those harboring drug-resistant SMO mutations [92–94]. Accordingly, itraconazole, an FDA-approved triazole antifungal agent, operates on the Hh signaling at the level of SMO at a different site than cyclopamine. Single and combined therapy with itraconazole and ATO inhibits cellular growth of MB and BCC *in vitro* and *in vivo*, both in wild type and in drug-resistant SMO^{D477G} mice. In fact, the problem of resistance establishment to cyclopamine or cyclopamine mimics, due to some human SMO point mutations (such as D473H and E518K), is becoming even more prevalent. These characteristics strongly encourage the synergic therapy with itraconazole and ATO for the treatment of *de novo* Hh-dependent tumors or those with acquired resistance to cyclopamine [94]. Following these studies, the efficacy of ATO in targeting GLI transcription factors has been investigated in MB [93–95], pleural mesothelioma [96], malignant rhabdoid tumors [97], osteosarcoma [98], and many others cancer

types. Furthermore, growing evidence points to a role for ATO as a modulator of the proliferation and differentiation of cancer cells progenitors by impacting on Hh or Notch signaling [99]. It is worth mentioning that a pilot clinical trial study to monitor the efficacy of ATO in treating patients with BCC started in late 2013 (<https://clinicaltrials.gov/show/NCT01791894>).

Hedgehog pathway inhibitors (HPIs)

A cell-based high-throughput screening performed by Hyman and coworkers led to the identification of some chemically unrelated small molecules (namely, HPI-1, HPI-2, HPI-3, and HPI-4) (Figure 3), which inhibit the Hh pathway downstream of SMO by directly antagonizing GLI (Table 1) [100]. Collectively, HPIs have demonstrated multiple and different mechanisms of action (Figure 4). HPI-1 has been observed to target post-translational events of GLI processing/activation downstream of SMO and has been shown to inhibit the growth of hepatocellular carcinoma and MB cells expressing drug-resistant SMO, especially when encapsulated in nanoparticles (Table 1) [101]. In contrast, HPI-2 and HPI-3 have been associated with an altered trafficking of GLI1 and with increased stability of GLI2, which in turn impairs the conversion of full-length GLI2 to a transcriptional activator. Finally, HPI-4 has been found to perturb ciliogenesis by an unclear mechanism, and to decrease human chondrosarcoma cell proliferation, invasion, and migration [102]. Only HPI-1 and HPI-4 have been shown to inhibit the proliferation of

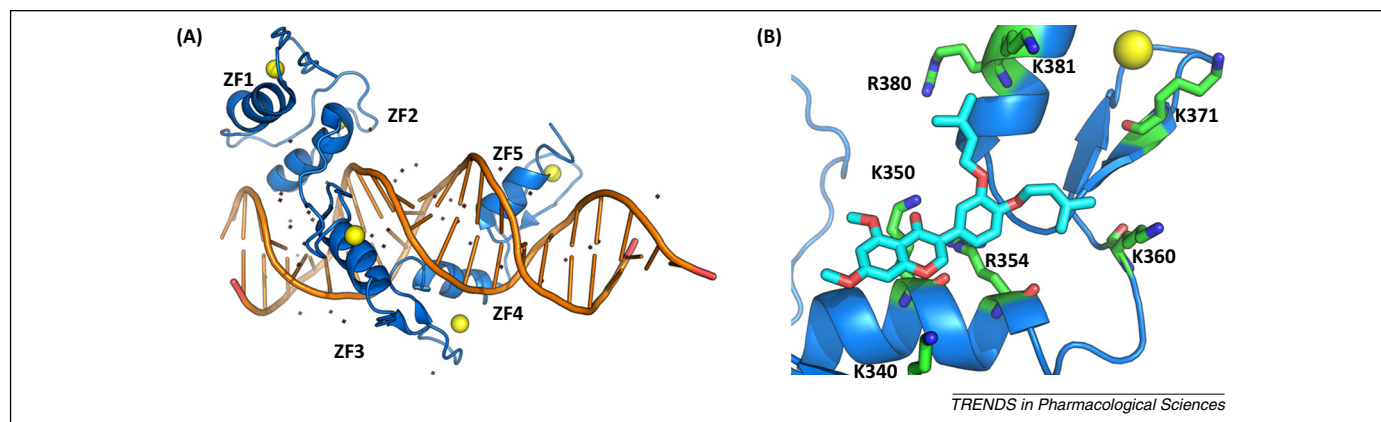


Figure 5. Structural details of GLI1/DNA interaction. **(A)** X-ray crystallographic structure of GLI1 ZF domain in complex with DNA, PDB ID: 2GLI; crystallographic water molecules are shown as red dots. **(B)** Details of the predicted binding pose of GlaB (cyan stick) within ZF4 and ZF5. Key residues for GLI1 transcriptional functions are shown as green sticks. Metal ions are shown as yellow spheres. Abbreviations: GlaB, Glabrescione B; GLI, glioma-associated oncogenes; ZF, zinc finger.

cerebellar granule neuron precursors. The multiple effects of HPIs on Hh signaling have highlighted the complexity of Hh/GLI regulation and have offered different opportunities for pharmacological modulation of aberrant Hh signaling even though their efficacy *in vivo* has not been evaluated yet.

Glabrescione B (GlaB)

Natural products have historically had a significant impact in Hh modulation (i.e., cyclopamine). Among the various chemical classes of natural products, isoflavones have displayed a noticeable pharmacophoric preference for Hh targets, as underlined by genistein and its derivatives in differentiated pancreatic cancer cells and CSCs [103,104]. Recently, our own research group has established a multidisciplinary drug discovery program focusing on the identification of natural products as direct GLI1 antagonists. Starting from the crystallographic structure of the GLI1-ZF domain in a complex with DNA [105], we have performed a mixed computational and experimental structure-based study to identify K350, R354, R380, and K381 residues as the strongest hot spots for GLI1/DNA interaction and transcriptional functions (Figure 5) [106]. This information was subsequently used to discover pharmacological agents able to interfere with this basic process. By virtual screening of a natural compounds library against these hot spots and monitoring Hh inhibition at GLI1 level, we have identified GlaB (Figures 3 and 5B, Table 1), an isoflavone naturally occurring in *Derris glabrescens*, as a potent Hh inhibitor acting downstream of SMO. NMR and (EMSA) Electrophoretic Mobility Shift Assay experiments have confirmed that GlaB binds to GLI1 in correspondence of ZF4 and ZF5 affecting GLI1/DNA interaction and also emphasizing the role of K350 and K340 in this event [106] (Figures 4 and 5). GlaB has proved to inhibit Hh signaling in multiple cancer cells and CSCs, as well as the *in vivo* MB and BCC tumor growth in orthotopic xenograft mice and in allograft mice models, respectively (Table 1) [106]. Our work has raised the importance to investigate the mechanism of GLI1-mediated transcription, and the identification of the structural requirements of GLI1/DNA interaction have highlighted their relevance for pharmacological interference of GLI1 signaling by direct GLI1 antagonists.

Concluding remarks

A critical goal in Hh-dependent tumor biology is the discovery of novel small molecules blocking the pathway at a downstream level. The rationale for the development of these pharmacological agents is based on (i) the heterogeneity of molecular defects sustaining the pathway activation; and (ii) the need to overcome the resistance to clinically available SMO antagonists.

Targeting GLI effectors represents a promising therapeutic strategy for cancer treatment. This is particularly relevant for certain tumors, such as MB, since, although classified into four distinct molecular groups (Hh- or Wnt-type and 3rd or 4th), common signals, such as placental growth factor (PIGF)/neuropilin axis, responsible of GLI1 upregulation, are shared by all subtypes and are important for tumor growth [107].

Transcription factors are generally considered as challenging targets in drug discovery for many different reasons, including the lack of deep hydrophobic pockets that accommodate small molecules and the highly charged surface. Nevertheless, big pharmaceutical companies are currently running Phase I clinical trials with small molecules targeting transcription factors such as Notch (Bristol-Myers Squibb and Ely Lilly) and p53 (Roche and Sanofi) [108], although no drugs have been approved yet by the FDA. Beside these targets, cMYC, NF- κ B, signal transducer and activator of transcription (STAT)3, STAT5, AP1, hypoxia-inducible factor (HIF1), and GLI1 are among the most promising transcription factors for the development of new anticancer drugs. GLI functions are finely tuned by a number of molecular interactions and postsynthetic modifications (i.e., GLI1 phosphorylation, gene copy number amplification, BRD4-driven epigenetic activation, deubiquitylation, deacetylation, or activation by aPKC ϵ /k or p70S6K or RAS/ERK) that, if dysregulated, are responsible for the resistance to anti-SMO drugs frequently observed in Hh-driven tumor initiation, progression and relapse [16,26, 109]. Interestingly, GLI-processing and activating post-translational events are pharmacologically targetable, even though by means of combination therapies. For this reason, the challenge in this field is the understanding of the molecular mechanisms that regulate GLI-mediated transcription. In this regard, the recently reported identification of the structural requirements of GLI1/DNA interaction,

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stands as a promising tool for discovering small molecules capable of inhibiting Hh pathway by directly targeting GLI [106]. The discovery of the natural compound, Glab, able to impair Hh oncogenic activity by inhibiting GLI1/DNA interaction, provides a proof-of-principle for the therapeutic relevance of such an approach, focused on the unique downstream GLI1 transcriptional effector rather than on multiple upstream oncogenic deregulated signals. Since the GLI proteins share highly conserved sequence of their ZF domain, targeting GLI1/DNA interaction could also interfere with GLI3/DNA binding, counteracting the GLI3 repressor function and resulting in severe side effects. However, the use of Hh antagonists is thought in a context of aberrant Hh pathway activation, where GLI repressor forms are absent, so bypassing the possibility of nonspecific effects on negative regulatory activity of GLI factors. Therefore, drugs specifically designed to modulate GLI/DNA interaction would provide valuable insights for developing and optimizing GLI antagonists, which would promise a more effective treatment of Hh-dependent tumors.

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