Chemistry & Biology
Brief Communication

Small Molecule Antagonists in Distinct Binding Modes Inhibit Drug-Resistant Mutant of Smoothened

Haiyan Tao,[1,5] Qihui Jin,[1] Dong-In Koo,[2] Xuebin Liao,[1] Nathan P. Englund,[1] Yan Wang,[1] Arun Ramamurthy,[3] Peter G. Schultz,[2] Marion Dorsch,[3,6] Joseph Kelleher,[3] and Xu Wu[1,4,*]
[1]Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121, USA
[2]Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA
[3]Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA
[4]Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129, USA
[5]Present address: Orphagen Pharmaceuticals, San Diego, CA 92121, USA
[6]Present address: Sanofi-Aventis, Cambridge, MA 02139, USA
*Correspondence: xwu@cbrc2.mgh.harvard.edu
DOI 10.1016/j.chembiol.2011.01.018

SUMMARY

Several small molecule antagonists for Smoothened (Smo) have been developed, and achieved promising preclinical efficacy in cancers that are dependent on Hedgehog (Hh) signaling. However, in a recent clinical study, a drug-resistant D473H SMO mutant was identified that is thought to be responsible for cancer relapse in a patient with medulloblastoma. Here, we report two Smo antagonists that bind to distinct sites, as compared to known antagonists and agonists, and inhibit both wild-type and mutant Smo. These findings provide an insight of the ligand-binding sites of Smo and a basis for the development of potential therapeutics for tumors with drug-resistant Smo mutations.

INTRODUCTION

Hedgehog (Hh) signaling is a key signal transduction pathway regulating embryonic development and tissue regeneration (Hooper and Scott, 2005; Ingham and McMahon, 2001). In mammalian cells, binding of Hh ligands (Shh, Ihh, and Dhh) to the receptor Patched1 (Ptch1) relieves the inhibition of a GPCR-like protein Smoothened (Smo). Pathway activation ultimately leads to the activation of the Gli family of transcription factors (Gli1 and Gli2) and induces the expression of downstream target genes (Ingham and McMahon, 2001).

It has been shown that aberrant regulation of Hh signaling is involved in developmental defects and cancers, such as cyclopia, Gorlin's syndrome, basal cell carcinoma (BCC), and medulloblastoma (Epstein, 2008; Rubin and de Sauvage, 2006; Scales and de Sauvage, 2009; Yauch et al., 2008). Therefore, small molecule agonists and antagonists of Hh signaling could be useful therapeutics for the treatment of degenerative diseases and cancers. Most of the known small molecule regulators of the Hh pathway bind to Smo, including agonists (purmorphamine, Hh-Ag 1.2 (SAG) and its derivative Hh-Ag 1.5) and antagonists (cyclopamine, SANT1, CUR61414, GDC-0449, NVP-LDE225, IPI-926, etc.) (Chen et al., 2002b; Frank-Kamenetsky et al., 2002; King, 2002; Mahindroo et al., 2009; Pan et al., 2010; Taipale et al., 2000; Williams et al., 2003; Wu et al., 2004). Notably, GDC-0449 showed promising efficacy in phase I/II clinical trials in the treatment of patients with BCC and medulloblastoma (Rudin et al., 2009; Von Hoff et al., 2009). However, in the phase I clinical study of GDC-0449, a drug-resistant mutation in SMO was identified from the relapsed tumor samples. The D473H mutation, or D477G mutation discovered from a mouse tumor resistant to GDC-0449, has no effect on Hh signal transduction but disrupts the binding of antagonist GDC-0449 (Rudin et al., 2009; Yauch et al., 2009). This finding highlights the need for new Hh inhibitors with alternative mechanisms.

Several known Smo antagonists, including GDC-0449 and KAAD-cyclopamine, strongly compete with ^{3}H-labeled Hh-Ag 1.2 (SAG) or its derivative, Hh-Ag 1.5, in membrane-binding assays (Frank-Kamenetsky et al., 2002; Rominger et al., 2009). In a cell-based Gli-responsive element-Luciferase (Gli-Luc) reporter assay, these antagonists also showed a significant decrease in potency when higher doses of agonists (SAG or Hh-Ag 1.5) were used to induce pathway activation (Table 1) (Miller-Moslin et al., 2009; Pan et al., 2010). These results suggest that GDC-0449 and KAAD-cyclopamine strongly compete with SAG and bind to an overlapping binding site, or allosterically displace SAG. It is likely that antagonists with the same binding mode as GDC-0449 might lose pathway inhibition efficacy in cells harboring the SMO:D473H mutant (or D477G mutant for mouse Smo) (see Table S1A available online), raising the concern that relapse might develop quickly as a result of the acquisition of the drug-resistant D473H mutation in tumors treated with this type of antagonist. Therefore, identification of small molecule antagonists that can inhibit the D473H (or mouse D477G) mutant Smo would be important for cancer drug discovery (Dijkgraaf et al., 2011).

RESULTS AND DISCUSSION

Identification of Hh Antagonists

By high throughput screening, we identified two Hh signaling inhibitors from commercial compound libraries (approximately 50,000 compounds from Maybridge and ChemDiv), named

Table 1. IC_{50} (nM) Values of Hh Antagonists in TM3-Gli-Luc Reporter Assay and Control Assays

Assay	ALLO-1	ALLO-2	KAAD-Cyclopamine
TM3-Gli-Luc: 1 nM Hh Ag1.5	33	5.6	9.7
TM3-Gli-Luc: 25 nM Hh Ag1.5	55	5.9	160
TM3-Gli-Luc: recombinant ShhN	320	39	60
TM3-Gli-Luc-Smo-WT	410	41	81
TM3-Gli-Luc-Smo:D477G	1000	83	1700
TM3 cell viability	>10 μM	>10 μM	3.1 μM
293 Wnt-responsive TopFlash	>10 μM	>10 μM	ND
TM3 SV40-Luc	>10 μM	7.1 μM	ND
293 NFκB responsive-Luc	>10 μM	>10 μM	ND

See also Table S1. ND, not determined.

ALLO-1 and ALLO-2, which inhibit Smo agonist Hh-Ag 1.5-induced luciferase expression in TM3-Gli-Luc cells, a stable clone of mouse TM3 cells expressing Gli-Luc reporter (see Supplemental Experimental Procedures for detailed information; Figure 1A) (Miller-Moslin et al., 2009; Pan et al., 2010). The potency of the compounds did not change when either low dose (1 nM) or high dose (25 nM) of Hh-Ag 1.5 was used, in contrast to other known Smo antagonists that are strong SAG or Hh-Ag 1.5 competitors (Chen et al., 2002b; Miller-Moslin et al., 2009). ALLO-1 inhibited Gli-Luc activity with an IC_{50} around 50 nM, and ALLO-2 had an IC_{50} around 6 nM (Figures 1B and 1C and Table 1). Both compounds also inhibited recombinant N-terminal Shh ligand (ShhN)-induced pathway activation with IC_{50} values of 320 and 39 nM, respectively (Figure 1D and Table 1). The inhibition of the Gli-Luc reporter was not a result of cytotoxicity or nonspecific transcriptional or translational inhibition because these compounds showed >100-fold window in a cell viability assay and did not inhibit other luciferase reporters under the control of constitutively active or Hh nonresponsive promoters, such as SV-40 promoter, Wnt-responsive SuperTopFlash, and NF-κB responsive element (Figures 1B and 1C and Table 1). Their inhibitory activities in human HEPM and mouse NIH 3T3 cells were further confirmed by the inhibition of human *GLI1* and *PTCH1* or mouse *Gli1* mRNA expression induced by ShhN, as determined by qRT-PCR (Figures 1E and 1F; Figure S1). ALLO-1 and ALLO-2 showed IC_{50} values of 805 and 40 nM in mouse *Gli1* qRT-PCR assays, respectively. Both compounds dose dependently inhibited the ShhN-induced proliferation of mouse cerebella granule neuron progenitors (CGNPs), with IC_{50} values of 410 and 22 nM, respectively, similar to that of the pathway inhibition in reporter assays (Figure 1G; Figure S1). Furthermore, both compounds inhibited the proliferation of *Ptch1*$^{+/-}$*p53*$^{-/-}$ mouse medulloblastoma cells in a dose-dependent manner, with IC_{50} values of 0.47 and 0.12 μM, respectively (Figure 1H). Although ALLO-2 shares structural similarities with protein kinase inhibitors, it showed no significant inhibition at 5 μM with respect of a panel of 99 kinases (Invitrogen kinase profiling, Table S1B).

ALLO-1 and ALLO-2 Are Smo Antagonists with Different Binding Modes

Neither ALLO-1 nor ALLO-2 inhibits Hh pathway activation induced by siRNA knockdown of Suppressor of Fused (SuFu), suggesting that these compounds act at a level upstream of SuFu in the signal transduction cascade (Figure S2). To determine whether these compounds directly target Smo, we then carried out membrane-binding assays using ^{3}H-labeled agonist (Hh-Ag 1.5) or antagonist (cyclopamine). In contrast to other known Smo antagonists (Chen et al., 2002b; Miller-Moslin et al., 2009; Pan et al., 2010; Rominger et al., 2009), ALLO-1 and ALLO-2 did not compete with [^{3}H]Hh-Ag 1.5 (Figure 2A), consistent with their noncompetitive characteristics in reporter assays. However, ALLO-2 competed with [^{3}H]cyclopamine, with an IC_{50} of 2.8 nM, whereas ALLO-1 did not (Figure 2B). We also tested whether these compounds compete with BODIPY-cyclopamine, a fluorescent derivative of cyclopamine, using an imaging-based fluorescent-binding assay (Chen et al., 2002a; Pan et al., 2010). Both ALLO-1 and ALLO-2 effectively competed with BODIPY-cyclopamine in CHO-K1 cells overexpressing wild-type Smo, with IC_{50} values of 140 and 2.8 nM, respectively (Figure 2C; Figure S2). Because the fluorescent-binding assay was done with fixed cells, the competition of binding does not implicate a conformational change or the recruitment of effector proteins (Chen et al., 2002a; Taipale et al., 2000). It was reported that BODIPY-cyclopamine and KAAD-cyclopamine have significantly improved potency compared to cyclopamine in Hh signaling inhibition (Chen et al., 2002a); therefore, an additional binding site that accommodates the BODIPY or KAAD moiety may exist (or is induced) adjacent to the cyclopamine-binding site. Given the fact that ALLO-1 competes with BODIPY-cyclopamine, but not [^{3}H]cyclopamine, it is possible that the binding site of ALLO-1 might be located in the site accommodating the BODIPY moiety. ALLO-1, BODIPY, and KAAD all contain a hydrophobic phenyl ring; thus, the binding site accommodating these motifs may be hydrophobic. It is known that cyclopamine competes with SAG or Hh-Ag 1.5, suggesting that the binding sites of cyclopamine and Hh-Ag 1.5 overlap, at least partially (Chen et al., 2002a; Rominger et al., 2009). Because ALLO-2 competes with [^{3}H]cyclopamine, but not [^{3}H]Hh-Ag 1.5, we speculate that the ALLO-2 binding site does not overlap with Hh-Ag 1.5 but may partially overlap with cyclopamine. Taken together, these experiments suggest that both ALLO-1 and ALLO-2 bind to Smo but might have different binding modes from previously known Smo antagonists. It was previously hypothesized that SAG or Hh-Ag 1.5-binding sites may be different from cyclopamine or other antagonists' binding sites (Chen et al., 2002a; Frank-Kamenetsky et al., 2002; Rominger et al., 2009). Our data suggested that antagonists such as ALLO-1 and ALLO-2 could occupy sites distinct from the SAG or Hh-Ag 1.5-binding sites and are noncompetitive with the SAG binding. Therefore, multiple binding sites for different compounds might exist and partially overlap with each other in Smo.

ALLO-1 and ALLO-2 Inhibit Drug-Resistant Smo Mutant

Because ALLO-1 and ALLO-2 bind to Smo differently than GDC-0449 and other Smo antagonists, we hypothesized that they might be active against the drug-resistant Smo mutant. We overexpressed both wild-type mouse Smo and the D477G mutant Smo with GFP tagged at the C termini in the TM3-Gli-Luc reporter cell line, and determined pathway inhibition with these compounds. The wild-type and D477G Smo variants were

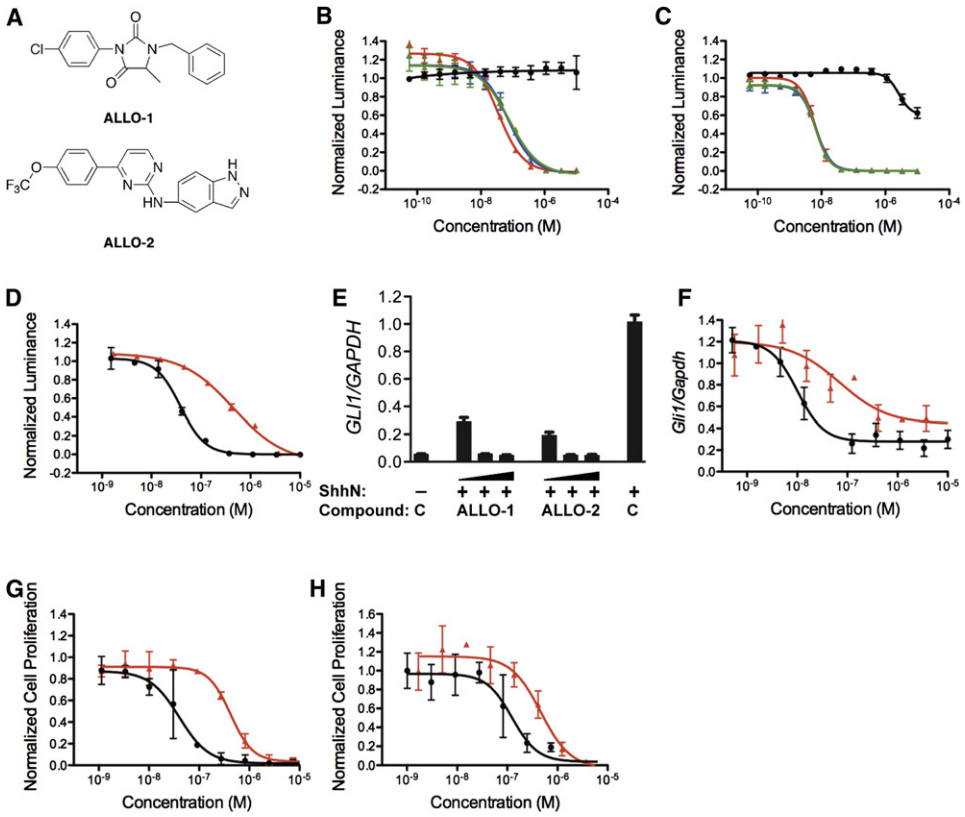

Figure 1. ALLO-1 and ALLO-2 Inhibit Hh Signaling

(A) The structures of ALLO-1 and ALLO-2.

(B and C) The inhibition of Gli-Luc reporter activity by ALLO-1 (B) and ALLO-2 (C) in TM3-Gli-Luc cells stimulated with 1 nM (red), 5 nM (blue), or 25 nM (green) Smo agonist Hh-Ag 1.5; and the inhibition to luciferase activity in TM3 cells expressing SV40-Luc (black) (n = 3). Values are mean ± standard deviation (SD). Represent at least three independent experiments.

(D) The inhibition of Gli-Luc reporter activity by ALLO-1 (red) and ALLO-2 (black) in TM3-Gli-Luc cells stimulated with recombinant ShhN (200 ng/ml) (n = 3). Values are mean ± SD. Represent at least three independent experiments.

(E) Both ALLO-1 (20, 2, and 0.2 μM) and ALLO-2 (5, 0.5, and 0.05 μM) inhibit recombinant ShhN (200 ng/ml) induced *GLI1* expression in human HEPM cells (C, DMSO control; n = 2; mean ± SD). Represent two independent experiments.

(F) Both ALLO-1 and ALLO-2 inhibit recombinant ShhN-induced *Gli1* expression in NIH 3T3 cells dose dependently. mRNA expression was normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an internal control (n = 3; mean ± SD).

(G) Both ALLO-1 (red) and ALLO-2 (black) inhibit ShhN-induced mouse CGNPs proliferation in a dose-dependent manner (n = 2; mean ± SD).

(H) Both ALLO-1 (red) and ALLO-2 (black) inhibit *Ptch1*$^{+/-}$*p53*$^{-/-}$ mouse medulloblastoma cell proliferation in a dose-dependent manner (n = 3; mean ± SD). All assays were normalized using DMSO treatments (control).

See also Figure S1.

expressed at similar levels, as determined by fluorescent imaging (Figure S3). Consistent with previous reports, GDC-0449 has an IC_{50} of 7.8 μM in TM3 cells overexpressing D477G mutant Smo, whereas it inhibits TM3-Gli-Luc cells overexpressing wild-type Smo with an IC_{50} of 42 nM (Figure 3A; Table S1). In contrast to GDC-0449, both ALLO-1 and ALLO-2 inhibit wild-type and the D477G mutant with only ~2-fold shift in IC_{50}, suggesting that the D477G mutation does not significantly interfere with the binding of ALLO-1 and ALLO-2 to Smo (Figures 3B and 3C and Table 1). Furthermore, ALLO-1 and ALLO-2 inhibit both wild-type and D473H mutant human SMO with similar potencies (Figure S3). Interestingly, SANT1 also inhibits Hh signaling in cells overexpressing the drug-resistant Smo mutant, with less severe (~10-fold) loss of potency when compared to GDC-0449 and cyclopamine (>20-fold IC_{50} shift) (Table S1). It was reported that SANT1 allosterically inhibits SAG-induced pathway activation, consistent with our hypothesis that compounds with different binding modes might inhibit GDC-0449-resistant D477G mutant more effectively (Chen et al., 2002b; Rominger et al., 2009). Furthermore, we tested whether ALLO-1 and ALLO-2 could inhibit the proliferation of *Ptch1*$^{+/-}$*p53*$^{-/-}$ mouse medulloblastoma cells harboring the drug-resistant D477G mutant Smo. We infected *Ptch1*$^{+/-}$*p53*$^{-/-}$ mouse medulloblastoma cells with lentivirus carrying wild-type Smo or D477G mutant Smo expression vectors, and treated cells with Smo antagonists. Consistent with the reporter assay results, GDC-0449 inhibited the proliferation of medulloblastoma cells overexpressing wild-type Smo but was less potent in cells overexpressing D477G mutant Smo, with IC_{50} values of 23 nM for wild-type Smo and 3.2 μM for D477G mutant Smo expression cells. In contrast, ALLO-1 and ALLO-2 showed similar antiproliferation activities on both cell lines (Figures 3D–3F). ALLO-1 has

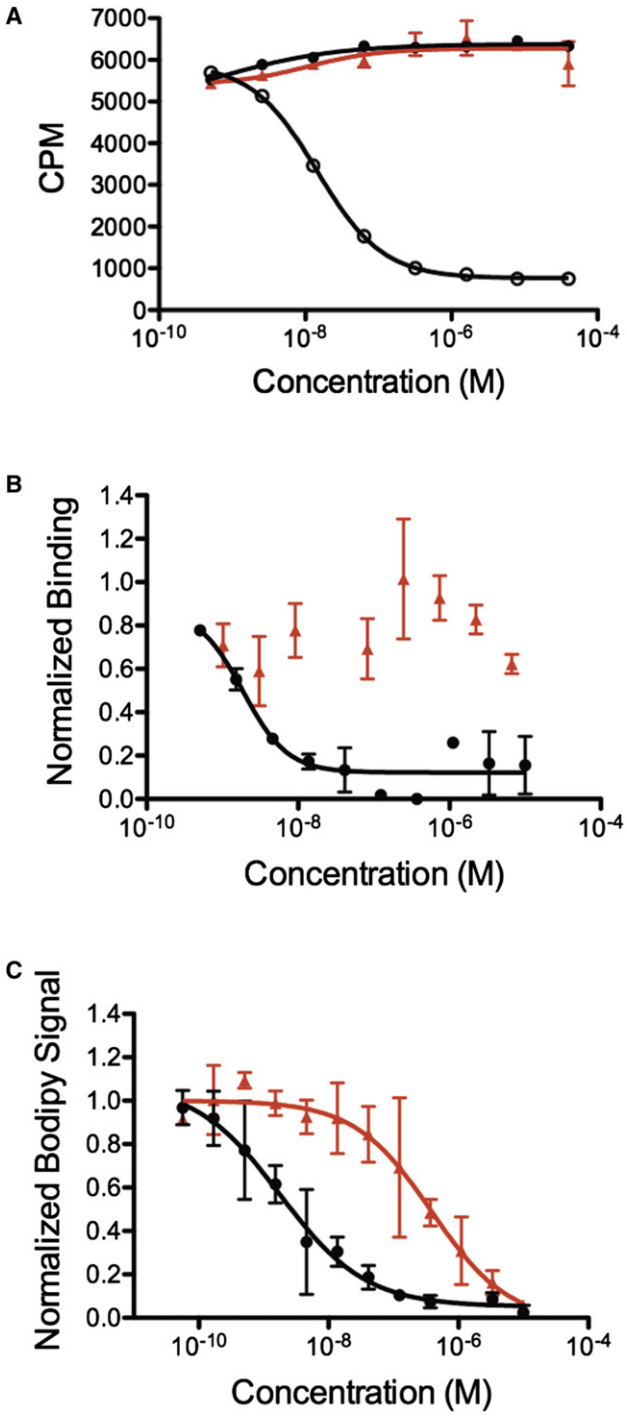

Figure 2. ALLO-1 and ALLO-2 Bind to Smo

(A) ALLO-1 (red triangles) and ALLO-2 (black dots) do not compete with [^{3}H] Hh-Ag 1.5, whereas a control compound (NVP-LDE225, black circles) (Pan et al., 2010) showed a dose-dependent competition (for ALLO-1 and ALLO-2, n = 2; mean ± SD).

(B) ALLO-2 (black), but not ALLO-1 (red), competes the binding of [^{3}H]cyclopamine to Smo (n = 2; mean ± SD).

(C) ALLO-1 (red) and ALLO-2 (black) compete the binding of BODIPY-cyclopamine to Smo (n = 2; mean ± SD). All assays were normalized using DMSO treatments (control).

See also Figure S2.

IC_{50} values of 489 nM for wild-type Smo and 1.2 μM for D477G Smo expression cells. ALLO-2 has IC_{50} values of 132 nM for wild-type Smo and 440 nM for D477G Smo expression cells. Finally, we tested whether ALLO-1 and ALLO-2 can inhibit the Hh signaling activated by a previously known oncogenic Smo:W539L (SmoM2) mutant, to which cyclopamine significantly loses its antagonist activity (Chen et al., 2002a, 2002b). ALLO-1 inhibited SmoM2-induced *Gli1* expression in NIH 3T3 cells, whereas ALLO-2 did not (Figure S3). Interestingly, as we showed in membrane-binding assays, ALLO-1 does not compete with [H^3]cyclopamine to bind to Smo. These results suggest that W539L mutation might disrupt cyclopamine binding, but antagonists that are noncompetitive with cyclopamine can bind to and inhibit SmoM2 mutant.

SIGNIFICANCE

Although, to our knowledge, there is no high-resolution structural information or detailed mutagenesis study of Smo available to date, our report here suggest that there are multiple ligand-binding sites in Smo that accommodate the binding of diverse compounds, as ALLO-1 and ALLO-2 do not compete with SAG and Hh-Ag1.5 and behave differently for competing cyclopamine. Oncogenic mutations such as W539L and drug-resistant mutations such as D473H might disrupt one small molecule-binding site, but ligands that bind to other sites are not affected, although additional Smo mutations could arise for these ligands in tumors. In summary our work describes two Smo antagonists with distinct binding modes and gives a new insight for the Smo ligand-binding sites. Derivatives of these compounds with appropriate pharmacological properties could be useful tools to explore therapies for tumors with SMO mutations in vivo.

EXPERIMENTAL PROCEDURES

Constructs

Drug-resistant D477G Smo expression vectors were generated by using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) from the wild-type Smo construct using the following primers: 5′-CTT CAG CTG CCA CTT CTA TGG CTT CTT CAA CCA GGC TG, and 5′-CAG CCT GGT TGA AGA AGC CAT AGA AGT GGC AGC TGA AG. Smo-GFP expression vector was constructed by subcloning eGFP ORF from pEGFP-N1 construct (Clontech, Mountain View, CA, USA) into the Smo expression vectors, using primers forward (5′-GCA TAC GTC GCT AGC AGG ATC GGT GAG CAA GGG CGA GGA G) and reverse (5′-GCA TAC AGC GTC GAC TTA CTT GTA CAG CTC GTC C) with NheI and SalI (New England Biolabs, Ipswich, MA, USA) digestion. The lentiviral Smo (wild-type and D477G mutant) expression vectors were generated by cloning of a 3 kb fragment containing wild-type or mutant Smo ORF digested by NdeI and XbaI restriction enzymes (New England Biolabs) to a pLVX-IRES-Neo lentiviral vector (Clontech). SmoM2 mutant construct is kindly provided by James Chen, Stanford University. Human SMO D473H mutant was generated as reported previously (Buonamici et al., 2010).

Lentiviral Particle Production and Cell Infection

Lentiviral particles carrying Smo-GFP and Smo:D477G-GFP genes were generated in 293T cells (ATCC, Manassas, VA, USA) by cotransfecting packaging vectors (VSV-G, REV, and RRE; Clontech) and the Smo vectors using Fugene 6 transfection reagent (Roche Applied Science) following manufacturer's recommendation. Viral supernatants were collected 48 hr post-transfection and purified using Amicon filters (Millipore, Billerica, MA, USA). TM3-Gli-Luc cells were infected with serial dilutions of lentivirus supernatant, and

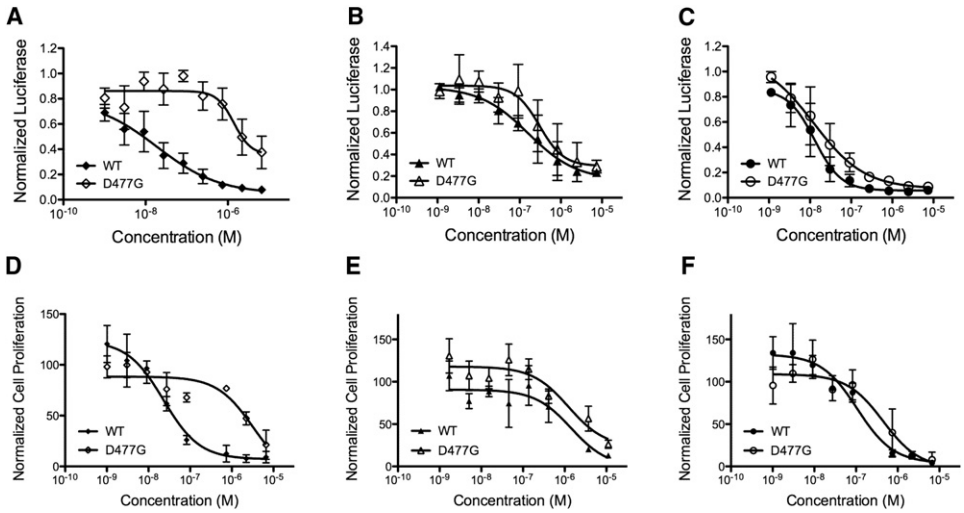

Figure 3. ALLO-1 and ALLO-2 Inhibit Drug-Resistant Smo:D477G Mutant

(A–C) The inhibition of Gli-Luc reporter activity by GDC-0449 (A), ALLO-1 (B), and ALLO-2 (C) in TM3-Gli-Luc cells overexpressing wild-type Smo-GFP (filled symbols) or Smo:D477G-GFP (open symbols). Represent three independent experiments.

(D–F) The inhibition to cell proliferation by GDC-0449 (D), ALLO-1 (E), and ALLO-2 (F) to medulloblastoma cells overexpressing wild-type Smo-GFP (filled symbols) or Smo:D477G-GFP (open symbols). All assays were normalized to the DMSO treatments (control).

See also Figure S3.

the clones with low but even Smo-GFP expression level were picked for further experiments. The cells expressing desired Smo-GFP were collected using BD FACSVantage SE cell sorter and analyzed using BD FACSDiva software (BD Biosciences, San Jose, CA, USA). Expression of GFP was confirmed using a Nikon Eclipse TE300 fluorescent microscope.

Cell-Based Reporter Assay

TM3 cells are from ATCC, and the reporter cell line (TM3-Gli-Luc) was generated as reported before (Miller-Moslin et al., 2009; Pan et al., 2010) and is briefly described in the Supplemental Experimental Procedures. TM3-Gli-Luc cells are cultured in F12 Ham's/DMEM (1:1) medium containing 5% horse serum, 2.5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). Cells were harvested by trypsin treatment, and were plated in 384-well assay plates in 40 μl of F12 Ham's/DMEM (1:1) containing 2% FBS with a density of 5000 cells/well. Hh antagonists were dissolved in DMSO with serial dilutions and were then added to the assay plate using 100 nl Pintool head (GNF, San Diego, CA, USA). Hh-Ag 1.5 (synthesized in house), or recombinant ShhN (200 ng/ml; R&D Systems, Minneapolis) or mouse Shh conditioned medium (10 μl, prepared by using 293 EcR Shh cell line obtained from ATCC under a license agreement between Novartis and Johns Hopkins University, following the protocols suggested by ATCC) in 10 μl of assay media was added 30 min later. The assay plates were incubated at 37°C for 48 hr and were assayed using Bright-Glo reagent (Promega, Madison, WI, USA) according to manufacturer's protocol. Luminance signals were read using Chemiluminescence Imaging Plate Reader (CLIPR) System (Molecular Devices, Sunnyvale, CA, USA). Luminance reading for TM3-Gli-Luc assay was normalized based on DMSO control (100% of pathway activity) and a known Smo antagonist (GDC-0449 at 10 μM, 0% of pathway activity). IC_{50} values, defined as the inflection point of the logistic curve, were determined by nonlinear regression using the Prism 4 software (GraphPad, La Jolla, CA, USA). The reporter assay of TM3-Gli-Luc cells infected with wild-type, D477H mutant Smo mutant was carried out with the same protocol.

TaqMan Gene Expression Assay

Endogenous mouse *Gli1* and *Ptch1* or human *GLI1* and *PTCH1* gene expression was performed using mouse NIH 3T3 or human embryonic palatal mesenchymal (HEPM) cells (ATCC). NIH 3T3 and HEPM cells were cultured in 6-well plate and treated for 48 hr with recombinant ShhN (200 ng/ml) and Smo antagonists or DMSO control in EMEM medium containing 0.5% FBS. Total RNAs were harvested using TRIzol reagent (Invitrogen), extracted using chloroform, and purified using RNeasy Mini Spin Column (QIAGEN, Valencia, CA, USA) according to manufacturer's protocol. RNAs were reverse transcribed by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). TaqMan Gene Expression Assays (Applied Biosystems; Mm00494645_m1 for mouse *Gli1*, Mm00436026_m1 for mouse *Ptch1*, Hs00171790_m1 for human *GLI1*, and Hs00181117_m1 for human *PTCH1*) were used for quantitative expression analyses, and *GAPDH* TaqMan assays with VIC/MGB Probe (Applied Biosystems; 4352339E for mouse; 4326317E for human) were used as endogenous controls. Real-time PCR was performed using the TaqMan Gene Expression Master Mix and ABI Prism 7900HT fast real-time PCR system (Applied Biosystems).

Fluorescence-Binding Assays

Fluorescence competition-binding assays using BODIPY-cyclopamine were conducted as described (Pan et al., 2010). Briefly, binding assays were conducted in 384-well plates using fixed CHO-K1 cells stably expressing mouse Smo. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed, covered in PBS containing 0.5% FBS, and incubated with 20 nM BODIPY-cyclopamine (Toronto Research Chemicals, Ontario, Canada) and Smo antagonists for 4 hr at 37°C. The treated cells then were washed with PBS three times, nuclear stained with Hoechst 33258, and analyzed by ImageXpress® Ultra imaging system (Molecular Devices, Carlsbad, CA, USA).

Membrane-Binding Assays

Membrane-binding assays were conducted as described (Miller-Moslin et al., 2009) in 96-well plates. Briefly, Smo membranes were prepared from CHO-K1 cells that were stably transfected with cDNA encoding mouse Smo. Binding buffer (50 mM Tris buffer [pH 7.5], 5 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin), 0.17 mg/ml of Smo membranes, 7 nM [^{3}H]cyclopamine, or 0.3 nM [^{3}H]Hh-Ag 1.5 with various concentrations of Smo antagonists in a total volume of 150 μl were incubated at 37°C for 3 hr, and the assays were terminated by filtration through 96-well fiberglass FB filtration plates precoated with 0.1% BSA (Millipore, Bedford, MA, USA), and then the filtration plates were washed three times with washing buffer (50 mM Tris buffer [pH 7.5], 5 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin, and 0.05% (2-Hydroxypropyl) cyclodextrin). The plate was added with scintillation cocktail and read on a TopCount Microplate Scintillation Counter (Perkin Elmer, Waltham, MA, USA).

CGNP Proliferation Assay

CGNPs were isolated from 4-day-old (P4) C57BL/6 mice as described (Hatten et al., 1998). CGNPs were cultured on poly-D-lysine-coated 384-well plates in Neurobasal medium containing 2% B-27 supplement, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% Penicillin/Streptomycin supplement (Invitrogen) at 2×10^5 cells per well. Recombinant ShhN (200 ng/ml) and antagonists were added. Cell proliferation assay was performed using Click-iT EdU Cell Proliferation Assay kit (Invitrogen) following manufacturer's protocol. Briefly, cells were pulsed with 5 μM EdU (5-ethynyl-2′-deoxyuridine; Invitrogen) for 12 hr, and then were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.5% Triton X-100, washed, and incubated with detection cocktail containing 500 nM Alexa Fluor 488 azide, 1 mM $CuSO_4$, and 5 mM ascorbic acid for 30 min at room temperature. The treated cells were then washed with PBS, nuclear stained with Hoechst 33258, and analyzed by ImageXpress® Ultra imaging system (Molecular Devices, Mountain View, CA, USA).

Medulloblastoma Proliferation Assay

Tumors derived from *Ptch*$^{+/-}$*p53*$^{-/-}$ transgenic mice were serially passaged as fragments in nude mice. *Ptch*$^{+/-}$*p53*$^{-/-}$ medulloblastoma cells were isolated from mouse allograft as described (Buonamici et al., 2010). Medulloblastoma cells were cultured on a nonadherent 10 cm Petri dish in Neurobasal Medium containing 2% B-27, 1% N2 supplement, and 1% Penicillin/Streptomycin (Invitrogen) with 1×10^7 cells per dish. After 3 days of culture, cells were then infected with lentiviral supernatants carrying either wild-type or D477G mouse Smo-GFP vectors. Expression levels of Smo were confirmed by RT-PCR. Cell proliferation was assayed as described above using Click-iT EdU Cell Proliferation Assay kit (Invitrogen).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at doi:10.1016/j.chembiol.2011.01.018.

ACKNOWLEDGMENTS

We thank Dr. Jun Liu for constructs and helpful discussions; Reem Javed, Richard Soden, Lindsey Aimone, Anlai Wang, and Kathy Hsiao for technical support; Christopher Trussell for cell sorting; Dr. James K. Chen for providing mouse SmoM2 construct, and Drs. Silvia Buonamici, Shifeng Pan, Stefan Peukert, Matthew Tremblay, Charles Cho, and Luke Lairson for critical comments of the manuscript. This work is supported by the Novartis Research Foundation (X.W.); H.T., Q.J., X.L., N.P.E., Y.W., A.R., M.D., J.K., and X.W. are employees of Novartis. H.T., D.-I.K., N.P.E., Y.W., A.R., M.D., and J.K. performed biological experiments. H.T., X.L., and Q.J. synthesized and characterized compounds. H.T., P.G.S., and X.W. analyzed data. H.T. and X.W. conceptualized the project and wrote the manuscript, and all authors read and edited the manuscript.

Received: September 1, 2010
Revised: January 11, 2011
Accepted: January 13, 2011
Published: April 21, 2011

REFERENCES

Buonamici, S., Williams, J., Morrissey, M., Wang, A., Guo, R., Vattay, A., Hsiao, K., Yuan, J., Green, J., Ospina, B., et al. (2010). Interfering with resistance to smoothened antagonists by inhibition of the PI3K pathway in medulloblastoma. Sci. Transl. Med. *2*, 51ra70.

Chen, J.K., Taipale, J., Cooper, M.K., and Beachy, P.A. (2002a). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. *16*, 2743–2748.

Chen, J.K., Taipale, J., Young, K.E., Maiti, T., and Beachy, P.A. (2002b). Small molecule modulation of Smoothened activity. Proc. Natl. Acad. Sci. USA *99*, 14071–14076.

Dijkgraaf, G.J., Alicke, B., Weinmann, L., Januario, T., West, K., Modrusan, Z., Burdick, D., Goldsmith, R., Robarge, K., Sutherlin, D., et al. (2011). Small molecule inhibition of GDC-0449 refractory Smoothened mutants and downstream mechanisms of drug resistance. Cancer Res. *71*, 435–444.

Epstein, E.H. (2008). Basal cell carcinomas: attack of the hedgehog. Nat. Rev. Cancer *8*, 743–754.

Frank-Kamenetsky, M., Zhang, X.M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F.Y., Jones, S., Shulok, J., et al. (2002). Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. J. Biol. *1*, 10.

Hatten, M.E., Gao, W.-Q., Morrison, M.E., and Mason, C.A. (1998). The Cerebellum: Purification and Coculture of Identified Cell Populations (Cambridge, MA: MIT Press).

Hooper, J.E., and Scott, M.P. (2005). Communicating with Hedgehogs. Nat. Rev. Mol. Cell Biol. *6*, 306–317.

Ingham, P.W., and McMahon, A.P. (2001). Hedgehog signaling in animal development: paradigms and principles. Genes Dev. *15*, 3059–3087.

King, R.W. (2002). Roughing up Smoothened: chemical modulators of hedgehog signaling. J. Biol. *1*, 8.

Mahindroo, N., Punchihewa, C., and Fujii, N. (2009). Hedgehog-Gli signaling pathway inhibitors as anticancer agents. J. Med. Chem. *52*, 3829–3845.

Miller-Moslin, K., Peukert, S., Jain, R.K., McEwan, M.A., Karki, R., Llamas, L., Yusuff, N., He, F., Li, Y., Sun, Y., et al. (2009). 1-amino-4-benzylphthalazines as orally bioavailable smoothened antagonists with antitumor activity. J. Med. Chem. *52*, 3954–3968.

Pan, S., Wu, X., Jiang, J., Gao, W., Wan, Y., Cheng, D., Han, D., Liu, J., Englund, N.P., Wang, Y., et al. (2010). Discovery of NVP-LDE225, a potent and selective Smoothened antagonist. ACS Med. Chem. Lett. *1*, 130–134.

Rominger, C.M., Bee, W.L., Copeland, R.A., Davenport, E.A., Gilmartin, A.G., Gontarek, R.R., Hornberger, K.R., Kallal, L.A., Lai, Z., Lawrie, K., et al. (2009). Evidence for allosteric interactions of antagonist binding to the Smoothened receptor. J. Pharmacol. Exp. Ther. *329*, 995–1005.

Rubin, L.L., and de Sauvage, F.J. (2006). Targeting the Hedgehog pathway in cancer. Nat. Rev. Drug Discov. *5*, 1026–1033.

Rudin, C.M., Hann, C.L., Laterra, J., Yauch, R.L., Callahan, C.A., Fu, L., Holcomb, T., Stinson, J., Gould, S.E., Coleman, B., et al. (2009). Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449. N. Engl. J. Med. *361*, 1173–1178.

Scales, S.J., and de Sauvage, F.J. (2009). Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. Trends Pharmacol. Sci. *30*, 303–312.

Taipale, J., Chen, J.K., Cooper, M.K., Wang, B., Mann, R.K., Milenkovic, L., Scott, M.P., and Beachy, P.A. (2000). Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature *406*, 1005–1009.

Von Hoff, D.D., LoRusso, P.M., Rudin, C.M., Reddy, J.C., Yauch, R.L., Tibes, R., Weiss, G.J., Borad, M.J., Hann, C.L., Brahmer, J.R., et al. (2009). Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. N. Engl. J. Med. *361*, 1164–1172.

Williams, J.A., Guicherit, O.M., Zaharian, B.I., Xu, Y., Chai, L., Wichterle, H., Kon, C., Gatchalian, C., Porter, J.A., Rubin, L.L., and Wang, F.Y. (2003). Identification of a small molecule inhibitor of the hedgehog signaling pathway: effects on basal cell carcinoma-like lesions. Proc. Natl. Acad. Sci. USA *100*, 4616–4621.

Wu, X., Walker, J., Zhang, J., Ding, S., and Schultz, P.G. (2004). Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. Chem. Biol. *11*, 1229–1238.

Yauch, R.L., Gould, S.E., Scales, S.J., Tang, T., Tian, H., Ahn, C.P., Marshall, D., Fu, L., Januario, T., Kallop, D., et al. (2008). A paracrine requirement for hedgehog signalling in cancer. Nature *455*, 406–410.

Yauch, R.L., Dijkgraaf, G.J., Alicke, B., Januario, T., Ahn, C.P., Holcomb, T., Pujara, K., Stinson, J., Callahan, C.A., Tang, T., et al. (2009). Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. Science *326*, 572–574.

