Discovery, Molecular and Pharmacological Characterization of GSA-10, a Novel Small-Molecule Positive Modulator of Smoothened

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Received December 20, 2012; accepted February 8, 2013

ABSTRACT

Activation of the Smoothened (Smo) receptor mediates Hedgehog (Hh) signaling. Hh inhibitors are in clinical trials for cancer, and small-molecule Smo agonists may have therapeutic interests in regenerative medicine. Here, we have generated and validated a pharmacophoric model for Smo agonists and used this model for the virtual screening of a library of commercially available compounds. Among the 20 top-scoring ligands, we have identified and characterized a novel quinolinecarboxamide derivative, propyl 4-(1-hexyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamido) benzoate, (GSA-10), as a Smo agonist. GSA-10 fits to the agonist pharmacophoric model with two hydrogen bond acceptor groups and four hydrophobic regions. Using pharmacological, biochemical, and molecular approaches, we provide compelling evidence that GSA-10 acts at Smo to promote the differentiation of multipotent mesenchymal progenitor cells into osteoblasts. However, this molecule does not display the hallmarks of reference Smo agonists. Remarkably, GSA-10 does not recognize the classic bodipy-cyclopamine binding site. Its effect on cell differentiation is inhibited by Smo antagonists, such as MRT-83, SANT-1, LDE225, and M25 in the nanomolar range, by GDC-0449 in the micromolar range, but not by cyclopamine and CUR61414. Thus, GSA-10 allows the pharmacological characterization of a novel Smo active site, which is notably not targeted to the primary cilium and strongly potentiated by forskolin and cholera toxin. GSA-10 belongs to a new class of Smo agonists and will be helpful for dissecting Hh mechanism of action, with important implications in physiology and in therapy.

Introduction

The Hedgehog (Hh) signaling pathway is implicated in growth and patterning during development. This pathway regulates stem cell maintenance and repair in adult tissues. Aberrant Hh signaling linked to gene pathway mutations is associated with severe physiologic consequences, such as birth defects and with development of cancer, including basal cell carcinoma and medulloblastoma. Hh signaling also supports the tumor microenvironment (Heretsch et al., 2010; Low and de Sauvage, 2010; Ng and Curran, 2011). Hh ligands activate the pathway through binding to the twelve-transmembrane receptor Patched (Ptc), leading to the derepression of Smoothened (Smo), a seven-transmembrane protein presumably belonging to the G protein–coupled receptor superfamily. Smo signals through a complex transduction machinery that includes the Gli family of transcription factors, resulting in the expression of Hh target genes, including Gli1 and Ptc. Trafficking of proteins involved in Hh signaling up and down the primary cilium has rapidly emerged as a key step in the processing of the Hh signal. Ptc is proposed to be localized to the cilium in the absence of its ligand and to inhibit signaling by excluding Smo from this organelle. After ligand binding, simultaneous removal of Ptc and localization of Smo to cilia occur (Goetz and Anderson, 2010; Ruat et al., 2012).

Smo has been identified as a molecular target for the action of antagonists aimed at blocking the Hh pathway. Smo inhibitors, such as GDC-0449 and LDE225, are candidates for the treatment of cancers associated with dysfunction of Hh signaling, and the search for Smo antagonists is under intense study (Heretsch et al., 2010; Low and de Sauvage, 2010; Ng

ABBREVIATIONS: AP, alkaline phosphatase; BC, Bodipy-cyclopamine; CTX, cholera toxin; DMSO, dimethyl sulfoxide; GCPs, cerebellar granule cell precursors; HEK, human embryonic kidney; Hh, Hedgehog; Smo, Smoothened; PCR, polymerase chain reaction; Ptc, Patched; PTX, pertussis toxin; qPCR, quantitative PCR; RT-PCR, reverse-transcription PCR.
and Curran, 2011). On the other hand, Hh proteins modulate the
HH pathway activities of mature neurons, and stimulation of
the HH pathway may have therapeutic interest (Traiffort et al., 2010).
High-throughput screening of chemical libraries has led to the
identification of the Smo reference agonists SAG, a chloro-
benzothiophene (Brunton et al., 2009; Chen et al., 2002;
Frank-Kamenetsky et al., 2002), and purmorphamine, a pu-
rine derivative (Fig. 1A) (Wu et al., 2002; Sinha and Chen,
2006). SAG has been shown to act as a neuroprotective agent
in neonates displaying glucocorticoid-induced neonatal celi-
bellar injury, which suggests the potential clinical interest of
Smo agonists (Heine et al., 2011). SAG and purmorphamine
have been used for modulating various patterning events in
embryonic stem cells and adult neural precursor cells (Frank-
Kamenetsky et al., 2002; Danjo et al., 2011). Further
investigations are necessary for delineating the potential
interest of several glucocorticoids (Wang et al., 2010) and
oxysterols (Corcoran and Scott, 2006; Nachtergaele et al.,
2012) as Smo agonists. However, only a limited number of
Smo agonists have yet been characterized, and none has
reached clinical trials.

We report here the discovery of a novel Smo agonist,
a quinolinecarboxamide named GSA-10, belonging to a new
chemical class of Hh modulators, and that promotes the
differentiation of multipotent mesenchymal progenitor cells
into osteoblasts. Characterization of GSA-10 in cell-based
assays demonstrates that this molecule does not display the
hallmarks of reference Smo agonists and specifically allows
the pharmacological identification of a novel Smo active site,
which is not only targeted to the primary cilium. Moreover,
the results provide novel important insights into the
pharmacological properties of reference Smo antagonists
currently in clinical development for treating various cancers.

Materials and Methods
Pharmacophore Design and Virtual Screening. The chemical
structures of purmorphamine and SAG agonists were used to build
the six-feature pharmacophoric model for Smo agonists by means of
the software Discovery Studio (version 3.0; Accelrys Software Inc.,
San Diego, CA). In particular, the common feature hypothesis
generation routine (HipHop, formerly belonging to the Catalyst
software) was applied to identify the common chemical features
shared by the compounds (called the pharmacophoric model). The
resulting pharmacophoric model was then used as a three-
dimensional query to mine in silico the Asinex (Moscow, Russia)
Gold, and Platinum Collections. Twenty compounds (structures
are available in Supplemental Table 1) were selected from the final
ranking list on the basis of their fit value and the uniqueness of the
molecular structure. Further details of the computational protocol
were previously described (Manetti et al., 2010).

Small Molecules. Potential Smo modulators identified from
virtual screening were purchased from Asinex. MRT-83, CUR61414,
and SAG were synthesized as described elsewhere (Solinas et al.,
2012). M25 (2-ethyl-N-((1-(2-methoxyphenyl)-1H-indazol-5-ylmethyl)
butanamide) was prepared in accordance with a general procedure
reported for analogous 1H-indazoles (Dessole et al., 2009). Analytical
data for M25 were as follows: mp, 77°C (heptane/AcOEt); 1H NMR (CDCl3, 300 MHz) 6 8.18 (s, 1H, H(ar)); 7.68 (s, 1H, H(ar)); 7.45
(m, 2H, H(ar)); 7.29 (m, 2H, H(ar)); 7.11 (m, 2H, H(ar)); 5.73 (m, 1H,
NH); 4.58 (d, J = 5.4 Hz, 2H, NCH2Ar); 3.80 (m, 3H, OCH3); 2.06 (m,
1H, CH(ali)); 1.66–1.26 (m, 8H, CH2(ali)); 0.90 (m, 6H, CH3(ali));
LRMS-ESI(m/z) 380.2 (M+1). Cyclopamine and Bodipy-cyclopamine
were from Toronto Research Chemicals (North York, ON, Canada);
SANT-1 from ChemBridge Corporation (San Diego, CA); GDC-0449,
1DE225, and 1CHIR99021 from Selleck Chemicals (Houston, TX);
and purmorphamine from Calbiochem (Merck, Lyon, France).
Forskolin and cholera toxin (CTX) were from Sigma-Aldrich (Saint-
Quentin Fallavier, France), and pertussis toxin (PTX) was from
Calbiochem.

Synthesis of GSA-10. Proppyl 4-(1-hexyl-4-hydroxy-2-oxo-1,2-
dihydroquinoline-3-carboxamido)benzoate was prepared in
accordance with a general procedure already reported for analogous
quinolinecarboxamides (Jönsson et al., 2004), as follows: (mp
118–120°C; MeOH/H2O); 1H-NMR (400 MHz, d6-dimethyl sulfoxide
(DMSO)): δ 8.30 (d, J = 8.2 Hz, 2H), 7.77 (m, 2H), 7.70 (d, J = 8.2 Hz,
2H), 7.42–7.31 (m, 4H), 4.26 (t, J = 7.5 Hz, 2H), 3.41 (m, 2H), 1.50
(m, 4H), 1.38–1.10 (m, 6H), 1.07 (t, J = 7.3 Hz, 3H), 0.89 (t, J = 7.6
Hz, 3H); 13C-NMR (CDCl3, 100 MHz, d6-DMSO): δ 172.1, 169.7, 162.7,
118.7 (2C) 114.4, 112.7, 61.9, 42.7, 31.8, 29.6, 27.5, 22.9, 21.3, 11.9,
20.4 Hz, 3H), 0.89 (t, J = 7.6 Hz, 3H); LRMS-ESI(m/z) 380.2 (M+1).

Cell Culture and Reagents. Human embryonic kidney (HEK)
293 and C3H10T1/2 were obtained from ATCC, and the Shh-light2
and Shh-Ptc2 cells were from P.A. Beachy (Stanford University). The HEK-hSmo
line cell stably expressing the human Smo was described previously
(Roudaut et al., 2011). They were maintained in Dulbecco’s modified
Eagle’s medium supplemented with 10% fetal calf serum (plus zeocyn/
G418 for Shh-light2, plus G418 for HEK-hSmo). All cell culture
reagents were from Life Technologies (Saint Aubin, France). Unless
otherwise stated, other reagents are from Sigma-Aldrich.

Plasmids. The prTK-TK Renilla luciferase plasmid is described
elsewhere (Manetti et al., 2010) and was given by H. Sasaki. prK5-
SP-myc-Smo and prK5-Ptc have been used previously (Maedeu et al.,
2006). The Wnt reporter plasmid M50 Super8xTOPFlash (Tcf/Lef)
was provided by R. Moon, the pLNC Wnt-3aHA (Wnt3a) by
J. Kitajewski, and the pRIAS-BMP4 (BMP4) by C. Tabin. These
plasmids (#12456, #18030, and #14001, respectively) were obtained
through Addgene (Cambridge, MA). The BMP reporter Bre-Luc
plasmid was generously provided by P. Dijke.

Alkaline Phosphatase (AP) Assay. C3H10T1/2 cells were incu-
bated for 6 days in the presence of the studied compounds. For
inhibition assay, MRT-83 was used in presence of a defined
concentration of GSA-10 or SAG. Similarly, forskolin, PTX, or CTX
was mixed with the agonist before being added to the cells.
cell-based bioassay was performed as described previously (Manetti et al., 2010). All data are mean ± S.E.M. of quadruplicates and are from a representative experiment of n = 3.

**shRNA Selection and Infection.** Predesigned mouse pLKO.1 lentiviral shRNA against Smo (TRCN0000026245) or Ptc (TRCN0000042540; Open Biosystem, Huntsville, AL) was tested in HEK293 cells transfected by FuGENE (Roche Diagnostic, Mannheim, Germany) with pR5-SF-myc-Smo or pR5-Ptc (1:1) as described elsewhere (Roudaut et al., 2011). A pLKO.1 vector expressing nonrelevant shRNA was provided by A. Monteil (Montpellier, France) and used as control. Inhibition efficiency was estimated 48 hours later by Western blotting using C-myc antibody 9E10 (Sigma-Aldrich) for Smo or Ab130, a previously described polyclonal rabbit antiserum against Ptc (Bidet et al., 2011). The shRNAs vectors were used for production of lentiviral particles (lentivectors production facility /SFR BioSciences Gerland; Lyon Sud, UMS3444/US8, France). For lentiviral infection, C3H10T1/2 cells were seeded into 12-well plates (6 × 10⁴ cells/well) and infected the next day with lentiviral particles containing Smo, Ptc, or control shRNAs at a multiplicity of infection of 33 in the presence of polybrene (Sigma). Fresh medium was added 24 hours later, and the following day, cells were transferred into 6 cm Petri dishes in medium supplemented with 2 μg/ml of puromycin (Life Technologies). After 2 weeks of puromycin selection, cells were plated into 96-well plates without puromycin for a differentiation assay.

**RNA Preparation and Semiquantitative and Quantitative Polymerase Chain Reaction.** Total RNA was extracted from C3H10T1/2 cells with use of TRIzol Reagent, and cDNA synthesis was performed using the RT SuperScript II with random hexamer primers (Life Technologies). Semiquantitative polymerase chain reaction (PCR) was performed on 2720 thermal cycler (Applied Biosystems, Villeurbe sur Yvette, France). Primer sequences are available on request. Quantitative PCR (qPCR) was performed in duplicate on ABI Prism 7900HT sequence detection system (Applied Biosystems) with use of geometrical mean of three murine genes: glyceraldehyde 3-phosphate dehydrogenase, beta 2-microglobulin, and TATA-binding protein as endogenous control (Plateforme de qPCR, IMAGIF, GifYvette, France). Gene expression was calculated using the relative quantification method. References for the primers used are indicated in Supplemental Table 2.

**Luciferase Reporter Assays.** Gli-dependent reporter assay was realized as described elsewhere (Roudaut et al., 2011) in Shh-light2 cells, as described elsewhere (Roudaut et al., 2011). After cotransfection (1 hour) with the Tcf/Lef or BMP reporter plasmid, HEK293 cells (Chen et al., 2002) stably expressing the Gli-Firefly and renilla luciferase plasmid (together with BMP4 or Wnt3a-containing plasmids when indicated), HEK293 cells were incubated with the drug to be tested, and the luciferase activities were measured 48 hours later. All data are firefly luciferase activity reported to the Renilla control activity. At 10 μM, compounds modified the Renilla activity by less than 30%.

**Primary Cerebellar Cultures.** Isolation of cerebellar granule cell precursors (GCCPs) from P8 male rat and quantification of [³H]thymidine incorporation were performed as described elsewhere (Roudaut et al., 2011).

**Ciliary Smo Accumulation Assay and Bodipy-Cyclopamine Binding.** The protocols were adapted from those previously described (Roudaut et al., 2011). Cell culture slides were analyzed using a DMRXA2 microscope (Leica Microsystems, Nanterre, France) equipped with a Photometric Cool-Snap camera (Roper Scientific, Sterling, Germany). Images for counting were taken with a 20× objective.

**Data Analysis.** Means and S.E.M. were calculated using Excel 2007. Statistical analysis was performed using the Student’s t test. Statistical significance was considered for *P ≤ 0.05; **P ≤ 0.01; or ***P ≤ 0.001. Curve-fitting and IC₅₀ determinations were performed using GraphPad Prism 4.03.

## Results

**Identification of GSA-10 by Virtual Screening Using a Smo Agonist Pharmacophore.** Recently, we identified and developed a series of thioureas, ureas, and guanidines as novel potent Smo antagonists with use of a pharmacophore-based virtual screening strategy (Manetti et al., 2010; Roudaut et al., 2011; Solinas et al., 2012). We reasoned that we could exploit this strategy to identify novel Smo agonists starting from purmorphamine, SAG, and its derivatives as reference Smo agonists (Fig. 1A).

A pharmacophoric model based on the structure of these molecules was built in accordance with a previously described computational protocol (Manetti et al., 2010). This resulted in a model constituted by two hydrogen bond acceptor groups (HBA1-2) and four hydrophobic regions (HY1-4) (Supplemental Fig. 1). Analysis of the superposition pattern of SAG showed a good fit between the Smo agonist and the pharmacophoric model (Supplemental Fig. 1A). In particular, the chlorobenzothiophene moiety and the central phenyl ring matched the hydrophobic regions HY2-HY3-HY4 and HY1, respectively, and the amide carbonyl group and the pyridine nitrogen atom were hydrogen bond acceptors, filling the HBA2 and HBA1 features, respectively. As expected, purmorphamine was also able to fit the pharmacophoric model (Supplemental Fig. 1B). In detail, the hydrophobic features were mapped by the central phenyl (HY1), the cyclohexyl (HY2), and the naphthyl rings (HY3 and HY4). Moreover, the oxygen atom at the morpholine ring was accommodated in the HBA1, and the oxygen atom bridging the naphthyl ring and the purinyl heterocycle corresponded to HBA2.

The Asinex Gold collection of diverse drug-like molecules was screened virtually for fitting to the six-feature model developed for Smo agonists. Then, 20 compounds were visually selected and analyzed at 10 μM in a primary screening procedure using the differentiation of the mesenchymal pluripotent C3H10T1/2 cells into osteoblasts with use of measurement of AP enzymatic activity, which is a readout for Smo agonists activity (Sinha and Chen, 2006; Manetti et al., 2010). One compound, GSA-10 ([propyl 4-(1-hexyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamido)benzoate] (HY2), and the naphthyl rings (HY3 and HY4). Moreover, the oxygen atom at the morpholine ring was accommodated in HBA1, and the oxygen atom bridging the naphthyl ring and the purinyl heterocycle corresponded to HBA2.

The Asinex Gold collection of diverse drug-like molecules was screened virtually for fitting to the six-feature model developed for Smo agonists. Then, 20 compounds were visually selected and analyzed at 10 μM in a primary screening procedure using the differentiation of the mesenchymal pluripotent C3H10T1/2 cells into osteoblasts with use of measurement of AP enzymatic activity, which is a readout for Smo agonists activity (Sinha and Chen, 2006; Manetti et al., 2010). One compound, GSA-10 ([propyl 4-(1-hexyl-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamido)benzoate] (Fig. 1B), shows structural differences with the chlorobenzothiophene SAG and the purine derivative purmorphamine. However, GSA-10 fits well to the pharmacophoric model, with all six key features being satisfied by different functional groups (Fig. 2). The HBA1-2 groups of the pharmacophore are represented by the carbonyl oxygen of the ester side chain and by the carbonyl oxygen of the quinolinone fragment, respectively. The hydrophobic groups are matched by the central phenyl ring (HY1), the terminal methyl group of the N1 hexyl side chain (HY2), the condensed phenyl ring (HY3), and the C2-C3 atoms of the same chain (HY4). GSA-10 stimulated the AP response by more than 16-fold above the basal level (Fig. 3A; Supplemental Table 1). GSA-10 was as potent (half-maximal effective concentration EC₅₀, 1.2 μM) as purmorphamine (EC₅₀, 0.8 μM) in the differentiation assay. We observed that SAG was more potent (EC₅₀, 0.13 μM) than GSA-10, whereas SAG maximal response represented only 75% of GSA-10 maximal response (n = 18).

Both molecules induced the transcription of the AP gene in C3H10T1/2 cells, as measured by semiquantitative reverse-transcription PCR (RT-PCR) (Fig. 3B). AP was also identified...
shown). These results demonstrate that GSA-10 promotes the differentiation of multipotent mesenchymal progenitor cells into osteoblasts and, therefore, represents a novel potent osteogenic molecule.

GSA-10 Mediates Differentiation of Mesenchymal Cells through Smo. We then investigated the effects of both Smo and Ptc depletion in C3H10T1/2 cells on the GSA-10- and SAG-mediated differentiation response through the use of specific shRNAs (Fig. 3, C and D). The efficiency of Smo and Ptc shRNAs to induce Smo or Ptc depletion, respectively, was validated by Western blot analysis of membrane extracts from HEK-293 over-expressing these proteins (Supplemental Fig. 2). We compared the differentiation activity of GSA-10 and SAG with that of CHIR-99021, which was shown to mimic Wnt signaling and to induce osteoblast differentiation through GSK3 inhibition (Bennett et al., 2002). Smo shRNA blocked both GSA-10- (60%) and SAG- (85%) induced differentiation of C3H10T1/2 cells into osteoblasts, compared with control shRNA (Fig. 3C). These data are in agreement with the model that GSA-10 binds and activates Smo. Ptc shRNA increased both GSA-10- (2.4-fold) and SAG- (15-fold) induced AP activity, compared with control shRNA. These data are consistent with the model that GSA-10 binds and activates Smo. Ptc shRNA was validated by Western blot analysis of membrane extracts from HEK-293 over-expressing these proteins (Supplemental Fig. 2).

GSA-10 Does Not Display the Hallmarks of SAG at Hh Signaling. We further compared SAG and GSA-10 activity in other Hh cell-based assays. We first tested these compounds at a large range of doses in NIH3T3 cells stably transfected with a Gli-dependent firefly luciferase reporter (Taipale et al., 2000). This mouse embryonic fibroblast cell line has been widely used for measuring Hh signaling activity through Smo (Solinas et al., 2012). We observed that, as expected (Masdeu et al., 2006), SAG potentely stimulated Hh reporter gene transcription with an EC\textsubscript{50} of \(\sim 0.15\ \mu M\), whereas GSA-10 displayed no stimulatory effect, even at 10 \(\mu M\) (Fig. 4A). Abnormal Hh signaling in the cerebellum has been proposed to be responsible for medulloblastoma both in mice and in humans (Low and de Sauvage, 2010). Cerebellar GCPs proliferate in response to Hh pathway activation (Dahmane and Ruiz-i-Altaba, 1999; Roudaut et al., 2011). Increasing concentrations of SAG caused a dose-dependent increase in the proliferation of rat GCPs measured by \([\text{H}]\)thymidine incorporation and corresponding to a near 90-fold increase over basal level (EC\textsubscript{50} \(\sim 5\ \text{nM}\) (Fig. 4B). Relative to DMSO vehicle, the treatment by GSA-10 up to 10 \(\mu M\) did not significantly modify GCP proliferation (Fig. 4B).

Hh pathway activation has been demonstrated to depend on Smo accumulation at the primary cilium (Corbit et al., 2005; Rohatgi et al., 2007; Roudaut et al., 2011; Ruat et al., 2012). Thus, we assayed GSA-10 to induce accumulation of endogenous Smo in the primary cilium of C3H10T1/2 cells that are responsive to GSA-10 (Fig. 4, C and D). In DMSO vehicle-treated cells, Smo was not detected at the primary cilium visualized by the acetylated tubulin-positive signal (Fig. 4C). The number of Smo-positive cilia was increased from 3% for vehicle to 5.8% and 50% in cells treated with GSA-10 (10 \(\mu M\)) or SAG (1 \(\mu M\)), respectively (Fig. 4D). We also observed that Smo trafficking to the primary cilium was achieved by cyclopamine and purmorphamine, in agreement with previously published data (Wang et al., 2009; Wilson et al., 2009; Roudaut et al., 2011). The effect of GSA-10 on Smo trafficking to the primary

![Fig. 2. Proposed pharmacophoric model for GSA-10. Graphical representation of GSA-10 fitted to the proposed pharmacophoric model for Smo agonists. Pharmacophoric features are color coded: hydrogen bond acceptor groups (HBA1 and HBA2) in green; hydrophobic regions for aromatic rings (HY1 and HY3) in light blue; aliphatic group (HY2) in dark blue; generic hydrophobic (HY4) in cyan. Each of the HBA features is constituted by two green spheres; the smaller is filled by the hydrogen bond acceptor atom of the agonist, and the largest sphere is located at the end of a vector and represents the region where the corresponding hydrogen bond donor group of the receptor counterpart should be located. The atoms are color coded: black, carbon; gray, hydrogen; red, oxygen; and blue, nitrogen.](Image 1023 to 1026)
GSA-10 Does Not Stimulate Wnt and BMP Signaling. We analyzed then the ability of GSA-10 and SAG to modify the subcellular localization of Smo at the primary cilium. Remarkably, altogether these results demonstrate that GSA-10 does not induce Smo trafficking to the cilium nor modify the activity of SAG. 

Fig. 4. Modulation of Hh signaling by GSA-10. (A) Gli-luciferase reporter activity in Shh-light2 cells was measured at increasing concentrations of SAG or GSA-10 and plotted as fold change, compared with cells treated with vehicle alone. (B) Effect on rat cerebellar granule cell precursors proliferation. [3H]Thymidine incorporation is increased in response to SAG but not to GSA-10 and is expressed as fold change, compared with proliferation in vehicle treated cells. (C and D) Smo ciliary translocation analysis. (C) Representative immunofluorescent images of C3H10T1/2 cells treated overnight with vehicle (Veh), GSA-10 (10 μM), SAG (1 μM), purmorphamine (5 μM), or cyclopamine (Cyclo, 3 μM) or by a combination of SAG and GSA-10 were stained with antibodies against endogenous Smo (green) and acetylated (Ac) tubulin (red). Nuclei were stained with DAPI (blue). (D) The percentage of Smo-positive cilia was calculated for each drug in three independent fields for each point. Data are mean ± S.E.M. of three independent experiments. GSA-10 does not induce Smo trafficking to the cilium nor modify the activity of SAG. **P ≤ 0.01, compared with Veh.

GSA-10 Does Not Stimulate Wnt and BMP Signaling. We analyzed then the ability of GSA-10 and SAG to modify the Wnt and BMP signaling pathways reported to be implicated in C3H10T1/2 differentiation (Jackson et al., 2005; Kang et al., 2009). To this aim, HEK293 cells were transiently transfected with a Tcflf- or Bre-dependent firefly luciferase reporter together with a Renilla reniformis luciferase control reporter (Masdeu et al., 2006) (Fig. 5, A and B). GSA-10 (10 μM) and SAG (1 μM) did not significantly modify Tcflf- or Bre-dependent luciferase activities, which were increased significantly when Wnt3a and BMP4 were transfected in these cells, respectively. Together, these results suggest that GSA-10 does not modify the expression of Wnt and BMP target genes.

Opposite Effects of GSA-10 and SAG on Gli1 Transcript Levels. Because GSA-10 failed to induce Gli-reporter luciferase transcription, the mechanism of action of GSA-10 on the differentiation of C3H10T1/2 was puzzling. Therefore, we compared the effects of GSA-10 (10 μM) and SAG (1 μM) with DMSO vehicle on the transcription of genes likely to be regulated by Hh signaling, such as Gli1-3, Ptc1, and Smo. We also tested the effect of LiCl (20 mM), which activates Wnt signaling and induces C3H10T1/2 differentiation (Jackson et al., 2005). Semiquantitative PCR was done at various time points starting as early as 15 hours to 4 days after stimulation. As expected, an increase in Gli1 and Ptc1 transcription was observed in SAG-treated samples and was already maximal at 48 hours, whereas no significant change in Gli2-3 and Smo transcription was observed (Supplemental Fig. 4). By contrast, GSA-10 treatment did not increase the transcription of any of these genes but rather slightly decreased the transcription of Gli1 and Ptc1 at 48 hours (Supplemental Fig. 4). To confirm these data, we performed quantitative RT-PCR at 48 hours of treatment, the time when maximal decrease in Gli1 expression was seen for GSA-10, and we enlarged the list of tested genes linked to the Hh pathway (primer references in Supplemental Table 2). We observed that Gli1 (~59-fold), Hip (~48-fold), Ptc1 (5.9-fold), and Ptc2 (5.3-fold) were up-regulated after SAG treatment. We noticed a significant decrease of Gli1 (3.3-fold) transcript level after GSA-10 treatment in agreement with our previous semiquantitative PCR experiment, whereas the transcription of the other tested genes did not change significantly, compared with control sample (Table 1). These results indicate an opposite effect of GSA-10 and SAG on Gli1 transcript levels, which again suggests a different mechanism of action for the two molecules on Hh signaling.

GSA-10 and SAG Actions Are Differentially Regulated by cAMP Modulators. We then investigated the effects of forskolin, a positive regulator of the cAMP/PKA transduction pathways and a negative regulator of Hh signaling (Ruiz i Altaba, 1999; Wang et al., 2000), on GSA-10– and SAG-mediated differentiation of C3H10T1/2 cells. In these experiments, the 10-fold stimulation of AP activity induced by SAG (0.3 μM) was completely abolished when forskolin (10 μM) was added (Fig. 6A). More remarkably, the 5-fold increase of AP activity induced by GSA-10 (1 μM) was potentiated by ~3.8-fold in the presence of forskolin (Fig. 6A). We then constructed a dose-response curve of forskolin effect on GSA-10- and SAG-induced differentiation of C3H10T1/2 (Fig. 6B). Increasing concentrations of forskolin caused a dose-dependent potentiation of the AP response induced by GSA-10 (EC50, 0.7 μM), whereas an inhibition of this response was observed when SAG was used (IC50, 0.4 μM).

To identify whether modulation of Gα subunits impacts GSA-10- and SAG-induced differentiation of C3H10T1/2 cells,
we treated the cells with these molecules together with either CTX, which activates adenylate cyclase through Gs, resulting in the production of cAMP or pertussis toxin PTX, which impairs receptor–G protein interactions by ADP-ribosylating Gαi/o (Fig. 6, C and D). The dose-response curve to SAG was abolished by CTX and forskolin treatment, whereas it was potentiated by PTX with no significant modification of its EC50 but with a nearly 3-fold increase in the maximal stimulation. In contrast, EC50 of GSA-10 response was left shifted (from 1.1 to 0.4 μM) in the presence of forskolin and CTX, with an increase in the maximal stimulation (by ∼125%), but was not affected by PTX treatment. PTX was found to decrease SAG-induced activation of the Gli-luciferase reporter in Shh-light2 cells (Supplemental Fig. 5) as previously reported (Wilson et al., 2009). These results further argue that GSA-10 and SAG are two Smo agonists with different transduction mechanisms. Together, these data demonstrate that CTX and forskolin, two known activators of adenylate cyclase, are positive and negative regulators of GSA-10– and SAG-mediated C3H10T1/2 cell differentiation, respectively.

**GSA-10 and SAG Act Synergistically on the Differentiation of C3H10T1/2 Cells.** Several models could be proposed for GSA-10 and SAG action at Smo. First, the two molecules are interacting in an allosteric manner on Smo, and the final response could be synergistic or not. However, because of the opposite effects that forskolin exerts on SAG- and GSA-10–mediated AP response presented above, this seems unlikely. The second hypothesis would be that these molecules act on different forms of Smo independently in the cell, with the final response being additive or synergistic if different transduction mechanisms are involved.

To determine whether GSA-10 and SAG act synergistically or not on the differentiation of C3H10T1/2 cells, we established the dose-response curve corresponding to the AP activity induced by SAG in the presence or the absence of GSA-10. In the presence of GSA-10 (1 μM), the EC50 of SAG was shifted to the left by more than 14-fold, from 0.14 μM to 0.01 μM (Fig. 6E), providing strong evidence for a synergistic interaction between the two Smo agonists. In the reciprocal experiment, we observed that the EC50 of GSA-10 in the presence of SAG (0.3 μM) was also left-shifted by 2-fold, from 0.9 μM to 0.4 μM (Fig. 6F), further confirming the synergistic effect of the two molecules. Of interest, the maximal increase of AP activity obtained in the presence of GSA-10 and SAG was not additive but rather strongly potentiated in the two experiments (Fig. 6, E and F). Those data provide further evidence for a distinct mechanism of action for the two molecules. We did not detect any potentiation of GSA-10 and SAG when we analyzed the accumulation of Smo at the primary cilia in C3H10T1/2 cells, suggesting that the synergistic effect was not linked to modification of Smo translocation to the primary cilium (Fig. 4D).

**GSA-10 and SAG Exhibit Distinct Pharmacology at Smo.** We investigated the site of action of these molecules at Smo. Inhibition of Bodipy-cyclopamine (BC) binding to Smo has been used as an assay for the identification of Smo modulators, including both agonists and antagonists (Chen et al., 2002; Sinha and Chen, 2006). Thus, the GSA-10 potential inhibition of BC binding to human Smo (hSmo) stably expressed in HEK293 cells (HEK-hSmo) was investigated and compared with the one of SAG (Roudaut et al., 2011). As expected, SAG (3 μM) inhibited BC binding to Smo, and GSA-10 did not, even at 30 μM, a concentration 25-fold higher than its EC50 (Fig. 7A). These data indicate that GSA-10, unlike SAG, does not compete with the BC site at Smo.

We then tested whether the differentiation of C3H10T1/2 cells observed after addition of GSA-10 and SAG could be blocked by antagonizing Smo. The concentration of SAG and GSA-10 used in these experiments was near their respective EC50. The potent Smo antagonist MRT-83 (Roudaut et al., 2011; Solinas et al., 2012) displayed a similar potency to block

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**TABLE 1**

Expression level of Hh pathway genes tested by qPCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>SAG</th>
<th>GSA-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Cdo</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Gli1</td>
<td>58.6**</td>
<td>0.3*</td>
</tr>
<tr>
<td>Gli2</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Gli3</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Ptc1</td>
<td>5.9**</td>
<td>0.6</td>
</tr>
<tr>
<td>Ptc2</td>
<td>5.3**</td>
<td>1.8</td>
</tr>
<tr>
<td>Hip</td>
<td>48.4**</td>
<td>1.1</td>
</tr>
<tr>
<td>Smo</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Shh</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Dhh</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Ihh</td>
<td>0.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*P = 0.05; **P = 0.01.
GSA-10 (1 μM) and SAG (0.1 μM) response (IC_{50}, ~38 and 11 nM, respectively) (Fig. 7, B and C). MRT-83 showed a competitive antagonism at both molecules. Increasing concentrations of GSA-10 and SAG led to a progressive rightward shift in the MRT-83 inhibition curves and an approximately 3–6-fold increase in their IC_{50} (Fig. 7, B and C). The ability of MRT-83 to competitively inhibit GSA-10 and SAG-mediated AP response is consistent with a direct action of each molecule on Smo for pathway activation.

The pharmacological characterization of SAG and GSA-10 agonist sites, called here Smo^{SAG} and Smo^{GSA-10}, respectively (Fig. 8), was then performed using a panel of potent Smo antagonists of different structures (Supplemental Fig. 6; Fig. 7, D and E; Table 2) (Ng and Curran, 2011). LDE225, SANT-1, and M25 blocked GSA-10- and SAG-induced AP activity with similar potencies. These drugs displayed IC_{50} in the nanomolar range, indicating that they are potent antagonists at both Smo^{SAG} and Smo^{GSA-10}. GDC-0449, which was with MRT-83, the most potent antagonist at Smo^{SAG}, was found as a low affinity antagonist at Smo^{GSA-10} with an IC_{50} of 3300 nM. CUR61414 and cyclopamine inhibited SAG-induced cell differentiation with IC_{50} of 330 and 620 nM, respectively. However, at 10 μM, they blocked less than 50% of GSA-10–induced response, clearly indicating that they lost their antagonist potency. The loss of cyclopamine antagonist property is also consistent with the lack of effect of GSA-10 to compete for the BC binding site observed previously. Therefore, our data establish, for the first time to our knowledge, the existence of two pharmacologically distinct Smo agonist sites that can be discriminated by the use of various antagonists; we identify potent antagonists of Smo^{GSA-10}, such as MRT-83, and demonstrate that reference
Smo antagonists, such as GDC-0449 and LDE225, currently in clinical trials, or cyclopamine, display different pharmacological properties at SmoSAG and SmoGSA-10.

The distinct and even opposite effects of GSA-10 and SAG on Gli-luciferase reporter activity and on Gli1 transcript levels, respectively (Fig. 4; Supplemental Fig. 4; Table 1), suggest that GSA-10 may display antagonist properties at SmoGSA-10. Therefore, we established in Shh-light2 cells the dose-response curve corresponding to the Gli-dependent luciferase reporter induced by SAG in the presence of GSA-10 (1 μM). We observed a marked reduction of the maximal response (42% ± 7%) without significant modification of it EC50 (0.13 ± 0.02 μM versus 0.17 ± 0.05 μM, mean ± S.E.M. of four independent experiments), indicating that GSA-10 behaved as a noncompetitive antagonist at this response level (Supplemental Fig. 7).

Discussion

To allow the characterization of new Smo modulators, we developed a Smo pharmacophore for agonists based on the structure of purmorphamine and SAG derivatives. We then used this pharmacophore for in silico screening of a virtual library of commercially available compounds and identified GSA-10, which does not display the hallmarks of a conventional Smo agonist. GSA-10 should be considered as a novel osteogenic molecule for multipotent mesenchymal cells and, therefore, may be of therapeutic interest in bone-related diseases.

Our molecular and biochemical studies provide strong evidence for the direct effect of GSA-10 at Smo. First, GSA-10, SAG, and purmorphamine share a common pharmacophore, despite some notable differences in their chemical structures. Second, GSA-10- and SAG-induced C3H10T1/2 cell differentiation were impaired in the presence of selective Smo shRNA. Third, the powerful Smo inhibitor MRT-83 blocks GSA-10- and SAG-mediated AP response in C3H10T1/2 cells in a competitive manner and with similar IC50 values. Moreover, several Smo antagonists of different structures, such as LDE225, SANT-1, and M25, antagonize GSA-10 activity with IC50 in the nanomolar range. Therefore, these molecules, such as MRT-83, should constitute interesting drug scaffold for further developing more potent and selective drugs acting at SmoGSA-10. Our data also clearly indicate the presence of two distinct binding sites for SAG and for GSA-10 on Smo that we have named SmoSAG and SmoGSA-10, respectively (Fig. 8). GSA-10 and SAG differ in their capacity to act at the canonical BC binding site previously identified on Smo, because GSA-10 does not recognize this site and SAG and purmorphamine do. Moreover, we provide a pharmacological discrimination of GSA-10- and SAG-induced responses at Smo, as shown by the highly reduced sensitivity of GSA-10 to several Smo reference antagonists, including GDC-0449, CUR61414, and cyclopamine. Because GDC-0449 and LDE225 display different antagonist potency at SmoSAG and SmoGSA-10, it will be important to see to what extent their effects in cancer, as well as those of other Smo antagonists in clinical trials, are related to inhibition of SmoSAG and to identify whether blockade of this binding site participates in the adverse effects of these molecules in humans (Sekulic et al., 2012; Tang et al., 2012).

Smo has been shown to adopt several conformations, both in the cytoplasm and in the cilium. Various classes of Smo modulators stabilize distinct conformations. For example, cyclopamine drives Smo from the cytoplasm into the cilium in an inactive state, whereas SAG and purmorphamine stabilize an active conformation in the cilium (Rohatgi et al., 2009; Rominger et al., 2009; Wilson et al., 2009; Yang et al., 2009; Belgacem and Borodinsky, 2011; Roudaut et al., 2011).

One simple model is that SAG and GSA-10 act to stabilize different active forms of Smo (Fig. 8) that promote, in a synergistic manner, the differentiation of multipotent mesenchymal progenitor cells into osteoblasts. The first active form, SmoSAG, is addressed to the primary cilium after stimulation by SAG, leading to activation of the Hh pathway and transcription of Hh-related genes. SmoGSA-10 response is negatively regulated by forskolin and CTX, in agreement with previous reports using the evaluation of the Gli-luciferase reporter activity (Wilson et al., 2009). On the other hand, we hypothesize that GSA-10 stabilizes a yet unidentified Smo active form, called SmoGSA-10, which does not translocate to the primary cilium and is strongly potentiated by forskolin and CTX. An alternative model would be that GSA-10 interacts with an unknown protein acting downstream of Ptc and upstream of Smo, to promote the cellular secretion of an, as yet, unidentified Smo ligand that would be responsible for C3H10T1/2 cell differentiation. Such ligand would have
the biologic and pharmacological properties described above for GSA-10. It should differ from the oxysterol derivatives proposed to interact with Smo and to induce its trafficking to the primary cilium (Nachtergaele et al., 2012).

Moreover, we show that GSA-10 displays no significant agonist BMP and Wnt signaling activity despite the structural resemblance of Smo with the Frizzled receptors (Schulte, 2010).

To our knowledge, GSA-10 is the first small-molecule Smo agonist that does not promote Smo translocation to the primary cilium. This feature questions the proposed theory according to which Smo localization to the cilium is necessary (but not sufficient) for activation of the Hh pathway (Wilson et al., 2009). In agreement with this questioning, the Shh-mediated chemotactic response that requires neither de novo gene transcription nor Gli protein functions was found to be mediated by Smo located outside the primary cilium (Chinchilla et al., 2010; Bijlsma et al., 2012).

We speculate that the potentiation of GSA-10–mediated AP response by forskolin and CTX reflects a direct effect on adenylyl cyclase and, thus, PKA stimulation. In Drosophila, PKA-mediated Smo phosphorylation at multiple serine/threonine residues in the Smo carboxyl-terminal cytoplasmic tail is proposed to antagonize arginine clusters responsible for Smo inactivation. Such a conformational switch is responsible for Smo cell surface accumulation and activation (Zhao et al., 2007). Because arginine clusters but not the PKA sites are conserved in vertebrates and are critical for maintenance of Smo in an inhibited state, further investigation is required to clarify the mechanism of action of forskolin and CTX at vertebrate Smo and, in particular, their opposite effects on SmoSAG and SmoGSA-10 conformations.

During development, forskolin has been shown to inhibit Hh signaling in brain, feather bud, tooth, and testis (Fan et al., 1995; Hynes et al., 1995; Cobourne et al., 2001; Yao and Capel, 2002). This is consistent with the negative effect of forskolin and CTX on differentiation of C3H10T1/2 cells by SAG (present data) and on activation of the Hh reporter (Fan et al., 1995; Epstein et al., 1996; Hammerschmidt et al., 1996; Pan et al., 2009). On the other hand, a positive role for cAMP and PKA in Smo regulation has been observed both in Drosophila and in mammals (Jia et al., 2004; Zhao et al., 2007; Milenkovic et al., 2009; Wilson et al., 2009). Both forskolin and PKA treatments induced extra digits in vertebrate limbs, suggesting that they display a positive role in Hh signaling (Tiecke et al., 2007). If SmoGSA-10 may be physiologically relevant, nevertheless, an endogenous Hh-stabilized SmoGSA-10 form mediating such response yet remains to be demonstrated.

In vitro studies in insect or mammalian cells have previously indicated that Smo specifically stimulates GTP binding to Gαi family members (Riobo et al., 2006) and can also interact with Gα15 protein (Masdeu et al., 2006). The absence of PTX effect on the GSA-10–induced response suggests that Gαo proteins are not implicated in this response. Whether SmoGSA-10 interacts with other G-proteins and β-arrestin at the cell membrane merits further work.

Investigation of the effect of GSA-10 on GCPs does not result in an increase of cell proliferation, as observed with SAG, suggesting either that GSA-10 is not able to stabilize a Smo-active form in these cells or that such active form is not linked to GCP proliferation. Consequently, GSA-10 mechanism of action differs from that of selected glucocorticoids proposed to bind Smo, to activate Gli, and to promote mouse GCP proliferation (Wang et al., 2010). Numerous other physiologic roles are associated with Hh signaling and, potentially, with Smo modulation in the developing or adult brain. These ones include proliferative and survival activities toward neural stem cells, axonal chemotaxation implicated in guidance of commissural axons (Charron and Tessier-Lavigne, 2005), retention activity for adult neural precursors (Angot et al., 2008), or control of oligodendroglial precursors with potential therapeutic interest in demyelinating diseases (Traftort et al., 2010; Ferent et al., 2013). In addition, Hh also mediates pathfinding of commissural axons through a noncanonical pathway involving Smo but not related to gene transcription (Yam et al., 2009). Finally, a Smo-dependent noncanonical pathway involving Camkk2/Ampk and recognizing the classic Hh antagonist cyclopamine as a potent selective partial agonist, was reported to induce acute glucose uptake in vitro and in vivo (Teperino et al., 2012). Of interest, we found that GSA-10 serves also as an antagonist to SAG-induced Gli-dependent luciferase, further showing the level of complexity of several drugs acting at Smo with important mechanistic and, potentially, therapeutic significance.

Together with the recent report showing activation of the noncanonical Smo-Ampk axis by both SAG and cyclopamine (Teperino et al., 2012), the present work adds a new level of complexity by which a cell can establish downstream signaling diversity and, consequently, cell type specificity. As a whole, our findings demonstrate a significant variability in Smo conformations induced by different ligands that has clearly important implications for the development of novel and more selective therapeutic agents.

Acknowledgments

The authors thank Gisèle Froment, Didier Nègre, and Caroline Costa from the lentivectors production facility/SFR Biosciences Gerland–Lyon Sud (UMS3444/US8) and Plateforme de qPCR, IMAGIF, Gif/Yvette, France.

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**Conducted experiments:** Gorojankina, Faure, Hoch, Roudaut.

**Contributed new reagents or analytic tools:** Girard, Manetti, Schoenfelder, Solinas, Petricci.

**Performed data analysis:** Gorojankina, Faure, Hoch, Roudaut, Traftort, Schoenfelder, Girard, Mann, Manetti, Solinas, Petricci, Taddei, Ruat.

**Wrote or contributed to the writing of the manuscript:** Ruat.

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