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Antipsychotic Drugs Regulate Hedgehog Signaling by Modulation of 7-Dehydrocholesterol Reductase Levels[S]

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ABSTRACT

Recently we identified GANT61, a small-molecule antagonist of Gli transcription factors, which are the final effectors of the mammalian Hedgehog (HH) signaling pathway. Here we describe a diamine substructure of GANT61 that carries the biological activity and show that this part of the molecule is structurally related to *trans*-1,4-bis(2-chlorobenzaminomethyl)cyclohexane dihydrochloride (AY9944), an inhibitor of the enzymatic activity and transcriptional inducer of 7-dehydrocholesterol-reductase (Dhcr7, EC 1.3.1.21). Treatment of cells with the GANT61 diamine, AY9944, or overexpression of DHCR7 results in the attenuation of Smoothened-dependent and -independent HH signaling. Whereas GANT61 function is independent of Dhcr7, AY9944 does require up-regulation of endogenous Dhcr7. In line with these findings, Dhcr7-modulating antipsychotic (clozapine, chlorpromazine, haloperidol) and antidepressant (imipramine) drugs regulate HH signaling in vitro and in vivo. Modulation of HH signaling may represent a hitherto undiscovered biological (side) effect of therapeutics used to treat schizophrenia and depression.

Introduction

Hedgehog (HH) signaling activity is crucially required for embryonal processes such as pattern formation (Jiang and Hui, 2008). The best studied examples are the establishment of a dorsoventral gradient, which specifies cell identity in the neural tube or the patterning of the developing limb (Dessaud et al., 2008). Postnatal HH activity is spatially restricted and is involved in the maintenance of tissue stem cells such as those of the brain (e.g., hippocampus) and the cerebellum (Palma et al., 2005; Galvin et al., 2008).

Mammalian genomes contain three HH genes: Sonic (*Shh*), Indian (*Ihh*), and Desert (*Dhh*) Hedgehog. They all bind with comparable affinity to their common receptor Patched 1 (Ptch1), which in its unliganded state may function as a molecular pump transporting small molecules across the cell membrane (Taipale et al., 2002). One candidate cargo molecule is vitamin D_3, which negatively modulates the function of another HH signaling molecule, Smoothened (Smo) (Bijlsma et al., 2006). Hedgehog ligand binding to Ptch1 releases Smo from its inhibition and allows for the signal to be conveyed to downstream pathway components such as Suppressor of Fused (Sufu) and the transcription factors Gli2 and Gli3. Whereas Gli2 acts mainly as a transcriptional activator, Gli3 behaves mostly as a repressor. Eventually, HH-specific target genes are activated, including members of the HH pathway itself such as *Gli1*, *Hip1*, and *Ptch1*, leading to a feedback control of signaling strength. These target genes can be used experimentally as a direct measure of pathway activity because their expression status correlates with the level of HH signal transduction.

Vitamin D_3, which is synthesized from 7-dehydrocholesterol (7-DHC), is not the only link between the cholesterol biosynthetic pathway and Hedgehog signal transduction. In fact, a complex network of positive and negative interactions

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ABBREVIATIONS: HH, Hedgehog; Dhcr7, 7-dehydrocholesterol-reductase; GANT61, Gli-antagonist 61; AY9944, *trans*-1,4-bis(2-chlorobenzaminomethyl)cyclohexane dihydrochloride; Ptch1, Patched 1 receptor; Smo, Smoothened; SAG, Smoothened agonist; Sufu, Suppressor of Fused; 7-DHC, 7-dehydrocholesterol; SLOS, Smith-Lemli-Opitz syndrome; SREBP, sterol regulatory element-binding protein; MEF, mouse embryonic fibroblast; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; QPCR, quantitative polymerase chain reaction; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; SANT1, *N*-[(3,5-dimethyl-1-phenyl-1*H*-pyrazol-4-yl)methylene]-4- (phenylmethyl)-1-piperazinamine; siRNA, small interfering RNA; SSD, sterol-sensing domain.

seems to tie cholesterol biosynthesis and HH signaling together. The Hedgehog ligands themselves are cholesterol-modified, increasing their affinity for cell membranes and restricting their free dispersal (Li et al., 2006). Furthermore, oxidized versions of cholesterol (oxysterols) are potent inducers of Smo activity (Corcoran and Scott, 2006). In addition, overexpression of 7-dehydrocholesterol-reductase (Dhcr7, EC 1.3.1.21), the enzyme performing the last step in the cholesterol biosynthesis (converting 7-DHC into cholesterol), results in strong inhibition of the HH pathway in *Xenopus laevis* embryos (Koide et al., 2006). Although these data have not been duplicated in mammals, these findings are contrasting results from another group suggesting that overexpression of Dhcr7 would result in decreased 7-DHC levels (and thus decreased levels of the Smo inhibitor Vitamin D_3) and would therefore be HH pathway-stimulatory (Bijlsma et al., 2006). In summary, it seems that Dhcr7 possesses dual and opposing functions on HH signaling.

Dhcr7 is a nine-pass transmembrane protein residing in the endoplasmic reticulum, and mutations in the *DHCR7* gene are the underlying cause for Smith-Lemli-Opitz syndrome (SLOS), an autosomal recessive human disorder characterized by a failure to thrive, psychomotor retardation, and organ malformation (Kelley and Hennekam, 2000). The spectrum of SLOS phenotypes can be recapitulated in animal models in which pregnant animals receive the Dhcr7 inhibitor *trans*-1,4-bis(2-chlorobenzaminomethyl)cyclohexane dihydrochloride (AY9944) (Llirbat et al., 1997).

Here we show that the Dhcr7 inhibitor AY9944 displays structural similarity to the hexahydropyrimidine part of the GANT61 molecule. GANT61 was discovered previously in a cellular screen identifying small-molecule inhibitors of Gli1 and Gli2 (Lauth et al., 2007a). Although AY9944 does not show potent direct inhibitory activity of Gli transcription factors, it does block HH signaling induced at the level of Smo or by loss of *Sufu*. We show that AY9944 functions by induction of *Dhcr7* expression and not by its similarity to GANT61. On the other hand, we verify that GANT61 has Dhcr7-independent inhibitory potential.

Antipsychotic drugs such as clozapine, chlorpromazine, and haloperidol and antidepressants such as imipramine are able to activate the sterol regulatory element-binding proteins (SREBPs) and subsequently the transcription of SREBP-controlled genes such as *DHCR7* (Fernø et al., 2005; Raeder et al., 2006). We demonstrate that these *DHCR7*-regulating substances modulate the HH pathway in vitro and in vivo, raising the possibility that interference with the HH pathway might represent a previously unrecognized biological aspect of clinical drugs used to treat schizophrenia and depression.

Materials and Methods

Cell Lines and Regents. NIH3T3 cells, ShhL2 cells, and *Sufu*(−/−) mouse embryonic fibroblasts (MEFs) were cultured in DMEM (high glucose) and 10% heat-inactivated fetal bovine serum plus 1 mM sodium pyruvate. C3H10T1/2 cells were grown in DMEM (low glucose) plus 10% heat-inactivated fetal bovine serum and AsPC1 cells in F-12 Ham's (50%)/DMEM (low glucose) (37%) plus 1 mM sodium pyruvate and 0.1 mM nonessential amino acids. All cell line media contained penicillin/streptomycin.

The GANT61 hydrolysis product GANT61-D and compound D8 were obtained from Actar AB (Solna, Sweden). D8-D was purchased from TimTec Corporation (Newark, NJ). D8-A, AY9944, *N*-[(3,5-dimethyl-1-phenyl-1*H*-pyrazol-4-yl)methylene]-4- (phenylmethyl)-1-piperazinamine (SANT1), TPA, imipramine, clozapine, haloperidol, and chlorpromazine were purchased from Sigma- Aldrich (St. Louis, MO) and/or Calbiochem (San Diego, CA).

Cloning of Expression Constructs. A full-length human DHCR7 expression clone was purchased from RZPD/imaGenes GmbH (Berlin, Germany). Because this construct was poorly expressed, we transferred the coding sequence to a pEF6/V5-His-TOPO backbone (Invitrogen, Carlsbad, CA). The deletion construct DHCR7ΔC was constructed by means of PCR in the same plasmid backbone. All constructs were verified by sequencing. Stable cell lines were obtained by plasmid transfection using FuGENE 6 reagent (NIH3T3) or by Amaxa electroporation (Amaxa Biosystems, Gaithersburg, MD) [*Sufu*(−/−) MEFs] and subsequent antibiotic selection.

HH Reporter Assays. Cells grown to 50 to 60% confluence were transfected with a firefly luciferase Gli reporter plasmid (Lauth et al., 2007a) and a *Renilla reniformis* luciferase plasmid for normalization. The following day, the cells reached full confluence and were treated with 100 nM SAG for 48 h. Subsequently, cells were lysed, and luciferase activities were measured using the Dual Luciferase kit from Promega (Madison, WI).

C3H10T1/2 cells were grown to full confluence and exposed to SAG and the test compounds for 4 days. Subsequent cell lysis was done using the Passive Lysis Buffer (Promega). The lysate (75%) was used to measure alkaline phosphatase activity (Alkaline Phosphatase Blue Microwell Assay; Sigma), whereas 25% was used to measure protein concentration (Protein Assay; Bio-Rad Laboratories, Hercules, CA).

RNA Preparation and Real-Time PCR. RNA was prepared from cultured cells using the RNeasy kit from QIAGEN (Valencia, CA) with on-column DNaseI digest. Subsequently, cDNA was synthesized with oligo(dT) primers (Promega) and Superscript II reverse transcriptase (Invitrogen). QPCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR machine using TaqMan probes (Applied Biosystems, Foster City, CA; see supplemental information).

Immunoblotting. Confluent *Sufu*(−/−) cells were treated with test compound for 48 h in full growth medium. Subsequently, cells were lysed in SDS buffer, and proteins were separated on SDS gels and transferred onto polyvinylidene difluoride membranes. Detection of blotted proteins was by incubation of the membranes using the following antibodies: α-Hip1 (R&D Systems, Minneapolis, MN); α-Gli3 (Santa Cruz Biotechnology, Santa Cruz, CA); α-β-Actin (Sigma-Aldrich); and α-V5 (Invitrogen).

GANT61 Hydrolysis Study. A 10 mM stock solution of GANT61 was prepared in DMSO. Using the DMSO stock solution, 100 μM GANT61 was prepared in PBS, pH 7.0, or PBS, pH 2.0. The PBS solutions were incubated in the dark at room temperature. Aliquots of the solutions were analyzed at the time interval of 0, 4, 12, and 24 h by liquid chromatography-mass spectrometry. The corresponding peak areas were recorded for each analyte. The ratio of the GANT61 remaining at each time point relative to the amount determined at time 0, expressed as a percentage, is reported as chemical stability. The entire analysis was performed by Anthem Biosciences (Karnataka, India).

Animal Experiments. Male C57BL6 mice (25–30 g) were injected intraperitoneally with a single dose of either saline, imipramine (20 mg/kg), clozapine (30 mg/kg), haloperidol (2.5 mg/kg), or chlorpromazine (2.5 mg/kg). Animals were sacrificed 48 h after injection, and brain frontal cortices were dissected out for RNA preparation. All animal experiments were performed according to institutional and Swedish ethical guidelines.

Results

Identification of a Biologically Active Substructure of GANT61. We have described the hexahydropyrimidine derivative GANT61 as a small-molecule inhibitor of GLI1 and GLI2 (Lauth et al., 2007a). The Gli transcription factors constitute the final effectors of the HH signaling pathway, and pharmacological inhibitors are interesting candidates for a targeted anticancer therapy (Rubin and de Sauvage, 2006; Lauth et al., 2007c). Based on its structure, we predicted that GANT61 would be unstable under acidic conditions and would be subject to hydrolysis, giving rise to a diamine and a benzaldehyde product (Fig. 1a). To investigate whether the proposed hydrolysis products carried some HH-inhibitory potential, we made use of the compound D8, a GANT61 analog, which would also be capable of hydrolyzing to a diamine (D8-D) and an aldehyde (D8-A) and of which we could obtain hydrolysis products for testing (Fig. 1b).

We first treated ShhL2 cells (an NIH3T3 clone stably expressing HH luciferase reporter constructs) (Taipale et al., 2000) with the synthetic Smoothened agonist SAG (Chen et al., 2002) to induce HH signaling and exposed these cells to increasing concentrations of D8, D8-A, and D8-D (Fig. 1c). The phorbol ester and HH inhibitor TPA was included as positive control (Lauth et al., 2007b). As expected for a close GANT61 analog, compound D8 could antagonize SAG-induced HH signaling in a dose-dependent manner. It is noteworthy that only the diamine of D8 (D8-D), not the aldehyde (D8-A), capable of inhibiting signaling. Adding D8-A and D8-D simultaneously (D8-D+A) gave results that were identical with "D8-D only" treatment (Fig. 1c). Next, we wanted to verify this result by treating *Sufu*(−/−) MEFs. These cells

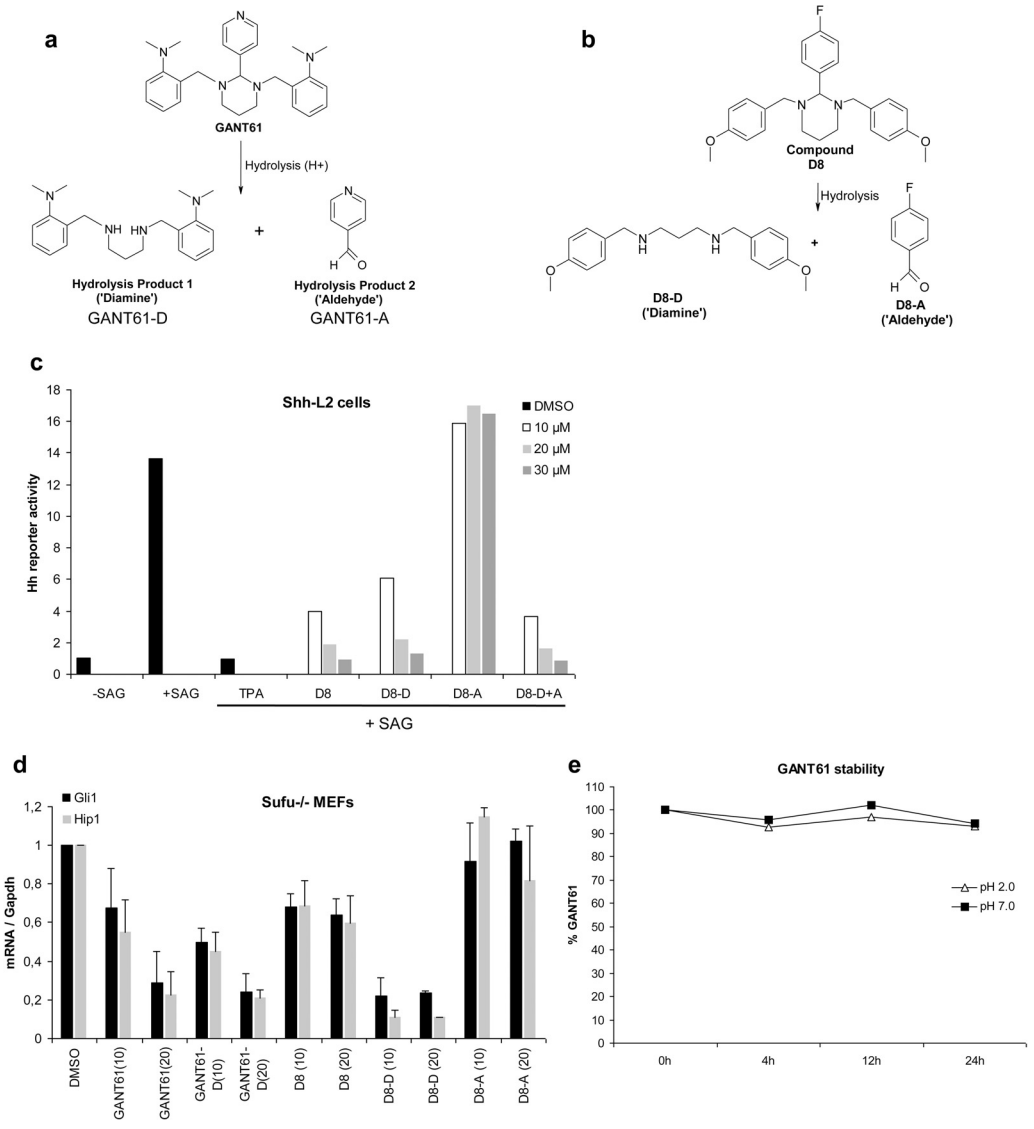

Fig. 1. Hydrolysis reaction of GANT61 and biological activity of its proposed hydrolysis products. a, predicted hydrolysis reaction of GANT61 into a diamine and a benzaldehyde under acidic conditions. b, structures of the GANT61 analog D8 and its predicted hydrolysis products. c, compound D8 and its substructure D8-D but not D8-A inhibit HH signaling in SAG-induced ShhL2 cells. As positive control, TPA (160 nM) was added. D8-D+A denotes the simultaneous addition of D8-D and D8-A. Treatment time was 48 h. Shown is one representative experiment of three. d, QPCR of *Sufu*(−/−) MEFs treated with the indicated compounds for 48 h. The bracketed numbers indicate the concentration (micromolar). $n = 3\pm$ S.D. e, GANT61 stability as a function of pH and time. Note that GANT61 is very stable even under strong acidic conditions. Shown is the mean of two independent experiments.

have a homozygous deletion of Suppressor of Fused leading to a strong Smo-independent activation of HH signaling (Svärd et al., 2006). As can be seen in Fig. 1d, D8 and its proposed diamine hydrolysis product (D8-D) but not the aldehyde D8-A could block signaling as measured by the induction of the HH target genes *Gli1* and *Hip1*. In agreement with the concept that the HH pathway inhibition is mediated by the diamine substructure, the corresponding GANT61 diamine (GANT61-D; Fig. 1a) was able to suppress signaling in *Sufu*(−/−) cells with the same efficacy as the parent compound GANT61 (Fig. 1d). We verified this finding by testing a series of GANT61-related hexahydropyrimidine derivatives, which would give rise to relatively similar diamines but structurally very different aldehydes. As can be seen in Supplementary Fig. 1, a and b, all GANT61 analogs inhibited HH signaling in a dose-dependent manner, arguing against an important role of the aldehyde portion of the molecule.

To elucidate the chemical stability of GANT61 in acidic pH, we performed liquid chromatography-mass spectrometry of GANT61 samples kept at neutral pH or at pH 2 for 0, 4, 12, and 24 h (Fig. 1e and Supplemental Fig. 2, a and b). GANT61 proved surprisingly very stable and showed no signs of hydrolysis at any pH or time point. In summary, despite the fact that the hexahydropyrimidine derivative GANT61 is chemically very stable in low pH, we were able to identify a substructure of GANT61, which seems to be exclusively responsible for the biological effects of GANT61 on the HH pathway.

GANT61-D Displays Structural Similarity to AY9944. Because we found that the diamines GANT61-D and D8-D were biologically functional and blocked HH signaling, we were intrigued by the observation that GANT61-D has some structural resemblance to AY9944, an inhibitor of 7-dehydrocholesterol-reductase (Dhcr7) (Fig. 2a). AY9944 has been reported previously to inhibit HH signaling at the level of Smo (Bijlsma et al., 2006), and we could verify inhibition by AY9944 in SAG-induced ShhL2 cells (Fig. 2b). We could also observe a weaker degree of inhibition of HH signaling in *Sufu*(−/−) cells in which pathway activity is independent of Smo function (Fig. 2, c and d). In addition, through its inhibitory effect on the cholesterol biosynthesis pathway, AY9944 gave rise to a significant feedback induction of Dhcr7 itself. This induction was not observed for GANT61, demonstrating that its affect on cholesterol synthesis is minimal (Fig. 2c). We verified the inhibition of HH signaling downstream of Smo by treating the pancreatic cancer cell line AsPC1 with GANT61 and AY9944. AsPC1 cells are insensitive to the SMO inhibitor SANT1 but express GLI1, indicating an activation of the pathway downstream of SMO. As can be seen in Fig. 2e, both GANT61 and AY9944 led to an inhibition of signaling as measured by a reduction in GLI1 mRNA levels. In line with previous results, only exposure to AY9944, but not GANT61, induced the cholesterol pathway genes *HMGCR* and *DHCR7*. Whereas the induction of the latter genes by AY9944 was dose-dependently increased, reduction of GLI1 levels was not, suggesting that the maximal inhibition by AY9944 has been achieved (Fig. 2e).

Because GANT61 was initially identified in a cellular screen designed for GLI inhibitors, we went on to investigate whether AY9944 showed some effect on transfected GLI1 or a dominant-active version of GLI2 (GLI2ΔN). However, whereas GANT61 potently inhibited GLI1 and GLI2ΔN function, AY9944 was ineffective in the same concentration range, suggesting that AY9944 blocks HH signaling upstream of the Gli transcription factors (Fig. 2f). In summary, the two structurally comparable compounds GANT61 and AY9944 seem to block HH signaling by different mechanisms.

Overexpression of DHCR7 Negatively Modulates Mammalian HH Signaling. Because AY9944 treatment leads to an induction of Dhcr7 expression and also inhibits the HH pathway, we wondered whether the increased Dhcr7 expression might mediate the antagonistic effect of AY9944 on the HH pathway. Dhcr7 has been shown previously to be a potent inhibitor of HH signaling in *X. laevis* embryos (Koide et al., 2006). To elucidate whether a similar mechanism applies to the mammalian situation, we cloned two different DHCR7 constructs (Fig. 3, a and b): Full-length DHCR7 and a C-terminally truncated version (DHCR7ΔC). This deletion mutant resembles the most frequent mutation in SLOS patients ($DHCR7^{IVS8-1G>C}$), leading to a partially truncated sterol-sensing domain and a significantly reduced enzymatic activity (Witsch-Baumgartner et al., 2000). Thus, this version can be used to address the relevance of DHCR7's reductase function.

In transient transfection experiments in NIH3T3 cells, we found that DHCR7 and DHCR7ΔC overexpression resulted in a 40 to 50% reduction of HH signaling (Fig. 3c). This result is in agreement with data from *X. laevis*, in which DHCR7-mediated inhibition of HH signaling is independent of DHCR7's enzymatic function (Koide et al., 2006).

To confirm the negative modulation of HH signaling by DHCR7, we generated NIH3T3 cells stably expressing DHCR7 and measured *Gli1* levels upon SAG induction. As can be seen in Fig. 3d, ectopic expression of DHCR7 resulted in an attenuation of HH pathway activity in these cells. In favor of an inhibitory potential of Dhcr7, cerebellar medulloblastomas (induced through genetic deletion of *Ptch1*) show a reduction in *Dhcr7* mRNA levels in preneoplastic and tumor cells compared with normal cerebellar granule cells (Fig. 3e) (Oliver et al., 2005). This is accompanied by an increase in *Gli1* mRNA levels, supporting our findings of an inverse relationship between *Dhcr7* and *Gli1* and in pathogenic situations.

To see whether overexpression of DHCR7 would dampen signaling in cells devoid of Suppressor of Fused, we stably expressed DHCR7 in *Sufu*(−/−) MEFs (Fig. 4a). QPCR analysis revealed that expression of SUFU as a positive control led to a strong down-regulation of *Gli1*, *Hip1*, and *Ptch1* levels, indicative of pathway inhibition. Expression of DHCR7 gave rise to a moderate but detectable suppression of signaling in *Sufu*(−/−) cells (Fig. 4a). Cells lacking *Sufu* express very low levels of Gli3 protein, suggesting that Sufu is required for Gli3 protein production or stability (Chen et al., 2009; Jia et al., 2009). Stable transfection of *Sufu*(−/−) MEFs with an SUFU plasmid restored expression of Gli3 with the truncated repressor form of Gli3 ($Gli3^R$) being more abundant (Fig. 4b). Expression of DHCR7 did not restore Gli3 protein levels, suggesting that the inhibitory mechanisms of Sufu and Dhcr7 are probably different.

The ability of DHCR7 to inhibit signaling in an Smo-independent manner was verified by transfection of the pancreatic cancer cell line AsPC1 with DHCR7 plasmid. AsPC1 cells are GLI1-positive, but they are resistant to SMO inhibitors such as SANT1, indicating a downstream activation of the

pathway (Fig. 2e). QPCR analysis demonstrated that transfection of these cells with DHCR7 expression plasmid resulted in an inhibition of HH signaling as measured by the decrease in *GLI1* and *PTCH* mRNA levels (Supplemental Fig. 3, a and b).

DHCR7 is a nine-pass transmembrane protein residing in the endoplasmic reticulum (Moebius et al., 1998). To elucidate whether overexpressed DHCR7 can also be seen in other cellular compartments (e.g., nucleus), in which it could theoretically interfere with HH signaling, we performed confocal microscopy on *Sufu*(−/−) cells stably expressing a tagged version of DHCR7 (Fig. 4c). However, no DHCR7 staining was observed in the nuclei of transfected cells and the pattern seen was in agreement with a predominant endoplasmic reticulum localization. Finally, we wanted to investigate the effect of DHCR7 and its deletion mutants on transfected GLI1, GLI2ΔN, and full-length GLI2. As can be seen in Fig. 4d, transfection with SUFU

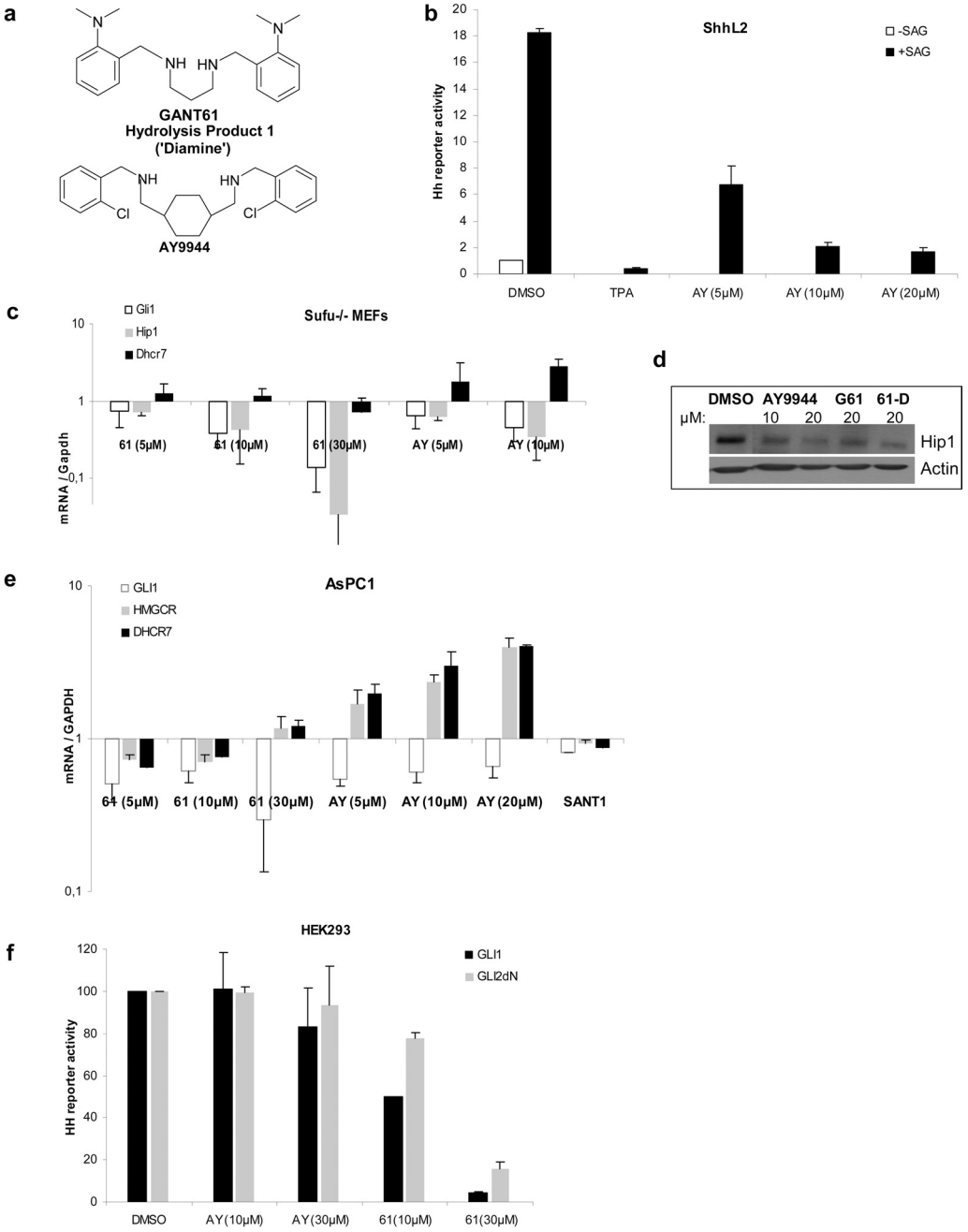

Fig. 2. The biologically active diamine substructure of GANT61 resembles the structure of AY9944. a, chemical structures of the Dhcr7 inhibitor AY9944 in comparison with the diamine part of GANT61. b, AY9944 (AY) blocks SAG-induced HH signaling in ShhL2 cells. TPA (160 nM) was included as positive control ($n = 3 \pm$ S.D.). c, GANT61 (61) and AY9944 (AY) inhibit HH signaling in *Sufu*(−/−) cells as measured by QPCR. Note that treatment with AY9944 induces Dhcr7 mRNA; $n = 3 \pm$ S.D. d, immunoblot showing reduction of Hip1 protein expression upon exposure of *Sufu*(−/−) MEFs to AY9944, GANT61 (G61), or GANT61-D (61-D). β-Actin levels are shown as loading control. e, AY9944 (AY) and GANT61 (61) reduce GLI1 mRNA levels in the pancreatic cancer cell line AsPC1, which is unresponsive to the Smo inhibitor SANT1 (0.2 μM, which is sufficient to fully block SAG-induced signaling in NIH3T3; data not shown). Note the strong induction of the cholesterol synthesis genes *HMGCR* and *DHCR7* by AY9944; $n = 3 \pm$ S.D. f, GANT61 (61) but not AY9944 (AY) can significantly inhibit transfected GLI1 and GLI2ΔN (GLI2dN) in a Gli-responsive luciferase assay in human embryonic kidney 293 cells; $n = 3 \pm$ S.D.

led to a strong inhibition of the transcription-inducing activity of all GLI constructs, whereas expression of any of the two DHCR7 variants did not result in a blockade of activity. In summary, we found that ectopic expression of DHCR7 attenuated the HH pathway in mammalian cells, which have activated signaling by Smo-dependent and -independent mechanisms.

Induction of Endogenous Dhcr7 by Antipsychotic Drugs Attenuates HH Signaling. It was reported previously that antipsychotic and antidepressant drugs augment DHCR7 levels in several cell types, such as glial and hepatic cells (Fernø et al., 2005; Raeder et al., 2006). DHCR7 induction in these cells occurs via activation of the SREBP, which is a master switch for the regulation of lipogenic genes. To elucidate whether members of these drug classes could affect HH signaling in a Dhcr7-dependent manner, we investigated the effects of the tricyclic antidepressant imipramine and the antipsychotics clozapine, haloperidol, and chlorpromazine (chemical structures are shown in Fig. 5a).

As can be seen in Fig. 5b, all of these drugs were capable of significantly inhibiting HH signaling in SAG-treated ShhL2 cells. To investigate whether the inhibition observed was indeed due to up-regulation of endogenous *Dhcr7*, we performed a rescue experiment in C3H10T1/2 cells (we used this cell line because it is HH-responsive and better transfectable with siRNA constructs than NIH3T3 cells). Knocking down endogenous *Dhcr7* levels and thus preventing the *Dhcr7* up-regulation mediated by the antipsychotic drugs should reduce the HH pathway inhibition. It is noteworthy that our siRNA construct targeting Dhcr7 resulted in a modest knockdown, which was sufficient to block the induction of *Dhcr7* by the antipsychotics. However, the knockdown was not efficient enough to completely eliminate Dhcr7 from the cell, which would have resulted in the stacking of 7-dehydrocholesterol and consequently the accumulation of the Smo antagonist vitamin D_3 (Bijlsma et al., 2006). As can be seen in Fig. 5c, left, all analyzed compounds augmented endogenous *Dhcr7* levels in C3H10T1/2 cells, and this induction was blocked by transfection of siRNA against *Dhcr7*. In this experiment, GANT61 (at 20 μM) induced *Dhcr7* expression, which is probably caused by its structural relatedness to AY9944. All compounds tested reduced *Gli1* levels to varying degrees (Fig. 5c, right side). It is noteworthy that when the induction of *Dhcr7* was blocked by transfection of siDhcr7, the capability to inhibit the HH pathway was greatly reduced. An exception was GANT61, which could still function

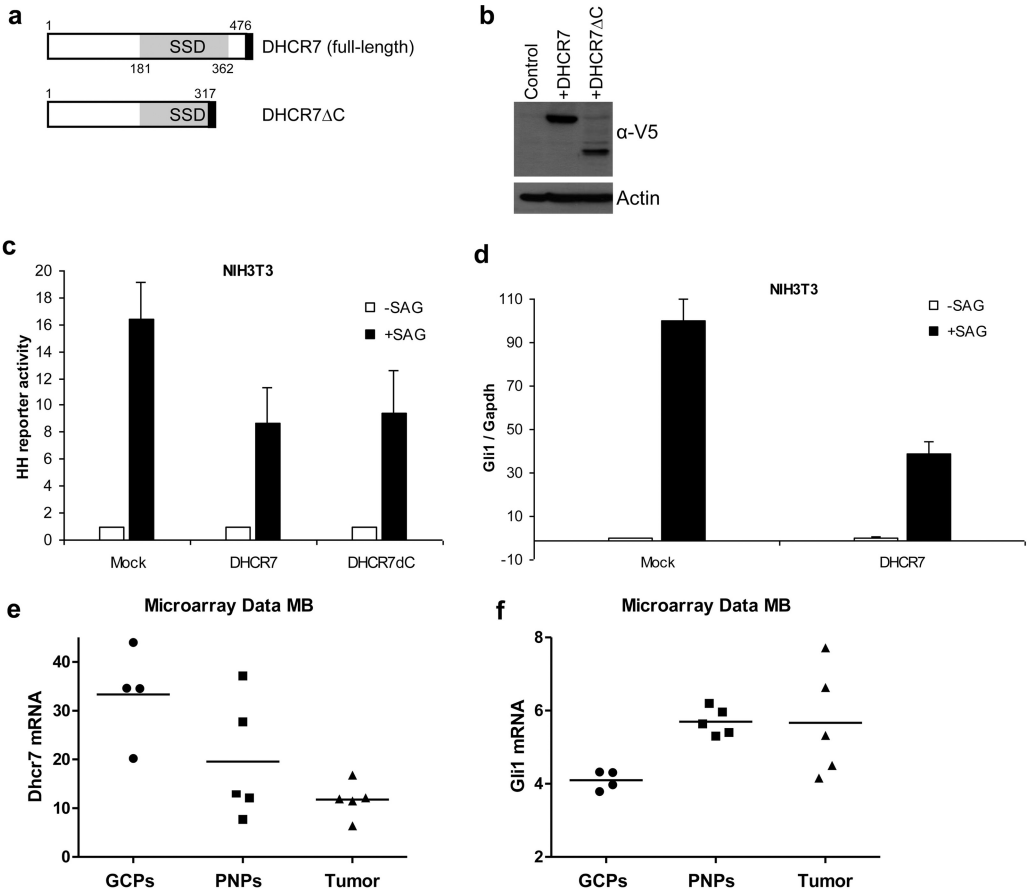

Fig. 3. Overexpression of DHCR7 results in attenuation of the HH pathway. a, scheme of the DHCR7 constructs used. DHCR7ΔC lacks the C-terminal part, which is required for catalytical activity. The black box indicates the V5 protein tag. b, immunoblot of NIH3T3 cells transiently transfected with V5-tagged DHCR7 and DHCR7ΔC. Control cells were nontransfected. c, Hedgehog reporter assay in NIH3T3 cells transiently transfected with the indicated constructs ($n = 3 \pm$ S.D.). d, QPCR analysis of NIH3T3 cells stably overexpressing full-length DHCR7; $n = 3 \pm$ S.D. e, microarray data from cerebellar cells of a mouse *Ptch1*(+/−) medulloblastoma model depicting the changes in *Dhcr7* mRNA levels. GCPs, normal granule cell precursors; PNP, preneoplastic cells; Tumor, tumor cells; MB, medulloblastoma. The data shown were taken from Gene Expression Omnibus (GEO) and were generated by Oliver et al. (2005). The mean value is indicated by a black bar. f, microarray data from cerebellar cells of a mouse *Ptch1*(+/−) medulloblastoma model depicting the changes in *Gli1* mRNA levels. The data shown were taken from GEO and were generated by Oliver et al., (2005). The mean value is indicated by a black bar.

as an HH pathway antagonist without *Dhcr7* induction. Taken together, these data show that the tested antidepressant/antipsychotics attenuate HH signaling by up-regulation of endogenous *Dhcr7*. Although GANT61 displayed a similar pattern in the tested cell line, it also possessed a strong *Dhcr7*-independent inhibitory function.

Because overexpression of DHCR7 and AY9944 treatment had a suppressive affect on HH signaling in *Sufu*(−/−) cells, we tested whether the antipsychotics/depressant showed a similar effect. QPCR analysis demonstrated that all of these compounds induced Dhcr7 expression and interfered with HH signaling, as measured by a reduction in *Gli1* and *Hip1* mRNA levels (Fig. 6a). Reduction of Hip1 was also verified on the protein level (Fig. 6b).

Next, we were interested to determine whether the neuroleptics were capable of interfering with a physiological process driven by endogenous HH pathway activity. The murine mesenchymal progenitor cell line C3H10T1/2 enters an osteogenic differentiation program with concomitant up-regulation of alkaline phosphatase upon activation of HH signaling. C3H10T1/2 cells were induced with SAG and were simultaneously treated with AY9944, imipramine, clozapine, chlorpromazine, or haloperidol. As can be seen in Fig. 6c, all compounds suppressed alkaline phosphatase expression in a dose-dependent manner, suggesting that HH-dependent physiological processes can be modulated by these drugs.

Antipsychotics Modulate HH Signaling in Neuronal Cells and Tissues. The primary target organ for antidepressants/antipsychotics is the brain. In an attempt to verify that the *DHCR7*-regulating drugs also function in neuronal cells, we applied these compounds to the glioblastoma cell line T98G. In line with the previous findings in non-neuronal cells, haloperidol, clozapine, and imipramine led to an up-regulation of endogenous *DHCR7* and a concomitant down-regulation of *GLI1* (Fig. 7a). Finally, we aimed at testing the effects of antipsychotics/antidepressants on HH signaling in an in vivo situation. Mice were treated intraperitoneally with saline, imipramine, haloperidol, chlorpromazine, or clozapine, and HH pathway activity in the brain was monitored by measuring *Gli1* mRNA in the frontal cortex. As can be seen in Fig. 7, b and c, an inverse correlation between *Dhcr7* and *Gli1* was also detected in these in vivo samples. Treatment with the antipsychotics/antidepressants unexpectedly resulted in a reduction of *Dhcr7* (Fig. 7b) and a subsequent induction of *Gli1* (Fig. 7c). This shows that despite the fact that the neuroleptic drugs behaved dissimilarly in the in vivo situation compared with in vitro experiments, the correlation between altered *Dhcr7* levels and HH/Gli signaling are similar.

Discussion

Hedgehog signaling activity is of crucial importance for several key steps in embryogenesis, and widespread HH activity can be detected during the development of numerous organs. In the adult organism, HH activity is associated with tissue maintenance and stem cell control and is therefore spatially restricted. Erroneous HH pathway activity in the adult organism has been implicated in the cause of several cancer types, such as basal cell carcinoma of the skin, cerebellar medulloblastoma, glioblastoma multiforme, and in tumors of the gastrointestinal tract (Rubin and de Sauvage,

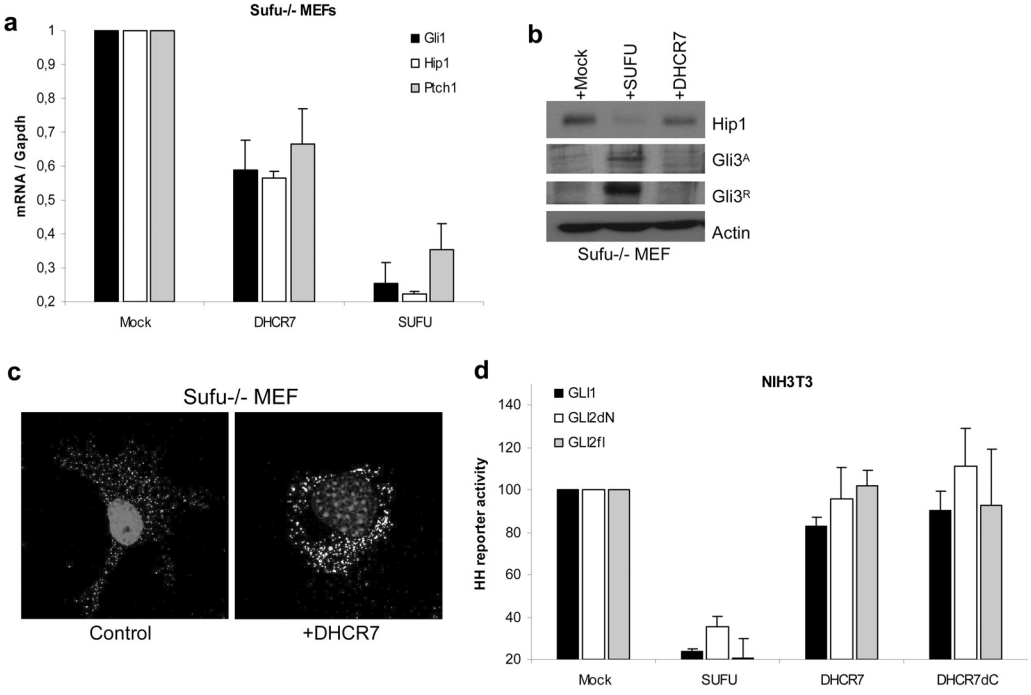

Fig. 4. DHCR7 possesses Smoothened-independent inhibitory properties. a, QPCR of HH pathway target genes in *Sufu*(−/−) cells stably transfected with the indicated constructs. SUFU was transfected as positive control ($n = 3 \pm$ S.D.). b, Western blot showing Hip1, Gli3 activator ($Gli3^A$), and Gli3 repressor ($Gli3^R$) levels in *Sufu*(−/−) cells stably expressing either DHCR7 or SUFU. Note that Gli3 becomes only visible after ectopic expression of SUFU. c, confocal fluorescence microscopy of *Sufu*(−/−) cells stably expressing V5-tagged DHCR7 (pseudocolored in white). Control cells are nontransfected. No DHCR7 localization could be seen in the nuclei (which appear solid light gray at left and punctate light gray at right). d, luciferase assay of NIH3T3 transfected with different GLI and DHCR7 constructs. SUFU was included as positive control. GLI2dn, GLI2ΔN; GLI2fl, full-length GLI2; $n = 3 \pm$ S.D.

2006). We identified previously GANT61, a small-molecule inhibitor of GLI1 and GLI2, which are the final effectors of the HH pathway and are overactivated in many tumors (Lauth et al., 2007a). Here, we identify the hexahydropyrimidine part of the molecule as the bioactive substructure of GANT61. A theoretically proposed hydrolysis reaction toward a diamine product could experimentally not be found, but the corresponding diamines were biologically as effective as the parent compound. Furthermore, the Dhcr7 inhibitor AY9944 bears a certain degree of structural resemblance to the GANT61 diamine. However, despite the fact that both compounds could block HH signaling when initiated at the level of Smo or Sufu, AY9944 was a poor inhibitor in situations of GLI overexpression. Although we interpret this result in a way that these two compounds have dissimilar mechanisms of action, we cannot rule out a possible effect of AY9944 on low levels of endogenous Gli.

AY9944 is a specific inhibitor of Dhcr7, the enzyme catalyzing the last step in the biosynthesis of cholesterol (Supplemental Fig. 4). Inhibition of cholesterol biosynthesis results in decreasing cholesterol levels, which are sensed by the cell via a sterol-sensing mechanism involving the SCAP/SREBP proteins, which are residing in the endoplasmic reticulum. Upon activation, SCAP/SREBP proteins are translocated to the Golgi compartment, in which SREBP is proteolytically cleaved and enters the nucleus to induce the transcription of lipogenic genes. Because one of these genes is *Dhcr7*, which itself has been shown to act as an antagonist of *X. laevis* HH signaling, it was unclear whether the inhibition observed with AY9944 is a result of the properties of the compound or the secondary up-regulation of *Dhcr7*. In support of the latter, we could show that overexpression of DHCR7 attenuates HH signaling in SAG-induced NIH3T3 and in *Sufu*(−/−) cells. It is noteworthy that *Dhcr7* levels are reduced in HH-induced mouse medulloblastoma cells, which is paralleled by an increase in *Gli1* levels, suggesting that the Dhcr7-Gli1 axis might even play a role in tumorigenesis.

It is noteworthy that the catalytic function of DHCR7 was not required for HH pathway inhibition, which is in agreement with data from *X. laevis* (Koide et al., 2006). Moreover, vitamin D_3 (derived from DHCR7's substrate 7-DHC) did not block signaling in *Sufu*(−/−) cells, and oxysterols (derived

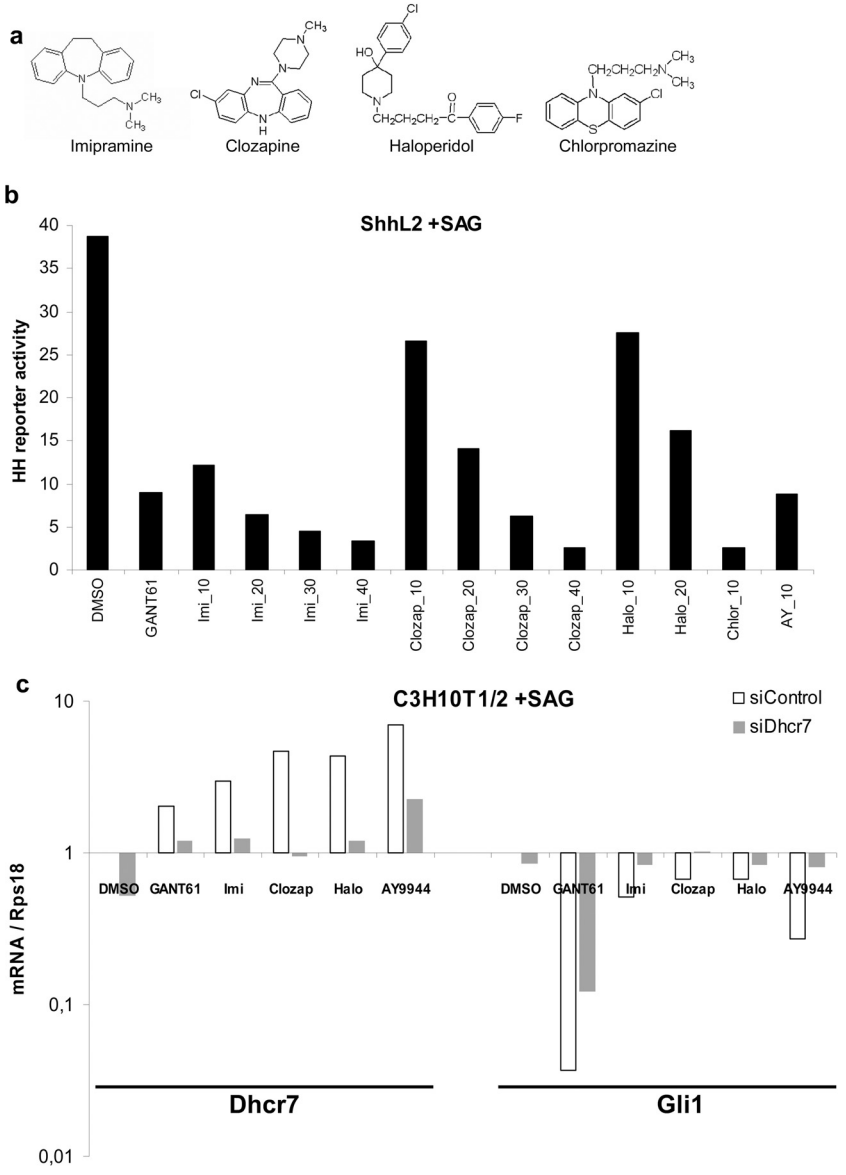

Fig. 5. Antipsychotics affecting DHCR7 levels modulate HH signaling. a, chemical structures of the antipsychotics clozapine, haloperidol, chlorpromazine, and the antidepressant imipramine. Note the structural dissimilarity of the compounds. b, HH reporter assay in ShhL2 cells treated with the indicated compounds. Shown is the fold induction of luciferase activity compared with noninduced cells. The concentration of the respective compound is given after the underscore (e.g., Imi_10 = imipramine 10 μM); Clozap, clozapine; Halo, haloperidol; Chlor, chlorpromazine; AY, AY9944. GANT61 (20 μM) was included as positive control. Chlorpromazine showed some toxicity and could not be used at higher doses. Shown is one representative experiment of three. c, rescue experiment in SAG-treated C3H10T1/2 cells. Cells were transfected with siRNA and subsequently treated with SAG and the indicated compounds for 48 h before *Gli1* and *Dhcr7* levels were measured by QPCR. Concentrations used were the following: GANT61, 20 μM; imipramine, 30 μM; clozapine, 40 μM; haloperidol, 20 μM; AY9944, 10 μM. Shown is one representative experiment of three. The DMSO-treated siControl sample was set to 1.

from DHCR7's product cholesterol) did not induce signaling in *Smo*(−/−) MEFs, underscoring the Smo-specificity of DHCR7's enzymatic substrates (7-DHC and thus vitamin D_3) and products (oxysterols) (data not shown). This suggests that in mammals, as in *X. laevis*, the inhibitory function of DHCR7 is independent of its enzymatic activity and is integrated most likely downstream of Smo. In contrast, the effects of its catalytic function (vitamin D_3/oxysterols) are acting on the level of Smo.

It is interesting to note that DHCR7 contains a sterol-sensing domain (SSD) and that another SSD-containing protein, PTCH, is able to inhibit HH signaling downstream of SMO (Rahnama et al., 2006). This raises the possibility that, although not required in *X. laevis* (Koide et al., 2006), in mammals, the SSD of DHCR7 might be mediating some aspects of the HH inhibition (Fig. 3c). However, future research is needed to address the exact mechanism of inhibition by DHCR7 and how its effects are linked to other interactions between the Hedgehog and the cholesterol biosynthesis pathways (Supplemental Fig. 4).

Antipsychotic drugs, which are used for treating schizophrenia and related conditions, are considered to act mainly as dopamine D_2 antagonists. Both "typical" neuroleptics, including haloperidol and chlorpromazine, and "atypical" neuroleptics, including clozapine, have high affinities for dopamine D_2 receptors. Clozapine has also a high affinity for

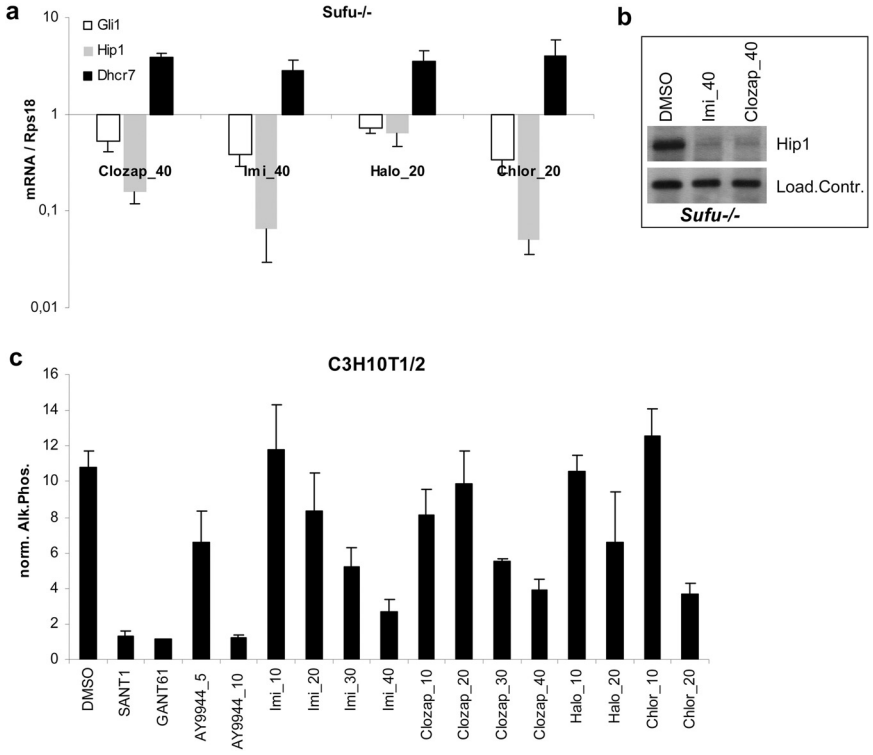

Fig. 6. Neuroleptics affect physiological HH pathway responses. a, QPCR of *Sufu*(−/−) cells treated for 48 h with the indicated compounds. Note that all compounds led to an induction of *Dhcr7* expression. The DMSO-treated sample was set to 1; $n = 3 \pm$ S.D. b, immunoblot showing the reduction of Hip1 protein in *Sufu*(−/−) MEFs after 48-h exposure to 40 μM imipramine or 40 μM clozapine. An unspecific band serves as loading control. c, C3H10T1/2 differentiation assay. Depicted is the fold induction of alkaline phosphatase activity (AP; normalized against total protein amount) upon SAG treatment relative to uninduced samples. Shown is one representative experiment (performed in triplicate ± S.D.) of three independent experiments. The micromolar concentration is given for each compound (e.g., Imi_10 = imipramine 10 μM); Clozap, clozapine; Halo, haloperidol; Chlor, chlorpromazine. GANT61 (20 μM) and SANT1 (0.2 μM) were included as positive controls.

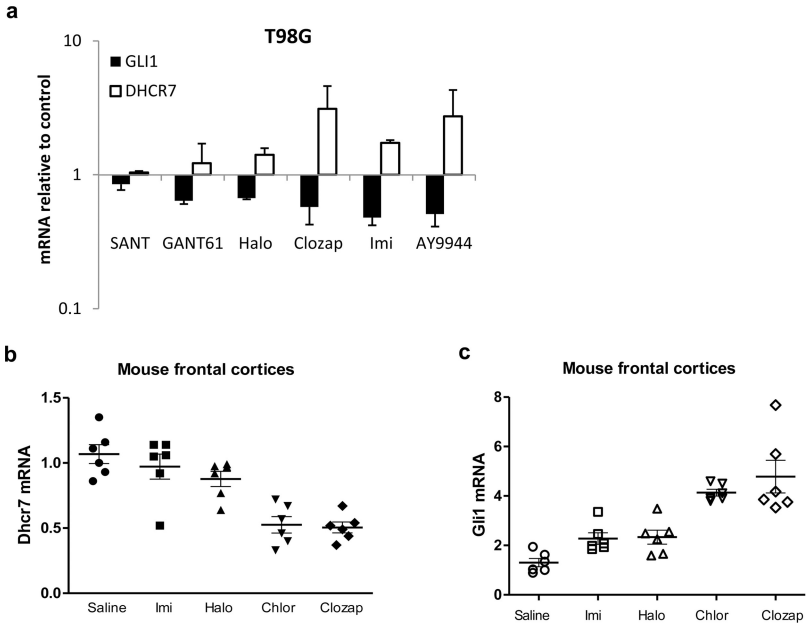

Fig. 7. Regulation of HH signaling by antipsychotics/antidepressants in neuronal cells and tissues. a, QPCR of T98G glioma cells treated for 48 h with the indicated compounds: SANT (0.2 μM); GANT61 (10 μM); haloperidol (10 μM); clozapine (40 μM); imipramine (40 μM); AY9944 (5 μM); $n = 3 \pm$ S.D. b, *Dhcr7*-QPCR of mouse brain (frontal cortex; $n = 6$) treated with the indicated compounds (for concentrations, see *Materials and Methods*). The black bar indicates the mean ± S.E.M. c, *Gli1*-QPCR of mouse brain (frontal cortex; $n = 6$) treated with the indicated compounds (for concentrations, see *Materials and Methods*). The black bar indicates the mean ± S.E.M.

several different serotonin receptors. The antidepressant imipramine targets preferentially serotonin (5-HT) and norepinephrine transporters and increases the synaptic availability of these monoamines by inhibiting their reuptake. The fact that these compound classes have distinct preferences for neuronal membrane proteins (which are not expressed in fibroblasts) makes a common receptor-based mechanism of action with regard to HH signaling unlikely. Furthermore, the described substances are structurally diverse, supporting the concept of action via an indirect effect on the HH pathway through up-regulation of *Dhcr7*. Indeed, we could show that blocking *Dhcr7* induction abolishes the negative effect of these drugs on the HH pathway. The transcriptional induction of lipogenic genes was also reported for other tricyclic antidepressants such as amitriptyline and clomipramine, raising the possibility that the HH effects described here are applicable to a larger group of clinical neuropharmaceuticals (Raeder et al., 2006).

Our finding that drugs used to treat schizophrenia or depression modulate HH signaling in numerous cell lines and in the brain raises the possibility that alterations in HH signaling might occur in the etiology of the disease or its therapy. Most of the psychotropic drugs are highly lipophilic, leading to accumulation in fatty tissues. As a consequence, 10 to 30 times higher concentrations of haloperidol and clozapine have been documented in the central nervous system compared with serum concentrations (Baselt and Cravey, 1995; Weigmann et al., 1999; Kornhuber et al., 2006). In liver tissue, concentrations of clozapine were shown to reach up to 30 μM (Baselt and Cravey, 1995), similar to what we have used for in vitro testing. Hence, the effect on HH signaling might as well be part of the spectrum of non-neuronal side effects of neuroleptics, including the metabolic syndrome.

In our in vivo experiment using intermediate concentrations of the drugs, *Dhcr7* was unexpectedly induced upon treatment with antipsychotics/antidepressants. It is currently unclear why the outcome was different from the in vitro situation, but it might involve aspects of different cell types, drug doses, altered cholesterol metabolism, or time points of analyses. With regard to the latter, it was shown that a single administration of clozapine led to an initial increase followed by a down-regulation of SREBP target genes in the liver (Fernø et al., 2009). Hence, the difference between our in vitro and in vivo data could result from the chosen time point at which we harvested the tissue. It will be interesting to see how the DHCR7/GLI levels are modulated in patients receiving long-term treatment with antidepressants/antipsychotics. Correlating to the in vivo down-regulation of *Dhcr7* in our experiment, HH signaling (*Gli1*) was up-regulated, supporting our finding of an inverse correlation of *Dhcr7* and *Gli1* levels.

With respect to schizophrenia, the underlying reasons are not fully elucidated, but one hypothesis is that the differentiation process of neural stem cells into more restricted descendants is altered, affecting the wider neural network in which these neurons integrate (Kalkman, 2009). It is noteworthy that SHH has been implicated in neural stem cell proliferation and in differentiation of several types of neurons, such as dopaminergic and serotonergic neurons (Stecca and Ruiz i Altaba, 2005; Dellovade et al., 2006). In addition, HH acts as a neuronal guidance cue in the developing nervous system (Charron and Tessier-Lavigne, 2005). Taken together, there is reason to suggest that hitherto unrecognized alterations in HH pathway activity may occur during the etiology and/or the current therapy of psychiatric disorders.

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