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Piperazic acid derivatives inhibit Gli1 in Hedgehog signaling pathway

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ABSTRACT

Piperazic acid, a non-proteinogenic amino acid, found in complex secondary metabolites and peptide natural substances, has shown down regulation of Gli1 expression in Hedgehog signaling pathway in cell based assays. Further structure activity relationship study indicated that amide derivatives of piperazic acid are more potent than piperazic acid itself, with little to no toxicity. However, other cellular components involved in the pathway were not affected. To the best of our knowledge, this is the first report on the inhibitory property of piperazic acid in this pathway. Hence, this molecule could serve as a useful tool for studying Hedgehog signaling.

Hedgehog (Hh) signaling plays a crucial role in orchestrating key steps involved in embryogenesis, adult tissue homeostasis and stem cell differentiation.[1] Hh ligand binds to its 12-pass transmembrane receptor, Patched1 (Ptch1), relieving its inhibitory effect on 7-pass transmembrane protein Smoothened (Smo). This de-repression eventually leads to the activation of Gli family of transcription factors, which regulates the transcription of Hh target genes including *Ptch1* and *Gli1*. Hh pathway has become the focus of intense study as its uncontrolled activation is implicated in the initiation and maintenance of various malignancies like basal cell carcinoma (BCC) and medulloblastoma and progression of pancreatic adenocarcinoma, prostate cancer and gastrointentestinal tumors.[2,3] Hence, Hh pathway inhibition has emerged as an attractive strategy in anticancer therapy. Since the discovery of cyclopamine, the first known inhibitor of hedgehog signaling pathway, by Beachy et al.[4,5], several small molecule modulators of this pathway have been reported,[6–11] which include hedgehog antagonists (Robotnikinin, 5E1)[12] and Gli transcriptional activity inhibitors (GANT58, GANT61,[13] JK184[14,15] and HPI4[16]). Recently, Waldmann et al. have reported Smo inhibitor derived from withanolides based natural product.[17] Among Smo inhibitors, GDC-0449 (Vismodegib) has been recently approved by the Food and Drug Administration (FDA) for the treatment of BCC, providing the first evidence of therapeutic benefit resulting from Hh signaling inhibition.[18]

Piperazic acids (hexahydropyridazine-3-carboxylic acid) are non-proteinogenic, cyclic α-hydrazino acids known to show remarkable biological activities and detected in various peptide based natural substances and secondary metabolites.[19] They were discovered by Hassall and coworkers as a component of monamycins (a group of cyclodepsipeptide natural products). Compounds containing this moiety are known to inhibit progression of the cancer cell cycle from G1 to S phase.[20,21] To the best of our knowledge, no reports have shown the effect of piperazic acid itself on cell cycle. As hedgehog antagonist cyclopamine is reported to arrest cell cycle at G0/G1 phase[22] and our group is focused on searching potent small molecule inhibitors of Hh signaling, we became interested to see the effect of piperazic acid along with its analogues on hedgehog pathway and cell cycle progression. Six-(**1**, **2**) and seven-membered (**3**, **4**) hydrazino acids were synthesized[23] and screened for Hh pathway inhibition. Interestingly, piperazic acid **1** (Fig. 1) showed downregulation of Gli-dependent signaling although its flow cytometry analysis in mouse fibroblasts did not show any significant effect on cell cycle (Fig. S1). In this

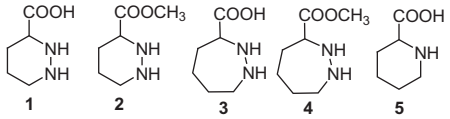

Figure 1. Structures of piperazic acid, ester and its 7-membered ring analogue; pipecolic acid.

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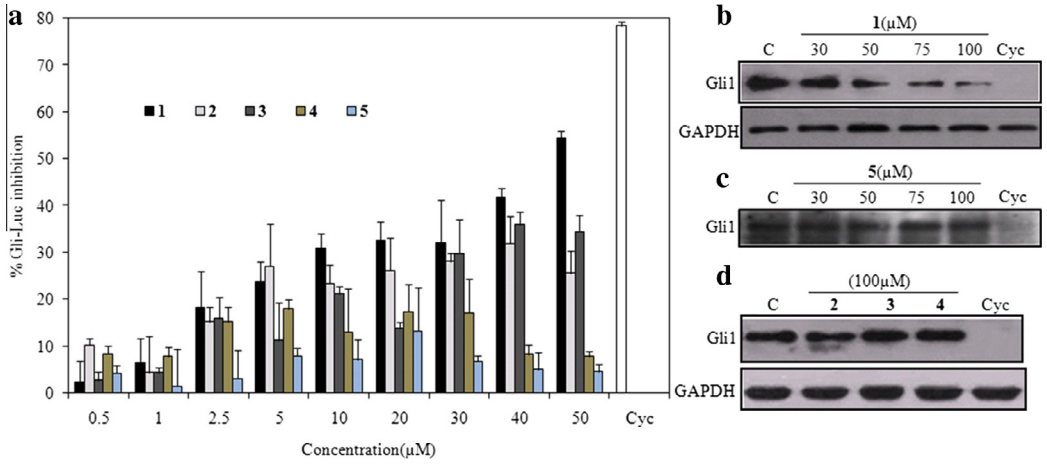


Figure 2. Screening of piperazic acid and its analogues for Hh signaling inhibition. (a) Luminescence plots showing percentage inhibition of Gli-dependent luciferase activity in Shh-LIGHT2 cells post 30 h treatment with indicated concentrations of **1**, **2**, **3**, **4** and **5**. Gli1 protein levels treated with varying doses of (b) **1**, (c) **5** and (d) 100 μM of **2**, **3** and **4**. Control set (C) denotes Shh-N stimulated cells treated with DMSO only. GAPDH is used as loading control. Data represents an average of three independent experiments performed in triplicate, with error bars denoting standard deviation (SD). 10 μM cyclopamine (Cyc) is used as a positive control.

Letter, we report the synthesis of piperazic acid derivatives and their ability to inhibit Gli-dependent luciferase activity and evaluation of their effects on Gli1 expression and other pathway components using cell-based assays. All the compounds used for biological assays were racemic mixtures.

Initially, compounds **1**–**5** were screened to evaluate their ability to inhibit hedgehog signaling using cell based luciferase assay (Fig. 2). Shh-LIGHT2 cells[24] (a clonal NIH-3T3 cell line stably transfected with a Gli-dependent firefly luciferase and constitutive *Renilla* luciferase reporters) were treated with varying compound concentrations for 30 h in the presence of Shh-N [N-terminal fragment of Sonic hedgehog (Shh) without cholesterol modification]-conditioned media. Interestingly, **1** showed upto 50% inhibition of Gli-dependent firefly luciferase activity at 50 μM dose whereas its methyl ester **2**, azepane acid **3** and its corresponding methyl ester **4** did not show significant luciferase inhibition. Next, to see whether 1-nitrogen of **1** was crucial for the compound's Hh pathway modulating activity, commercially available DL-pipecolic acid **5** (lacking 1-nitrogen) was chosen. **5**, on the other hand, did not show down regulation of firefly luciferase activity upto 50 μM. Cyclopamine, a known Smo antagonist, was used as positive control at a concentration of 10 μM. To validate the above results, immunoblot analysis of Gli1 levels was performed with various compound doses in Shh-LIGHT2 cell line. Results revealed that **1** down regulated Gli1 expression in a dose-dependent manner although **5** showed no effect on Gli1. These studies indicated an essential role of 1-nitrogen in the suppression of firefly luciferase expression and Gli1 levels by **1**. Compounds **2**, **3** and **4** did not perturb Gli1 levels even at 100 μM. Also, protein expression of other pathway components (SHH, SuFu, Ptch1 and Smo) was unaltered

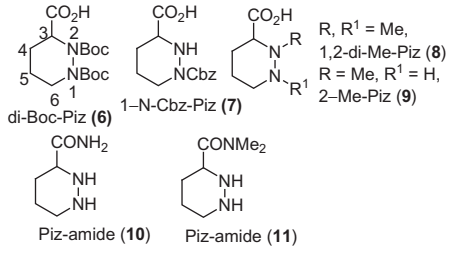
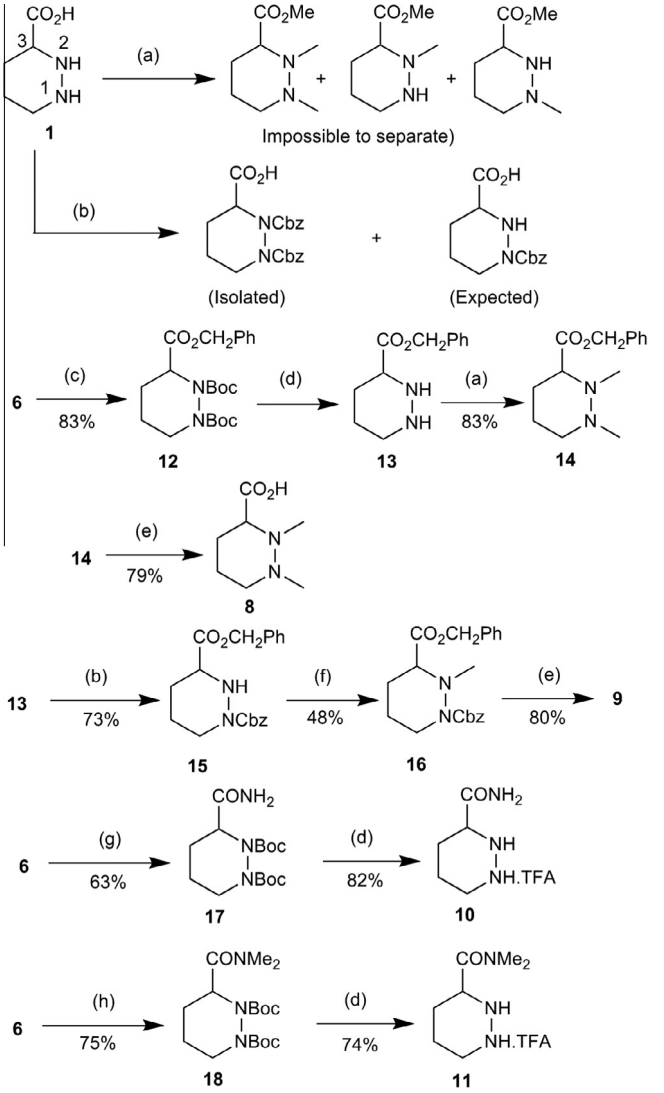


Figure 3. Derivatives of piperazic acid, modifications made either at 1/2-positions or at the carboxylic acid.



Scheme 1. Synthesis of piperazic acid derivatives. (a) K_2CO_3 (3 equiv), MeI (3 equiv), dry DMF, 12 h; (b) Cbz-Cl, Et_3N, DCM, 30 min; (c) $PhCH_2Br$ (1.2 equiv), K_2CO_3 (2.5 equiv), dry DMF, 12 h; (d) 20% TFA in DCM, 4 h; (e) 10% Pd/C, H_2 (1 atm), MeOH, 5 h; (f) K_2CO_3 (1.2 equiv), MeI (1.5 equiv), dry DMF, 12 h; (g) Dry CH_3CN, Et_3N, HBTU, NH_4Cl, rt, 6 h; (h) Dry DMF, Et_3N, HBTU, Me_2NH, THF, 12 h.

by **1** (Fig. S2). Further, cell viability tests were performed using MTT assay in Shh-LIGHT2 cells. No sign of cytotoxicity was observed post 40 h treatment with doses upto 150 μM under conditions used for Hh reporter assays (Fig. S3).

Thus, we decided to prepare the compounds di-Boc-Piz (**6**), δ-N-Cbz-Piz (**7**), di-Me-Piz (**8**), δ-N-Me-Piz (**9**) focusing our modifications at the α/β-nitrogen positions or compounds Piz-amide (**10**) and Piz-amide (**11**) at the carboxylic group (Fig. 3). These compounds were synthesized according to Scheme 1 and screened using luciferase assay as mentioned previously. Compounds **6** and **7** were synthesized following our previously reported procedures. Our initial attempt to synthesis di-Me-Piz (**8**) using MeI in the presence of K_2CO_3 in dry DMF gave a mixture of *N*-methylated esters which were impossible to separate by column chromatography. Our next attempt was to selectively protect the 1-N with Cbz, followed by methylation using HCHO/$NaBH_4$ method at the 2-position, however, selective Cbz protection of piperazic acid (**1**) was not achieved. Instead, we isolated di-Cbz-protected piperazic acid.

Finally we started with benzyl ester of piperazic acid **13** which was prepared from **6**. After methylation by MeI/K_2CO_3, it gave di-Me-Piz-OBz **14**. Hydrogenolysis of **14** by 10% Pd/C gave the desired dimethylated compound **8**. For the preparation of 2-methylpiperazine-3-carboxylic acid, mono Cbz-protected benzyl ester **15** was used as a starting material. Unlike free piperazic acid **1**, benzyl ester **13** underwent mono Cbz protection at the 1-position selectively to give compound **15**. After treatment with MeI/K_2CO_3, 2-methylated product **16** was obtained in the protected forms which after hydrogenolysis gave the desired compound **9**. None of the analogues **6**, **7**, **8** and **9** showed inhibitory activity in luciferase assay (Fig. S4).

We investigated the effect of amides **10** and **11** on hedgehog signaling which were then synthesized from **6** by the activation of carboxylic acid **6** using HBTU followed by the treatment with amine (Scheme 1). Interestingly, amide (**11**) exhibited higher activity than **1** and the other derivatives as seen from the luciferase assay, where it inhibited Gli-mediated transcription with an IC_{50} = 10 μM. Amide (**10**) also showed suppression of firefly expression (IC_{50} = 30 μM). Hence, we found that amide (**11**) is the best among the tested derivatives (Fig. 4a). The derivatives were nontoxic up to 150 μM when their cytotoxicity was evaluated using MTT assay in Shh-LIGHT2 cells (Fig. S3). Western blot analysis in the presence of the derivatives showed that amide (**10**) and amide (**11**) reduced Gli1 protein levels with amide (**11**) depicting better potency (Fig. 4b).

To understand the mechanism of action of **10** and **11** and examine the possibility of their interaction with Smo, luciferase assay was performed in the presence of varying concentrations of the compounds, where pathway stimulation was carried out using 500 nM SAG (a known agonist of the pathway which acts by binding to Smo).[5,24] Both compounds significantly suppressed Gli transcriptional activity even in the presence of SAG. This was ascertained by western blot analysis of Gli1 expression in presence and absence of compounds upon pathway activation by SAG. Both compounds inhibited Gli1 with **11** showing comparatively better inhibition (Fig. 5a and b). This indicated that the compounds act either independent of Smo binding or bind to a site other than the Smo heptahelical bundle (where SAG is known to bind) although protein levels of Smo remained unaffected (Fig. S2). Further, protein expression of other components of the pathway (SHH and SuFu) was unaffected post 24 h treatment with amide (**10**) and amide (**11**) when analyzed by western blotting (Fig. S2).

Next, their activity was evaluated in *Ptch1*$^{-/-}$ cells derived from mouse embryos where Ptch1 is functionally absent[25] and hence, hedgehog signaling is constitutively active. Pathway activation in this cell line is indicated by β-galactosidase activity as its expression is under control of the Ptch1 promoter and Ptch1 itself is a transcriptional target of Hh signaling. No significant suppression

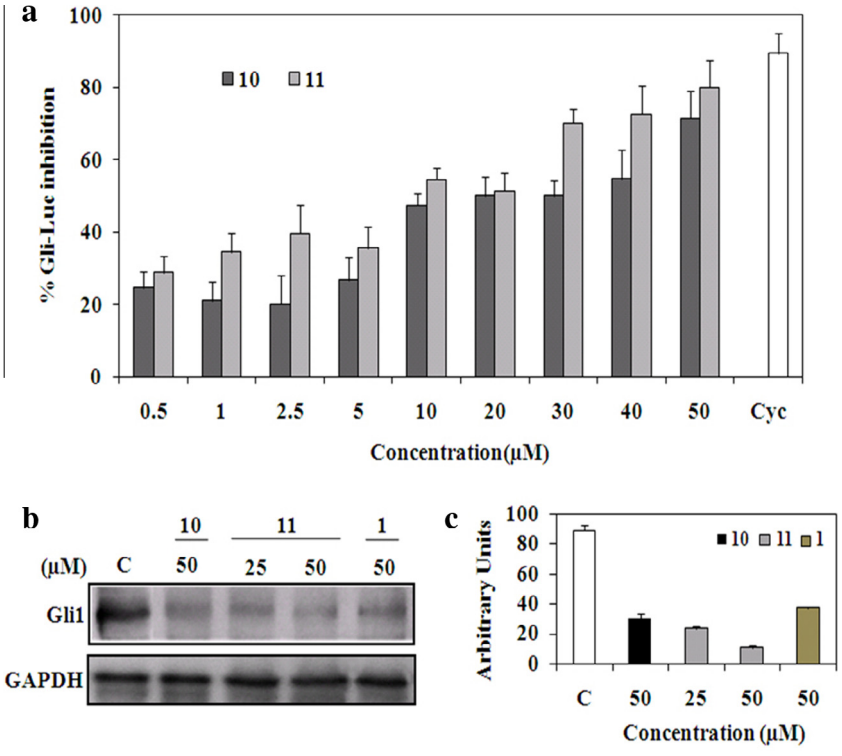


Figure 4. (a) Plot showing percentage inhibition of luciferase expression in presence of **10** and **11**. (b) Downregulation of Gli1 protein post 24 h treatment with **10**: 50 μM; **11**: 25, 50 μM; **1**: 50 μM. 10 μM cyclopamine (Cyc) is used as a positive control. (c) Plot showing quantification of Gli1 bands obtained in (b) using ImageJ software. Control set (C) denotes Shh-N stimulated cells treated with DMSO only. GAPDH is used as loading control. Data represents an average of three independent experiments performed in triplicate, with the error bars denoting standard deviation (SD).

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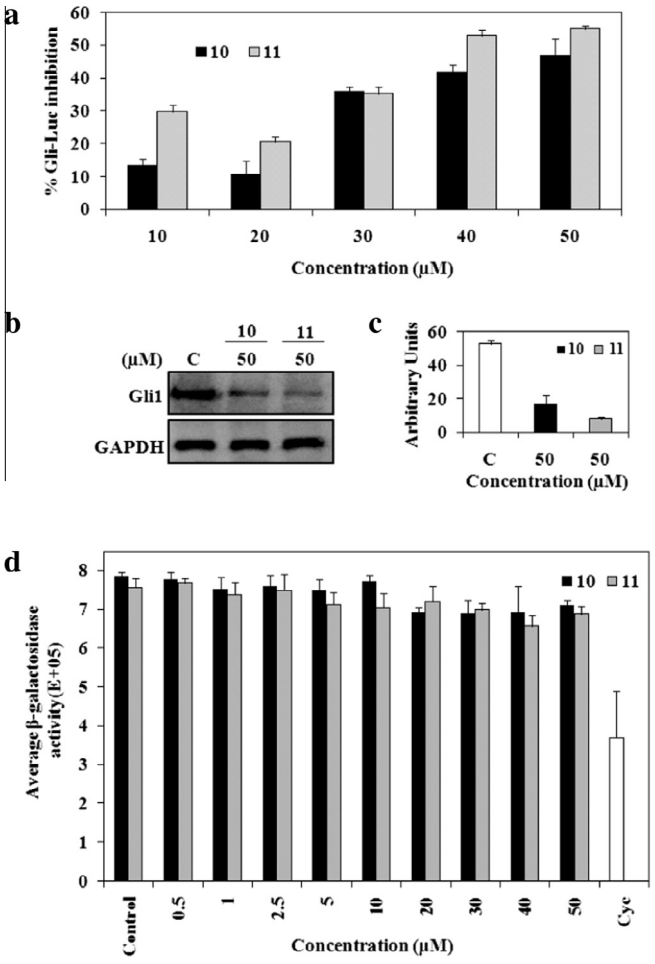

Figure 5. (a) Percentage inhibition of Gli-dependent firefly luciferase reporter gene activity by **10** and **11** when pathway is induced with 500 nM SAG. (b) Immunoblot showing Gli1 downregulation post compound treatment in presence of 500 nM SAG. (c) Plot showing quantification of Gli1 bands obtained in (b) using ImageJ software. Control set denotes SAG-stimulated cells treated with DMSO only. (d) Average β-galactosidase expression in *Ptch1*$^{-/-}$ cells post 40 h treatment with **10** and **11**. 10 μM cyclopamine (Cyc) is used as a positive control.

of β-galactosidase expression was observed post treatment with varying doses of compounds for 40 h (Fig. 5d).

Also, protein expression of Ptch1 was unaltered by the compounds as seen in Figure S2. These results suggested that the plausible target of compound action is likely to be a pathway component either upstream of Ptch1 or the receptor itself.

In conclusion we have shown that piperazic acid and its amide derivatives inhibit Gli1 expression in Hh signaling in a dose-dependent manner. They exhibit no inhibitory activity in *Ptch1*$^{-/-}$ cell line and are not functionally competitive with Smo agonist SAG. These results indicate that the compounds are probably working either in a Ptch1 dependent manner or targeting a component upstream of it. Robotnikinin[12] and RU-SKI 43[26] have been reported to work upstream of Ptch1 to inhibit Hh-pathway in which Robotnikinin is a Hh antagonist known to bind extracellular Sonic Hedgehog (Shh) protein and prevent its binding to Ptch1. It shows no pathway inhibition in *Ptch1*$^{-/-}$ but all of the inhibitory effect of Robotnikinin was eliminated when SAG was co-administered in Shh-LIGHT2 cells. RU-SKI 43 targets production of mature signaling competent Hh ligand by inhibiting Hhat (Hedgehog acyltransferase, required for N-terminal palmitoylation of Shh) and thereby blocking signaling. RU-SKI 43 shows no effect on the ability of SAG or a recombinant, hydrophobic variant of Shh to activate Gli1 signaling in Shh-LIGHT2. As Smo functions downstream of Shh/Ptch1, our compounds still show inhibition in the presence of Smo-agonist SAG and both, in the presence and absence of ShhN ligand. Hence, **10** and **11** appear to be mechanistically distinct from Robotnikinin and RU-SKI 43. As the compounds show activity in the presence of SAG, a possibility could be that SAG is unable to override their inhibitory effect or the repression of Hh signaling by **10** and **11** is non-selective. Extensive studies are being carried out in our laboratory to identify their specific target and further characterize the mechanism of inhibition. These initial findings represent our early effort in identifying piperazic acid-mediated Gli1 repression which could prove to be useful as a valuable probe of the pathway and diseases associated with its aberrant activity.

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Supplementary data

Supplementary data (detailed experimental methods, spectral data of the new compounds and biological assay procedures of cell culture and supporting figures of biological assays) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.008.

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