Bioorganic & Medicinal Chemistry Letters 26 (2016) 4423–4426

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# Piperazic acid derivatives inhibit Gli1 in Hedgehog signaling pathway

Harleen Khatra, Jayanta Kundu, Pragya Paramita Khan, Indranil Duttagupta [†], Sankha Pattanayak [‡], Surajit Sinha [*]

*Department of Organic Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700 032, India*

ARTICLE INFO

*Article history:*
Received 3 June 2016
Revised 1 August 2016
Accepted 3 August 2016
Available online 4 August 2016

*Keywords:*
Piperazic acid
Hedgehog signaling
Hedgehog-inhibitors
Gli
Shh-LIGHT2 cells

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

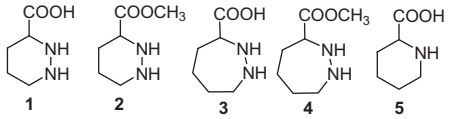



**Figure 1.** Structures of piperazic acid, ester and its 7-membered ring analogue; pipecolic acid.

* Corresponding author. Tel.: +91 33 2473 4971; fax: +91 33 2473 2805.
*E-mail address:* ocss5@iacs.res.in (S. Sinha).
† Present address: Institute of Radical Chemistry, Aix-Marseille University, Marseille Cedex 20, France.
‡ Present address: Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, California, CA 94305, USA.

http://dx.doi.org/10.1016/j.bmcl.2016.08.008
0960-894X/

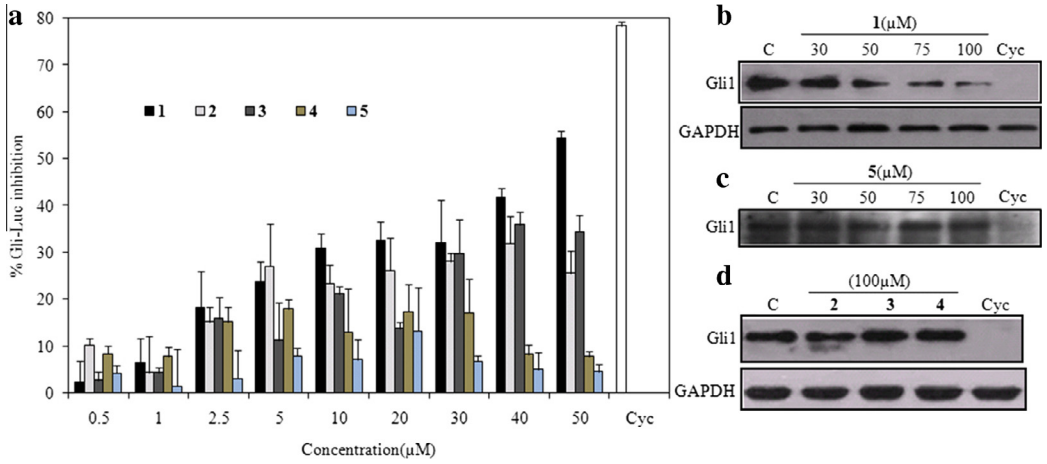



**Figure 2.** Screening of piperazic acid and its analogues for Hh signaling inhibition. (a) Luminescence plots showing percentage inhibition of Gli-dependent luciferase activity in Shh-LIGHT2 cells post 30 h treatment with indicated concentrations of **1**, **2**, **3**, **4** and **5**. Gli1 protein 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

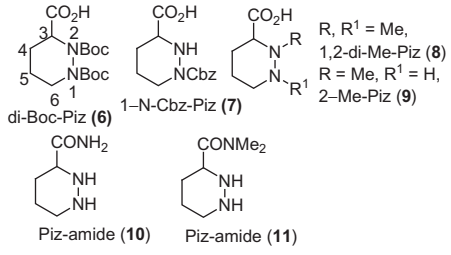
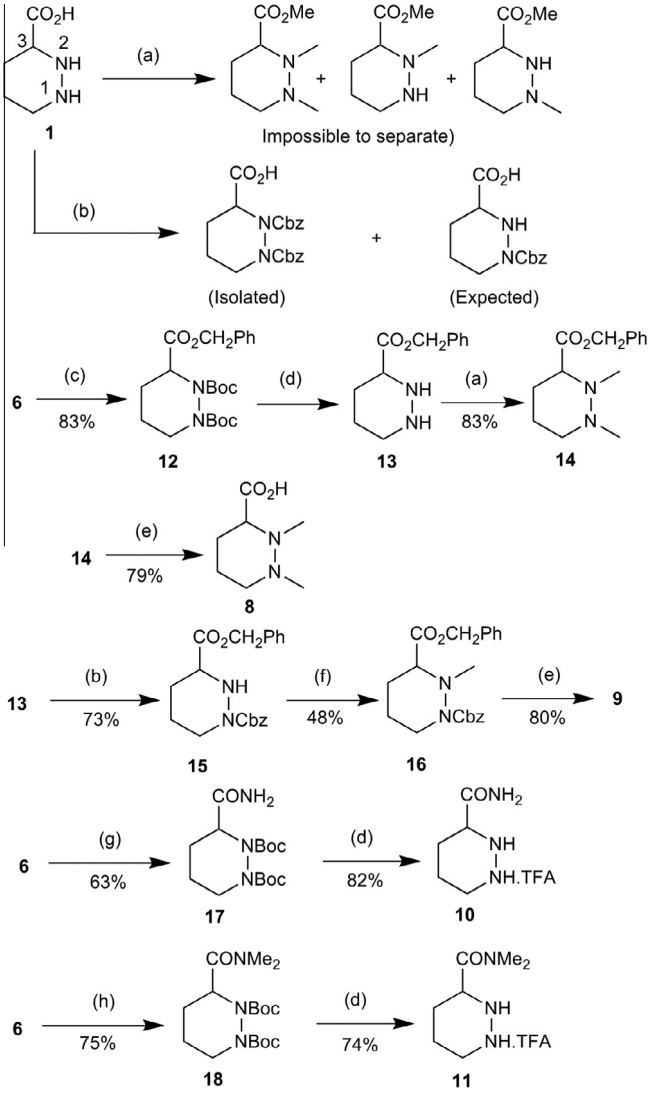



**Figure 3.** Derivatives of piperazic acid, modifications made either at 1/2-positions or at the carboxylic acid.




**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.