Bioorganic & Medicinal Chemistry Letters 20 (2010) 4932–4935

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Smoothened antagonists for hair inhibition

Jie Jack Li [a,*], Veerabahu Shanmugasundaram [a], Satya Reddy [b], Laura L. Fleischer [b], Zenquan Wang [b], Yvonne Smith [a], William G. Harter [a], Wen-Song Yue [a], Manju Swaroop [b], Ling Li [b], Christy Xiaodong Ji [b], Danielle Dettling [b], Bella Osak [c], Laura R. Fitzgerald [c], Robert Conradi [d,e]

[a] Department of Chemistry, Michigan Laboratories, Pfizer Global Research & Development, 2800 Plymouth Rd., Ann Arbor, MI 48105, USA
[b] Department of Dermatology Biology, Michigan Laboratories, Pfizer Global Research & Development, 2800 Plymouth Rd., Ann Arbor, MI 48105, USA
[c] Mechanistic and Target Biology, Michigan Laboratories, Pfizer Global Research & Development, 2800 Plymouth Rd., Ann Arbor, MI 48105, USA
[d] Department of Pharmacokinetics and Drug Metabolism, Michigan Laboratories, Pfizer Global Research & Development, 2800 Plymouth Rd., Ann Arbor, MI 48105, USA
[e] Department of Pharmaceutical Sciences, Michigan Laboratories, Pfizer Global Research & Development, 2800 Plymouth Rd., Ann Arbor, MI 48105, USA

ARTICLE INFO

Article history:
Received 23 April 2010
Revised 4 June 2010
Accepted 7 June 2010
Available online 11 June 2010

Keywords:
Smoothened antagonists
Hair
Photo-irritation
Aminomethylpyrazoles

ABSTRACT

A series of aminomethylpyrazoles were prepared and evaluated using cell-based Smoothened β-lactamase reporter assay and Smoothened binding assay. Potent Smoothened antagonists **10k** and **10l** were found to inhibit hair growth in vivo in the C3H/HeN mouse hair growth model. The more selective compound **10l** was tested negative in the 3T3 NRU assay, indicating a low risk for causing photo-irritation and was efficacious using the C3H/HeN mouse hair growth model although it was slightly less efficacious than that of the reference compound eflornithine (**7**).

Largely thanks to the pioneering work of Lewis,[1] Nüsslein-Volhard and Wieschaus[2] three decades ago, genetic experiments in *Drosophila* identified the Hedgehog (Hh) mutant. Mammals have three Hedgehog homologues: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh). The Hh signaling pathway is implicated in many different processes in vertebrate development including cartilage differentiation, myotome and sclerotome specification, *hair follicle development*, limb morphogenesis and the specification of different neuronal cell types.[3] Aberrant activation of the Hedgehog pathway has been identified as the likely cause of a number of tumors in humans such as basal cell carcinomas (BCCs) and primitive neurectodermal tumors (PNETs). It is now clear that multiple components of the Hh pathway can be altered in tumors.[4]

On the other hand, the seven-transmembrane protein Smoothened (Smo) mediates the cellular response to the Hedgehog protein signal and is involved in cell growth and differentiation during embryonic development. Stimulation of the Smo pathway is directly implicated in tissue maintenance and repair, but over-activation of Smo could lead to tumorigenesis. Beachy and co-workers at Johns Hopkins demonstrated that in unstimulated cells, the activity of Smo is repressed by the protein Patched (Ptc), which appears to be the receptor for the Shh ligand.[5] Therefore, blocking the pseudo-GPCR Smo would block the Hh signaling pathway.

Last decade saw great strides in the Smo field. As shown in Figure 1, using the first known Smo antagonist cyclopamine (**1**) as the starting point, Infinity Pharmaceuticals discovered IPI-926 (**2**), a potent and orally active Hh pathway antagonist now in phase I clinical trials in cancer patients.[6] Collaborating with Curis Inc., a pioneer in the Smo field, Genentech discovered Smo antagonist GDC-0449 (**3**).[7] On the basis of positive results in phase I clinical trials, **3** has been recently advanced to phase II trials to treat patients with locally advanced or metastatic solid tumors and BCC. Beachy's group also discovered potent Smo antagonists including SANT-1 (**4**) and SANT-2 (**5**), as well as agonists such as SAG (**6**).[8]

Interestingly, Hh signaling is also closely related to hair cycle regulations.[9–14] Shh-dependent activation of Gli2 is essential for embryonic hair follicle development.[10] Sustained Hh signaling is required for BCC proliferation and survival: conditional skin tumorigenesis recapitulates the hair growth cycle.[13] In knock-out (KO) mice (Shh−/−), hair follicle development was severely impaired in mutant animals.[14] The most compelling evidence emerged when small molecule agonists of the Hh signaling pathway was shown to modulate hair growth.[15]

Currently, the only prescription treatment for unwanted hair, eflornithine (**7**, Vaniqa®, Fig. 2), is only moderately efficacious in a small percentage of subjects.[16] Novel mechanisms with robust

* Corresponding author at present address: Discovery Chemistry, Bristol–Myers Squibb Company, 5 Research Parkway, Wallingford, CT 06492, USA. Tel.: +1 203 677 7225.
E-mail address: jack.li@bms.com (J. J. Li).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.06.033

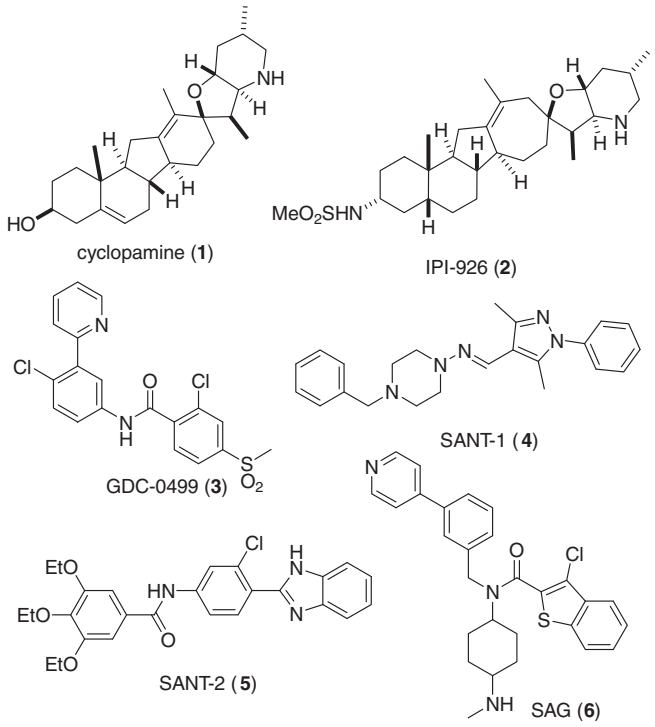


Figure 1. Known Smo antagonists **1**–**5** and Smo agonist **6**.

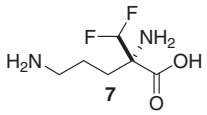


Figure 2. Eflornithine (**7**, Vaniqa®).

efficacy and long-term safety are still needed for this indication. We sought topical Smo antagonists to prevent or slow growth of unwanted hair.

At the outset of the project, the proof of concept (POC) was tested with known Smo antagonists for the treatment of unwanted hair. In the C3H/HeN mouse hair growth model[17] and using 30:70 propylene glycol/ethanol as the vehicle (Fig. 3), 3% of SANT-1 (**4**) showed spectacular efficacy in inhibiting hair growth while SANT-2 (**5**) was not active. In comparison, 13.9% of reference compound **7** showed only moderate efficacy after 14 days.

We at first developed a robust and sensitive functional cell-based assay that measures the activity of endogenous Smo using a β-lactamase transcriptional readout.[18] This is the first Smo reporter assay that utilizes β-lactamase reporter technology. This assay type has distinct advantages over other reporter technologies and can be used in a high-throughput mode to search for therapeutically relevant downstream Smo target effectors. The assay also allows a quick confirmation of the results by visual inspection without any special instrumentation.

With functional cell-based β-lactamase assay as the *primary assay*, a Smo binding assay was also developed as the *secondary assay* to evaluate if the compound binds to the Smo receptor. To that end, a construct of truncated Smo over-expressing cells mammalian expression system was prepared to gauge the compound's influence on the specific interaction between Smo and tritiated-SANT-2 (**5**).[19]

In addition to screening our compound library, an effort was undertaken to 'parachute-in' around known Smo antagonist

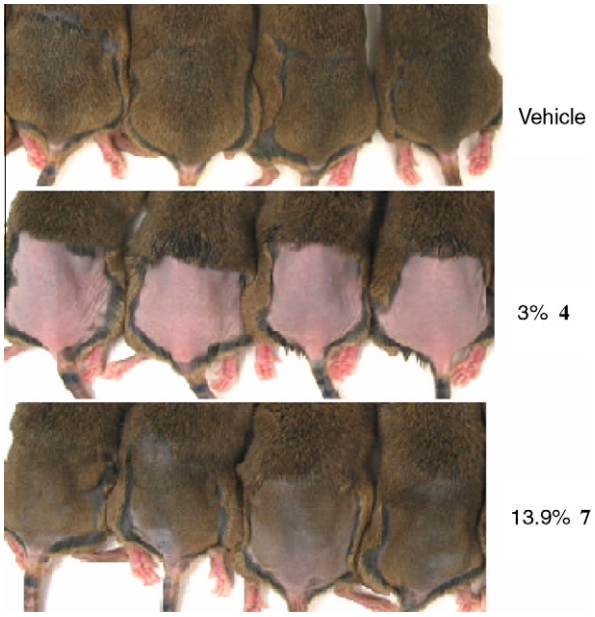
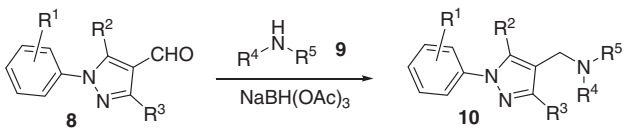


Figure 3. Day 14 for compounds **4** and **7** in the in vivo efficacy tests using the C3H/HeN mouse hair growth model.

SANT-1 (**4**) to develop our own Smo antagonists due to its robust in vivo efficacy in the mouse hair growth model. It was decided to replace the hydrazone functionality on **4** with amine due to the known hepatotoxicity and neurotoxicity associated with hydrazone-containing drugs.[20] Furthermore, the potential hydrazone-associated toxicities could be even more acute and pronounced for dermatological indications when the drug is exposed to sunlight. As shown in the Scheme 1, our Smo antagonists **10** were readily assembled by reductive amination of pyrazole-aldehyde **8** with amine **9**.

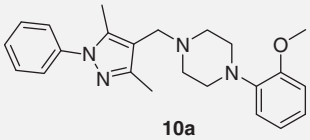
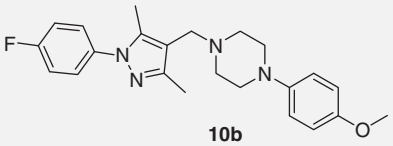
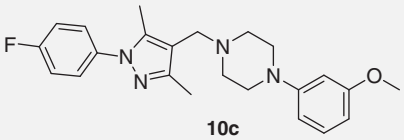
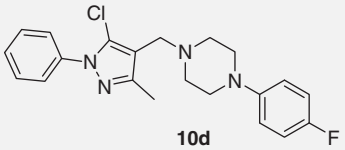
The structure–activity relationship (SAR) of the first-batch derivatives is listed in Table 1. All tri-substituted phenyl-pyrazoles (**10a**–**10e**) have significantly weaker activities in the cell-based assay in comparison to hydrazone **4**. Remarkably, while the *para*-methoxy-phenyl pyrazole **10b** has an IC_{50} of 2000 nM in the Smo binding assay, the corresponding *meta*-methoxy-phenyl pyrazole **10b** has an IC_{50} of 20 nM, a 100-fold increase! This observation suggests that the *meta*-methoxyl-phenyl moiety may represent a favorably tight binding to a particular pocket at the Smo receptor. Chloro-pyrazole **10d** is also very potent in the binding assay with an IC_{50} of 10 nM, but it was not further explored because it was tested positive in the 3T3 neutral red uptake (NRU) assay,[21,22] indicating a high risk for causing photo-irritation.

A breakthrough was made when di-substituted phenyl-pyrazole analogues were prepared in place of the tri-substituted phenyl-pyrazoles. As shown in Table 2, although 3-methyl-1-phenylpyrazoles **10e** and **10f** are inactive in both Smo cell-based assay and Smo binding assay, 3-methyl-1-phenylpyrazoles **10g**–**10l** showed significantly improved potency in both assays. Once again, *meta*-methoxyl analog **10j** is remarkably potent with an IC_{50} of 5 nM in the binding assay and 64.7 nM in the cellular assay. When tested in vivo using the C3H/HeN mouse hair growth model,



Scheme 1. Synthesis via reductive amination.

Table 1
SAR of the tri-substituted phenyl-pyrazole analogues

Compd	SMOTT[a] IC_{50} (nM)	SMOBA[b] IC_{50} (nM)
4	4.74	25
 **10a**	1870	>5000
 **10b**	1690	2000
 **10c**	823	20
 **10d**	1860	10

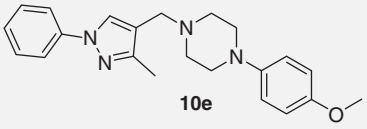
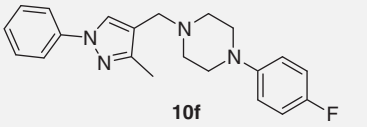
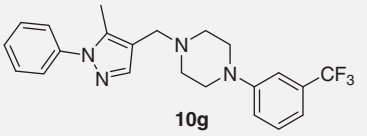
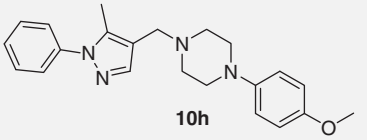
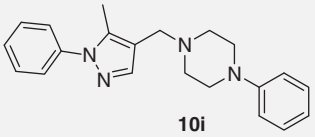
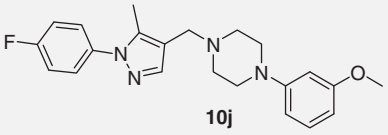
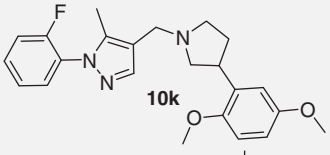
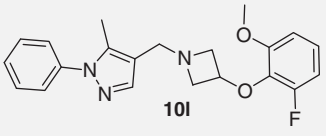
[a] SMOTT, Smoothened transient transfection cell-based assay.
[b] SMOBA, Smoothened binding assay.

it was found to be equipotent to reference compound **7**. Due to perceived toxicological liabilities that anilines are associated with drugs in general and topical drugs in particular (although the nitrogen atom of aniline **10j** here is in a piperazine ring), we sought to eliminate this potential toxicity. We found our answer in analogs **10k** and **10l**, both of which have good potency in cellular and binding assays. They were subsequently tested efficacious to inhibit hair growth in the C3H/HeN mouse hair growth model with a minimal efficacious dose (MED) of 3%.

With regard to selectivity, compound **10k** is less selective than **10l**. Among the GPCRs tested, **10k** is also active against 5-HT_{1A} (K_i = 103 nM), 5-HT_{2B} (K_i = 180 nM), 5-HT_7 (K_i = 15 nM), although it is not active against β_1, β_2, D_1, D_2, 5-HT_{2A} and calcium channel with K_i's all greater than 1 μM. On the other hand, **10l** is active only against α1A (K_i = 76 nM) and D_4 (K_i = 148 nM), but inactive against 5-HT_{1A}, 5-HT_{2A}, D_4, α1B, α2δ and dofetilide with K_i's all greater than 1 μM.

Ideally, topical drugs exert their desired effects locally but are rapidly inactivated via metabolism once they reach the systemic circulation, thereby reducing unwanted systemic effects. Preliminary pharmaco-kinetics data predicts that **10l** will be rapidly metabolized systematically because its half-life ($t_{1/2}$) is 9.7 min in rat liver microsomes (RLM) and 30.2 min in human liver microsomes (HLM). Its cell membrane penetration $Papp_{AB}$ was 11.3×10^{-6} cm/s at 10 μM. Unlike oral drugs, topical drugs are required to penetrate the skin to exert their pharmacological effects. We evaluated skin penetration of **10l** using human cadaver skin mounted onto Franz diffusion chambers.[23] Compound **10l** has high skin penetration with a ratio of 0.52 versus isobutylmethylxanthine (IBMX), a reference compound for skin penetration. It also has a good solubility of 79 μg/mL. Screening **10l** against a panel of CYP enzymes resulted in no significant binding and it had only 56% hERG inhibition at 3 μM concentration, indicating a low risk for QT_C prolongation, and thus cardiac liability. More significantly, **10l** was tested efficacious in vivo using the C3H/HeN mouse hair growth model although it was slightly less efficacious than that of the reference compound eflornithine (**7**).

Table 2
SAR of the di-substituted phenyl-pyrazole analogues

Compd	SMOTT[a] IC_{50} (nM)	SMOBA[b] IC_{50} (nM)
 **10e**	>5000	>5000
 **10f**	>5000	>5000
 **10g**	328	333
 **10h**	105	126
 **10i**	78.4	96
 **10j**	64.7	5
 **10k**	40.8	216
 **10l**	8.27	124

[a] SMOTT, Smoothened transient transfection cell-based assay.
[b] SMOBA, Smoothened binding assay.

In summary, a series of aminomethylpyrazoles were prepared and evaluated using cell-based Smo β-lactamase reporter assay and Smo binding assay. Potent Smo antagonists **10k** and **10l** were found to inhibit hair growth in vivo in the C3H/HeN mouse hair growth model. The more selective compound **10l** was tested negative in the 3T3 NRU assay, indicating a low risk for causing photoirritation. More significantly, **10l** was tested efficacious in the C3H/HeN mouse hair growth model although with slightly lower efficacy than that of the reference compound eflornithine (**7**).

References and notes

1. Lewis, E. B. *Nature* **1978**, *276*, 565.
2. Nüsslein-Volhard, C.; Wieschaus, E. *Nature* **1980**, *287*, 795.
3. For a review, see: Ingham, P. W. *EMBO J.* **1998**, *17*, 3505.
4. For a review, see: Borzillo, G. V.; Lippa, B. *Curr. Top. Med. Chem.* **2005**, *5*, 147.
5. Chen, J. K.; Taipale, J.; Cooper, M. K.; Beachy, P. A. *Genes Dev.* **2002**, *16*, 2743.

6. Tremblay, M. R.; Lescarbeau, A.; Grogan, M. J.; Tan, E.; Lin, G.; Austad, B. C., et al *J. Med. Chem.* **2009**, *52*, 4400.
7. Robarge, K. D.; Brunton, S. A.; Castanedo, G. M.; Cui, Y.; Dina, M. S.; Goldsmith, R., et al *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5576.
8. Chen, J. K.; Taipale, J.; Young, K. E.; Maiti, T.; Beachy, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14071.
9. For a review, see: Dlugosz, A. *J. Clin. Invest.* **1999**, *104*, 851.
10. Mill, P.; Mo, R.; Fu, H.; Grachtchouk, M.; Kim, P. C. W.; Dlugosz, A. A.; Hui, C. C. *Genes Dev.* **2003**, *17*, 282.
11. Oro, A. E.; Higgins, K. *Dev. Biol.* **2003**, *255*, 238.
12. Brancaccio, A.; Minichiello, A.; Grachtchouk, M.; Antonini, D.; Sheng, H.; Parlato, R.; Dathan, N.; Dlugosz, A. A.; Missero, C. *Hum. Mol. Genet.* **2004**, *13*, 2595.
13. Hutchin, M. E.; Kariapper, M. S. T.; Grachtchouk, M.; Wang, A.; Wei, L.; Cummings, D.; Liu, J.; Michael, L. E.; Glick, A.; Dlugosz, A. A. *Genes Dev.* **2005**, *19*, 214.
14. Sagai, T.; Masuya, H.; Tamura, M.; Shimizu, K.; Yada, Y.; Wakana, S.; Gondo, Y.; Noda, T.; Shiroishi, T. *Mamm. Genome* **2004**, *15*, 23.
15. Paladini, R. D.; Saleh, J.; Qian, C.; Xu, G.-X.; Rubin, L. L. *J. Invest. Dermatol.* **2005**, *125*, 638.
16. Jackson, J.; Caro, J. J.; Caro, G.; Garfield, F.; Huber, F.; Zhou, W.; Lin, C.-S.; Shander, D.; Schrode, K. *Int. J. Dermatol.* **2007**, *46*, 976.
17. C3H/HeN mouse hair growth model: Jiang, J.; Tsuboi, R.; Kojima, Y.; Ogawa, H. *Dermatology* **2005**, *32*, 243.
18. Osak, B.; Fitzgerald, L. R. *Assay Drug Dev. Technol.* **2007**, *5*, 785.
19. Experimental procedure for the Smo filtration binding assay: Smo membranes were prepared from HEK 293 cells that were stably transfected with cDNA encoding mouse or human Smo. Membranes were diluted in binding buffer (50 μM Tris pH 7.5, 25 mM $MgCl_2$, 1 μM EDTA, 0.1% BSA, and 100 mM NaCl). Test compounds were also prepared by making appropriate dilutions in DMSO using a final concentration of 1 μM. In a 96-well costar assay plate, 5 μg of membranes were incubated with 4.0 nM of tritiated-SANT-2 (**5**) in the presence of vehicle, cold competitor or test compound in binding buffer in a final volume of 250 μL. Reactions were incubated at rt for 2 h. Then reaction mixtures were harvested and washed with 6 mL of wash buffer (50 μM Tris, 0.1% BSA) into pre coated unifilter GF/B filter plates (Perkin–Elmer) using a semi-automated 96-well harvester Mach III (Tomtec). Plates were air dried. 50 μL of Microscint-20 (Perkin–Elmer) was added to each well, and counted for 3 min using Packard top counter. Specific binding was calculated by subtracting non specific binding from total binding. Initially, compounds were screened at 10 μM in duplicate plates. Compounds with >50% inhibition were titrated from 10 to 0.01 μM in triplicate to determine IC_{50}.
20. (a) Ortiz de Montellano, P. R.; Augusto, O.; Viola, F.; Kunze, K. L. *J. Biol. Chem.* **1983**, *258*, 8623; (b) Kalyanaraman, B.; Sinha, B. K. *Environ. Health Perspect.* **1985**, *64*, 179; (c) Nelson, S. D. *J. Med. Chem.* **1982**, *25*, 753; (d) Streeter, A. J.; Timbrell, J. A. *Drug Metab. Dispos.* **1983**, *11*, 179; (e) Timbrell, J. A. *Drug Metab. Rev.* **1979**, *10*, 125; (f) Prough, R. A.; Coomes, M. W.; Cummings, S. W.; Wiebkin, P. *Adv. Exp. Med. Biol.* **1982**, *136B*, 983.
21. Nam, C.; An, S.; Lee, E.; Moon, S.; Kang, J.; Chang, I. *ATLA, Altern. Lab. Anim.* **2004**, *32*, 693.
22. Spielmann, H.; Presented at *Alternatives to Animal Testing II*, Proceedings of the Second International Scientific Conference, Brussels, 1999, 15.
23. Franz diffusion chambers: Antille, C.; Tran, C.; Sorg, O.; Saurat, J.-H. *Exp. Dermatol.* **2004**, *13*, 558.