Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 349 (2006) 471–479
BBRC
www.elsevier.com/locate/ybbrc

# Identification and characterization of Hedgehog modulator properties after functional coupling of Smoothened to $G_{15}$

Christelle Masdeu [a], Hélène Faure [a], Josée Coulombe [a], Angèle Schoenfelder [b], André Mann [b], Isabelle Brabet [c], Jean-Philippe Pin [c], Elisabeth Traiffort [a], Martial Ruat [a,*]

[a] *CNRS, Institut de Neurobiologie Alfred Fessard-IFR 2118, UPR9040, Laboratoire de Neurobiologie Cellulaire et Moléculaire, Signal Transduction and Developmental Neuropharmacology team, 1 avenue de la Terrasse, Gif-sur-Yvette F-91198, France*
[b] *CNRS, UMR7081, Laboratoire de Pharmacochimie de la Communication Cellulaire, Université Louis Pasteur, Faculté de Chimie, UMR7081, Illkirch F-67401, France*
[c] *CNRS, UMR5203, INSERM U661, Université Montpellier, Institut de Génomique Fonctionnelle-IFR3, Département de Pharmacologie Moléculaire, Montpellier F-34094 Cedex 5, France*

Received 26 July 2006
Available online 22 August 2006

**Abstract**

The seven-transmembrane receptor Smoothened (Smo) transduces the signal initiated by Hedgehog (Hh) morphogen binding to the receptor Patched (Ptc). We have reinvestigated the pharmacological properties of reference molecules acting on the Hh pathway using various Hh responses and a novel functional assay based on the coexpression of Smo with the α subunit of the $G_{15}$ protein in HEK293 cells. The measurement of inositol phosphate (IP) accumulation shows that Smo has constitutive activity, a response blocked by Ptc which indicates a functional Hh receptor complex. Interestingly, the antagonists cyclopamine, Cur61414, and SANT-1 display inverse agonist properties and the agonist SAG has no effect at the Smo-induced IP response, but converts Ptc-mediated inactive forms of Smo into active ones. An oncogenic Smo mutant does not mediate an increase in IP response, presumably reflecting its inability to reach the cell membrane. These studies identify novel properties of molecules displaying potential interest in the treatment of various cancers and brain diseases, and demonstrate that Smo is capable of signaling through $G_{15}$.


*Keywords:* Patched; G protein; Phospholipase C; Inverse agonist; Hedgehog; Morphogen; Stem cells; Tumor suppressor

The seven-pass transmembrane (TM) protein Smoothened (Smo) is an essential component of the Hedgehog (Hh) signaling pathway in vertebrates. Binding of Hh proteins to the receptor Patched (Ptc), a protein displaying homology with the family of resistance-nodulation-division family of prokaryotic permeases, is believed to relieve the inhibition exerted by Ptc on Smo, allowing the transmission of the signal. In the absence of Ptc, Smo is thought to be constitutively active and control of Hh signaling in tissues relies on the cell capacity to modulate the interactions between Ptc and Smo that form the Hh receptor complex [1,2].

Besides its role during embryogenesis, Hh signaling is also involved in postnatal and adult tissue functions and participates in the regulation of stem cell fate and the modulation of neuronal activity in the adult brain [1–7]. Alteration of Hh signaling observed upon Ptc and Smo mutation contributes to the development of basal cell carcinoma (BCC), one of the most common human cancers, and medulloblastoma, a brain tumor with poor prognosis. Thus, a Smo mutant initially identified in a sporadic case of BCC and then in medulloblastoma has been shown to partially escape Ptc inhibition leading to a constitutively active

* Corresponding author. Fax: +33 1 69 82 36 39.
*E-mail address:* ruat@nbcm.cnrs-gif.fr (M. Ruat).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.07.216

Hh pathway associated with enhanced cell proliferation [4,8].

More recently, the role of Hh signaling has been unravelled in the development of a growing number of tumors occurring in the lung, esophagus, stomach, pancreas, biliary tract, breast or prostate [1,4]. Interestingly, Smo has been proposed as a molecular target for the action of antagonists aimed at blocking the Hh pathway. Such molecules are therefore considered as candidate drugs for the treatment of these cancers. Cyclopamine (Fig. 1), which can be isolated from the corn lily *Veratrum californicum*, blocks Hh signaling and slows down the growth of these tumors in various animal models [1,4,8]. Several other small molecules endowed with Hh inhibitor properties have been developed such as SANT-1 or Cur61414 (Fig. 1) and their pharmacological properties evaluated in various Hh cell-based assays [9–11]. They have been proposed to act at the level of the hydrophobic domain delineated by the seven putative TM domains of Smo. Another class of molecules including SAG (Fig. 1) derived from a chlorothiophen moiety act as Smo agonists when applied to Hh responding cells [9,10].

Whether Smo belongs to the superfamily of G-protein coupled receptors (GPCR) is still a matter of controversy. In drosophila cell cultures, RNA interference of a full spectrum of G protein subunits failed to compromise Hh signaling [12]. In contrast, when expressed in frog melanophores, human Smo has been shown to couple to G-protein αi subunit [13], although previous works had reported that Shh stimulation did not change the intracellular levels of cAMP in cultured mammalian cells [14]. Nevertheless, Gαi coupling has not been used to further investigate the pharmacological properties of Smo ligands, and this was also the case for the complex pathway implicating $G_{12}$ and the small GTPase RhoA shown to be involved in several responses to Shh [15].

Here, we develop a simple functional assay based on the coexpression of Smo with the α subunit of the $G_{15}$ protein. The resulting functional coupling allows us to identify novel properties of molecules proposed to interact with Smo and to show that a constitutively active mutant (W539L, SmoA1) does not display any significant coupling in this assay.

## Materials and methods

*Drugs.* Cyclopamine was from Dr W. Gaffield (Albany, USA). SANT-1 was purchased from ChemBridge Corporation (San Diego, CA, USA). SAG and Cur61414 were prepared as described [9–11]. ShhN was from Dr D. Baker (Biogen Idec, Boston, USA).

*Plasmid constructions and site-directed mutagenesis.* The plasmids pRK5, pRK5-$G_{15}$, pRK5-$G_{qzIC}$, pRK5-$G_{16}$, pRK5-mGluR5, and pRK5-SP-Myc have been described [16,17]. The plasmids encoding $G_q$, $G_{qG66D}$, $G_{qG66Di5}$, and $G_{qG66Ds5}$ were a kind gift from Dr E. Kostenis. DNA encoding the full-length mouse Smo was provided by Pr P.A. Beachy (The Johns Hopkins University School of Medicine, Baltimore, USA). To generate the Myc-Smo construct, a fragment encoding amino acid residues 35–107 of mouse Smo was amplified by PCR. The restriction fragment *Mlu*I–*Xho*I was subcloned into pRK5-SP-Myc. The remaining Smo nucleotide sequence was then subcloned into the latter recombinant plasmid using the restriction sites *Xho*I and *Sal*I. The tryptophan residue at position 539 was mutated into lysine in mouse wild type (WT) Smo by site-directed mutagenesis to give pRK5-SP-Myc-SmoA1. The pRK5-Ptc vector was generated using the full-length nucleotide sequence from Pr M. Scott (Stanford University School of Medicine, Stanford, CA). The

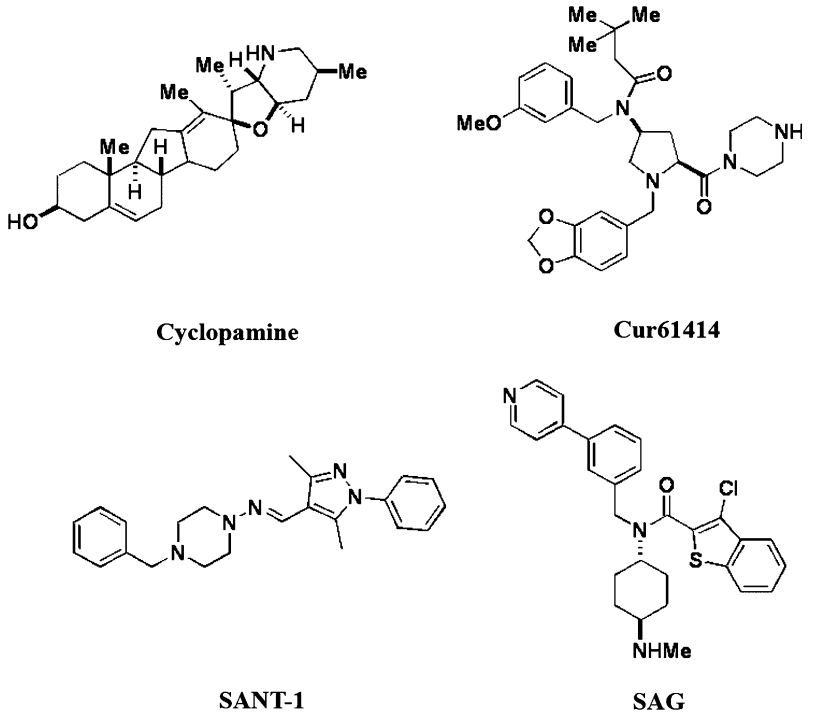


Fig. 1. Chemical structure of cyclopamine, Cur61414, SANT-1 and SAG.