European Journal of Pharmacology 667 (2011) 105–112

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and Cellular Pharmacology

Agonist-dependent effects of mutations in the sphingosine-1-phosphate type 1 receptor

Pieter B. van Loenen [a,1], Chris de Graaf [b,1], Dennis Verzijl [a], Rob Leurs [b], Didier Rognan [c], Stephan L.M. Peters [a], Astrid E. Alewijnse [a,*]

[a] Department of Pharmacology & Pharmacotherapy, Academic Medical Center, Amsterdam, The Netherlands
[b] Leiden/Amsterdam Center for Drug Research, Division of Medicinal Chemistry, Faculty of Science, VU University, Amsterdam, The Netherlands
[c] Structural Chemogenomics Group, UMR 7200 CNRS-UdS (Université de Strasbourg), Illkirch, France

ARTICLE INFO

Article history:
Received 16 March 2011
Received in revised form 5 May 2011
Accepted 22 May 2011
Available online 6 June 2011

Keywords:
G protein-coupled receptor
Sphingosine-1-phosphate (S1P)
$S1P_1$ receptor model
FTY720-P
SEW2871
Point mutations

ABSTRACT

The sphingosine-1-phosphate type 1 ($S1P_1$) receptor is a new target in the treatment of auto-immune diseases as evidenced by the recent approval of FTY720 (Fingolimod). The ligand-binding pocket of the $S1P_1$ receptor has been generally characterised but detailed insight into ligand-specific differences is still lacking. The aim of the current study is to determine differences in ligand-induced $S1P_1$ receptor activation using an *in silico* guided site-directed mutagenesis strategy. $S1P_1$ mutant receptors (modifications of residues $Y98^{2.57}$, $R120^{3.28}$, $F125^{3.33}$) were probed with a chemically diverse set of $S1P_1$ agonists (S1P, dihydro-S1P (dhS1P), *R*-, *S*- and racemic FTY720-P, VPC24191, SEW2871). Mutation of the $R^{3.28}$ residue generally results in a reduction of the potency of all ligands although the synthetic ligands including FTY720-P are less sensitive to these mutations. The $Y^{2.57}F$ mutation does not affect the potency of any of the ligands tested, but for all ligands except FTY720-P a significant decrease in potency is observed at the $Y^{2.57}A$ mutant. The $F^{3.33}A$ mutation significantly decreased the potency of FTY720-P and is detrimental for SEW2871 and VPC24191. The non-aromatic endogenous ligands S1P and dhS1P are less affected by this mutation. Our *in silico* guided mutagenesis studies identified new molecular determinants of ligand-induced $S1P_1$ receptor activation: 1) the flexibility of the polar head of the agonist to maintain a tight H-bond network with $R^{3.28}$ and 2) the ability of the agonist to make aromatic π-stacking interactions with $F^{3.33}$. Interestingly, FTY720-P has both chemical properties and is the only ligand that can efficiently activate the $Y^{2.57}A$ mutant.

1. Introduction

The sphingosine-1-phosphate type 1 ($S1P_1$) receptor, originally referred to as endothelial differentiation gene 1 receptor, is a G protein-coupled receptor, which belongs to the five-membered family of S1P receptors ($S1P_{1-5}$) (Chun et al., 2002). Pharmacological characterization of the $S1P_1$ receptor revealed a predominant coupling to and activation of $G_{i/o}$ proteins (Ancellin and Hla, 1999; Kon et al., 1999; Windh et al., 1999) although activation of $G_{12/13}$ proteins under some conditions cannot be excluded (Lee et al., 1998).

The $S1P_1$ receptor plays a crucial role of in vascular development and deletion of this receptor in mice resulted in embryonic lethality due to defects in vascular maturation (Liu et al., 2000). Besides its essential role in the vascular system (Alewijnse and Peters, 2008) the $S1P_1$ receptor plays a pivotal role in the immune system. T cell-specific deletion of $S1P_1$ and pharmacological intervention studies in mice indicated a role for the $S1P_1$ receptor in lymphocyte trafficking (Mandala et al., 2002; Rosen et al., 2003). Interestingly, the United States Food and Drug Administration recently approved a drug, FTY720, for the treatment of multiple sclerosis based on this mechanism of action (Brinkmann et al., 2010). The phosphorylated form of this drug, FTY720-P (Fig. 1), is a potent agonist for the $S1P_1$ receptor which most likely explains its immuno-modulating properties (Rosen et al., 2003). However, because FTY720-P additionally targets the $S1P_{3-5}$ receptor it cannot be excluded that effects on these receptor subtypes also contribute to the therapeutic effect observed in multiple sclerosis (Brinkmann, 2009).

Mutation studies of the $S1P_1$ receptor have primarily focused on the identification of residues involved in receptor activation and binding of the endogenous agonist S1P and the synthetic agonist SEW2871 (Deng et al., 2007; Fujiwara et al., 2007; Lim et al., 2004; Parrill et al., 2000; Wang et al., 2001). Investigation of the molecular determinants of $S1P_1$ receptor activation by other agonists, including *S*-FTY720-P, the active metabolite (Albert et al., 2005) of the recently approved drug Fingolimod (Brinkmann et al., 2010), is however scarce (Deng et al., 2007; Schürer et al., 2008). Site-directed mutagenesis studies showing agonist-specific effects can give a

* Corresponding author. Tel.: +31 205664884; fax: +31 20 6965976.
E-mail address: a.e.alewijnse@amc.uva.nl (A.E. Alewijnse).
[1] These authors contributed equally.

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2011.05.071

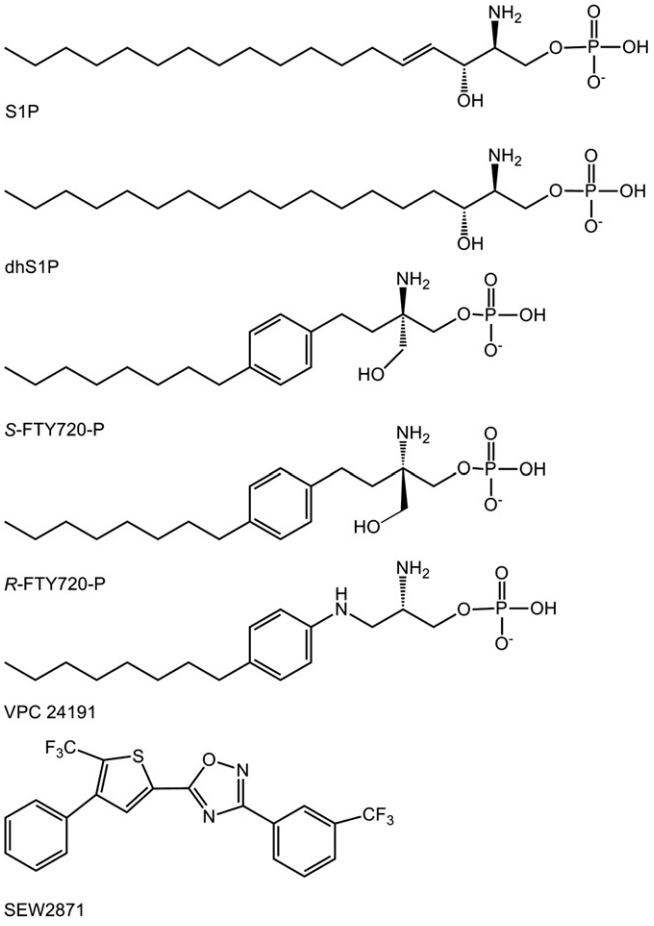

Fig. 1. Chemical structures of S1P, dhS1P, *S*-FTY720-P, *R*-FTY720-P, VPC24191 and SEW2871.

more detailed insight into the structure-activity relationships of $S1P_1$ agonists and can be used to elucidate $S1P_1$-ligand binding modes. The aim of the current study is to determine differences in ligand-induced $S1P_1$ receptor activation. Using an *in silico* guided site-directed mutagenesis strategy we have designed $S1P_1$ mutants which modulate the ability of ligands to stabilise active $S1P_1$ receptor conformations. We have probed these mutants (modifications of residues $Y98^{2.57}$, $R120^{3.28}$, $F125^{3.33}$) with a chemically diverse set of $S1P_1$ agonists, i.e. S1P, dihydro-S1P (dhS1P), *R*-, *S*- and racemic FTY720-P, VPC24191, and SEW2871 (Fig. 1) to identify differences in ligand-induced $S1P_1$ receptor activation. These studies will give more insight into the subtle differences in activation mode between various agonists and can eventually provide valuable information for the structure-based design of novel $S1P_1$ ligands.

2. Materials and methods

2.1. Materials

Forskolin, Pertussis toxin (PTX), bovine serum albumin (BSA), BSA fatty acid free, Hepes and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sphingosine-1-phosphate (S1P), dihydrosphingosine-1-phosphate (dhS1P) and (S)-Phosphoric acid mono-[2-amino-3-(4-octyl-phenylamino)-propyl] ester (VPC24191) were obtained from Avanti Polar Lipids Inc. (via Instruchemie B.V., The Netherlands). 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole (SEW2871) was obtained from Calbiochem (via VWR, Zwijndrecht, The Netherlands). 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol mono (dihydrogen phosphate) ester (FTY720-P) was synthesised according to previously described methods (Albert et al., 2005). Dimethylsulfoxide (DMSO) was obtained from Merck (Nottingham, UK). The LANCE™ cAMP 384 kit and White 384 Well optiplates were obtained from Perkin-Elmer (Zaventem, Belgium). Foetal Calf Serum (FCS), PBS, Hanks Balanced Salt Solution (HBSS), Penicillin/Streptomycin, cell dissociation buffer and Ham-F12 medium were obtained from Gibco (via Invitrogen, Breda, The Netherlands). Primers were obtained from Biolegio (Nijmegen, The Netherlands). Hygromycin B was obtained from PAA (Cölbe, Germany). Lipofectamine™ 2000, pOG44, pcDNA3.1/HisC, pcDNA5/FRT/TO, Anti-HisG antibody, Alexa Fluor® 488 goat anti-mouse IgM and CHO-Flp-In cells were obtained from Invitrogen (Breda, The Netherlands). BamHI, HindIII and XhoI were obtained from Fermentas Life Sciences (St. Leon-Rot, Germany).

2.2. Construction of $S1P_1$ receptor models

First, a preliminary high-throughput receptor model of only the 7 TM helices of $S1P_1$ was generated using the GPCRgen program (Bissantz et al., 2004) based on the high resolution crystal structure of bovine rhodopsin (Li et al., 2004). All currently available GPCR crystal structures have low sequence homology to $S1P_1$. The bovine rhodopsin crystal structure template was selected because the ligand binding pocket in bovine rhodopsin is deeper than the pockets in the adrenergic beta-1 (Warne et al., 2008) and beta-2 receptor (Cherezov et al., 2007), and adenosine A2A receptor (Jaakola et al., 2008) co-crystal structures, extending all the way towards F6.44, a known ligand binding residue in $S1P_1$ (Fujiwara et al., 2007). During the course of this study, the dopamine D3 (Chien et al., 2010) and CXCR4 chemokine receptor (Wu et al., 2010) structures were solved, but also these structures do not have a deep binding ligand pocket between TM helices 3–6 which is suitable for modelling $S1P_1$. Finally it should be noticed that a co-crystal structure of $S1P_1$ has been announced (http://www.receptos.com/receptos-pipeline-sphingosine.php), but this structure was not yet available during the preparation of this manuscript. The Ballesteros–Weinstein residue numbering scheme (Ballesteros and Weinstein, 1995) was used throughout this manuscript for $S1P_1$ transmembrane (TM) helices. This preliminary $S1P_1$ receptor model was minimised with AMBER 10 (http://ambermd.org/) using the AMBER03 force field (Wang et al., 2004) to relax the structure and remove steric bumps. The minimizations were performed by 1000 steps of steepest descent followed by conjugate gradient until the root mean square gradient of the potential energy was lower than 0.05 kcal mol^{-1} Ångstrom (Å). A twin cut-off (12.0, 15.0 Å) was used to calculate non-bonded electrostatic interactions and the non-bonded pair-list was updated every 25 steps. $S1P_1$ agonists, including *S*-FTY720-P, S1P, dhS1P, VPC24191, and SEW2871, were manually sketched in Chemsketch (http://www.chemaxon.com/). Starting from the Chemsketch file, 2-D sd structures were subsequently protonated using Filter2 (SciTegic Inc., San Diego, CA 92123-1365, USA) and converted into 3-D mol2 files with Corina 3.4 (http://www.molecular-networks.com/products/corina). *S*-FTY720-P was docked into this structure using PLANTS (Korb et al., 2007, 2009). Experimentally-driven receptor-ligand H-bond constraints (Wang et al., 2001) were used to guide the docking process in the receptor: 1) between one of the acidic phosphate oxygen atoms of *S*-FTY720-P and the NH_2 nitrogen atom of the guanidine group of $R^{3.28}$ (Wang et al., 2001) 2) between the protonated amine nitrogen atom of *S*-FTY720-P and one of the carboxylate oxygen atoms (OE1) of $E^{3.29}$ (Wang et al., 2001). Fifteen *S*-FTY720-P poses were generated and the top ranked docking pose accommodating the aliphatic chain in the hydrophobic pocket between TMs 3, 4, 5, and 6 (in line with experimental data (Deng et al., 2007; Fujiwara et al., 2007)) was selected. Extracellular loops (ecl) 1 and 3, intracellular loops (icl) 1–3, and helix 8 were constructed based on the bovine rhodopsin crystal structure (Li et al., 2004) using Modeller 9v1. Extracellular loop 2 (the $S1P_1$ receptor lacks the conserved $C^{3.25}$