



Selectivity and specificity of sphingosine 1-phosphate receptor ligands: “off-targets” or complex pharmacology?

Nigel J. Pyne* and Susan Pyne

Cell Biology Group, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK

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***Correspondence:**

Nigel J. Pyne, Cell Biology Group, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK.
e-mail: n.j.pyne@strath.ac.uk

A recent perspective published in *Frontiers of Pharmacology* by Salomone and Waeber (2011) discussed the selectivity and specificity of sphingosine 1-phosphate (S1P) receptor ligands. This perspective surveyed the use of various S1P receptor ligands and attempted to reconcile a number of inconsistencies in the predicted biological outcomes: these were interpreted as “off-target” effects. Therefore the perspective cautioned against the use of these S1P receptor ligands. Here we highlight the complex pharmacology of S1P receptors, which along with “inside-out” signaling might provide an alternative explanation for “off-target” effects.

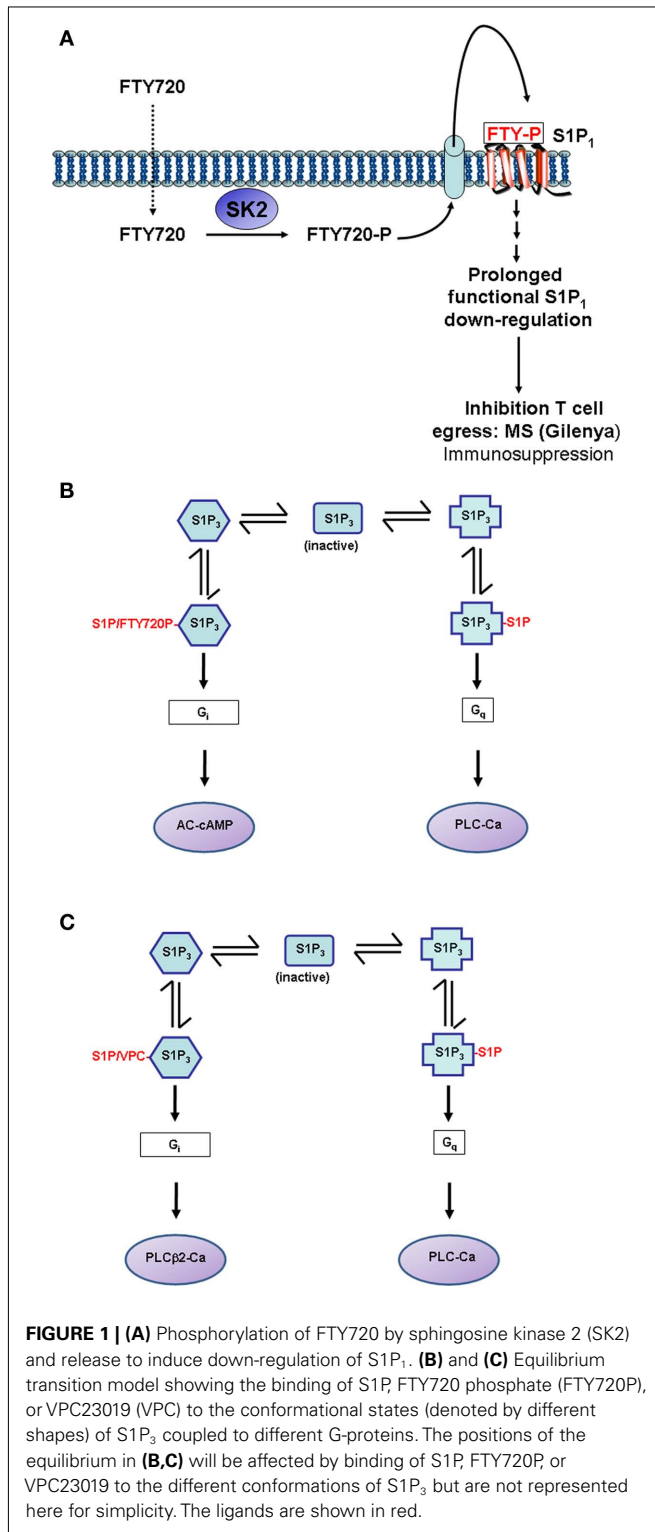
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S1P binds to five different GPCRs termed S1P₁₋₅ that exhibit differential coupling to various G-protein sub-types (Pyne and Pyne, 2010). The perspective by Salomone and Waeber (2011) focused on three S1P receptor ligands, JTE-013, BML-241 (CAY10444), and VPC23019. The major issues raised by these authors related to the findings that JTE-013 (which has been characterized as an S1P₂ receptor antagonist; Osada et al., 2002; Ohmori et al., 2003) inhibited not only the vasoconstrictor effect of S1P, but also that of the prostanoid analog U46619, endothelin-1, or high KCl (Salomone et al., 2008). In addition, BML-241 (which has been characterized as an S1P₃ receptor antagonist; Koide et al., 2002) inhibits increases in intracellular calcium mediated by P₂ receptor or α_{1A} -adrenoceptor stimulation and reduces α_{1A} -adrenoceptor stimulated contraction of mesenteric artery, while it failed to prevent the S1P₃-mediated reduction in forskolin-stimulated cyclic AMP accumulation (Jongsma et al., 2006). Moreover, VPC23019 (characterized as an S1P_{1/3} antagonist; Davis et al., 2005) unexpectedly potentiated S1P-stimulated contraction in intact basilar arteries (Salomone et al., 2008); a result confounded by the finding that S1P-stimulated vasoconstriction was reduced in tissue from S1P₃ knockout mice. In addition, VPC23019 behaves as a partial agonist in a S1P₃ calcium mobilization assay (Jongsma et al., 2009). These anomalies are used to suggest that “off-target” effects should be seriously considered as caveats to conclusions drawn, unless validated by siRNA knock down or genetic knock out of the S1P receptor being studied. If applied at sufficient concentrations, all small molecules are likely to have “off-target” effects. The issue here is that for the ligands mentioned above, none of the “off-targets” have been definitively identified, and we do not know whether a sufficient concentration has been reached. In addition, critical analysis requires defining the precise GPCR ligand

pharmacology for these reagents before a definitive appraisal of “off-target” effects can be made. In this regard, the pharmacology of GPCRs is not simple, and is made more complicated by the fact that an array of different conformational states of the same receptor might exist, each of which might bind ligands with markedly different affinities. Moreover, the different conformational states of a GPCR might also be differentially coupled to distinct G-proteins. Several examples of this critically important complex ligand pharmacology are discussed here.

FTY720 (or fingolimod) is an immunosuppressant that was very recently licensed by the Food and Drug Administration and the European Medicines Agency as Gilenya™. This sphingosine analog is taken up by cells, phosphorylated by sphingosine kinase 2 (SK2) and released as FTY720 phosphate. FTY720 phosphate binds to and activates S1P₁, S1P₃, S1P₄, and S1P₅, but not S1P₂ (Brinkmann et al., 2010). FTY720 phosphate also induces a later “functional antagonism” by promoting the polyubiquitination, endocytosis, and proteasomal degradation of S1P₁ (Gräler and Goetzl, 2004), which creates S1P₁ receptor null T-cells to induce lymphopenia. In contrast, S1P₁ receptors recycle in response to S1P. Therefore, S1P and FTY720 phosphate do not appear to bind to and stabilize the same conformation of S1P₁. These different conformational states are likely to exist in equilibrium, as binding of FTY720 phosphate to a specific S1P₁ conformation will increase its concentration by mass action such that the entire population of S1P₁ will be eventually degraded in response to FTY720 phosphate, and this is indeed the case (Figure 1A).

The concept that different conformational states of S1P receptors might exist is very important in terms of explaining the unusual pharmacology of receptor ligands that could otherwise be interpreted as “off-target” effects. For instance, FTY720 phosphate



binds to and activates a specific S1P₃-G_i coupled receptor conformation and inhibits S1P₃-G_q coupled receptor signaling (Sensken et al., 2008). These findings can be explained by a model in which S1P₃-G_q and S1P₃-G_i are in equilibrium. Thus, FTY720 phosphate may act as a “biased agonist” by binding exclusively to the S1P₃-G_i conformation which would reduce the concentration of

the S1P₃-G_q receptor conformation by mass action and thereby inhibit S1P₃-G_q signaling (**Figure 1B**). Therefore, it is possible that BML-241 is also a “biased” agonist of the S1P₃-G_i conformation that regulates adenylyl cyclase and thus, an antagonist of S1P₃-G_q signaling. If this is the case, then one would not necessarily expect BML-241 to prevent an S1P/S1P₃-mediated inhibition of forskolin-stimulated cyclic AMP accumulation.

We have evaluated the effect of BML-241 (CAY10444) on S1P₂, S1P₃, and S1P₄ each separately over-expressed in HTC4 cells. Our findings demonstrate that BML-241 inhibited S1P/S1P₃-mediated calcium mobilization in a concentration-dependent manner with a ~80% inhibition at 10 μM BML-241. BML-241 had no effect on S1P-stimulated calcium responses mediated by S1P₂ or S1P₄ (Long et al., 2010a). Although, BML-241 is a low potency antagonist of S1P₃, the extent to which it reduces S1P-stimulated calcium mobilization in HTC4 cells over-expressing S1P₃ is superior to that reported by Koide et al. (2002). Further support for an action of BML-241 on S1P₃ was obtained by our findings that S1P stimulation of ERK-1/2 in MCF-7 breast cancer cells is abolished by BML-241 and this is recapitulated by siRNA knockdown of S1P₃ (Long et al., 2010b). Interestingly, S1P stimulation of ERK-1/2 in MCF-7 cells is reduced by pertussis toxin, indicating involvement of S1P₃-G_i (Sukocheva et al., 2006). This might suggest increased complexity in the behavior of BML-241 which appears to antagonize a conformation of S1P₃-G_i that is coupled to the ERK-1/2 pathway in MCF-7 cells.

BML-241 inhibits increases in intracellular calcium mediated by P₂ receptor or α_{1A}-adrenoceptor stimulation in CHO cells and reduces α_{1A}-adrenoceptor stimulated contraction of mesenteric artery (Jongsma et al., 2006). However, the possibility that stimulation of these receptors activates sphingosine kinase (catalyzes synthesis of S1P from sphingosine) to promote S1P synthesis and release, which can then act on local S1P receptors (e.g., S1P₃) in the artery (termed “inside-out” signaling), cannot be excluded at this time. Indeed, we have also shown that BML-241 inhibits S1P- and anandamide-induced relaxation of endothelium intact coronary artery vessels (by blocking the “inside-out” signaling by S1P in response to anandamide). The latter is also reduced by VPC23019 (Mair et al., 2010), while W146, an S1P₁ selective antagonist (Wamhoff et al., 2008) had no effect (Mair et al., 2010). In addition to “inside-out” signaling, the alternative possibility that P₂ or α_{1A}-adrenoceptors might form functional heterodimers with S1P₃ and that BML-241 induces allosteric effects on P₂ or α_{1A}-adrenoceptor-mediated signaling via S1P₃ has not been excluded.

JTE-013 (Ohmori et al., 2003) is widely used as a S1P₂ antagonist *in vitro* and is effective *in vivo*, reducing streptozotocin-induced apoptosis of pancreatic β cells and incidence of diabetes (Imasawa et al., 2010) and IgE-triggered anaphylactic responses (Oskeritziyan et al., 2010). Both of these disease models are attenuated in S1P₂^{-/-} mice, thereby supporting a role for this receptor type, and the action of JTE-013 at S1P₂. As pointed out by Salomone and Waeber (2011), JTE-013 inhibited the specific binding of radiolabeled S1P with an IC₅₀ of 17 ± 6 and 22 ± 9 nM in CHO cells over-expressing human or rat recombinant S1P₂ respectively, and had no effect on S1P₁ or S1P₃ at concentrations in excess of 10 μM (Ohmori et al., 2003). We confirmed that JTE-013

inhibited S1P-stimulated calcium mobilization with an IC_{50} of $\sim 1 \mu M$ in HTC4 cells over-expressing S1P₂, with no significant effect on S1P-stimulated calcium mobilization in HTC4 cells over-expressing S1P₃ at concentrations as high as $10 \mu M$ (Long et al., 2010a). However, we also demonstrated that JTE-013 is not specific for S1P₂, and inhibits S1P-stimulated calcium mobilization in HTC4 cells over-expressing S1P₄ with a K_i of 237 nM (Long et al., 2010a). Moreover, S1P stimulation of ERK-1/2 in MDA-MB-453 breast cancer cells, which express endogenous S1P₂ and S1P₄, was inhibited by JTE-013 ($10 \mu M$) and this was recapitulated by siRNA knockdown of S1P₄, but not S1P₂ (Long et al., 2010a).

The ability of JTE-013 to antagonize S1P₄ might provide an alternative explanation for the finding that JTE-013 inhibits S1P-induced vasoconstriction in S1P₂^{-/-} mice (Salomone and Waeber, 2011). Although S1P₄ is reported to have a rather restricted tissue distribution confined to immune cells, this may not be the case as evidenced by its expression in breast cancer cells (Long et al., 2010a) or, alternatively, heterogenous tissue preparations may contain inflammatory cells that release vasoconstrictors in response to S1P, mediated by S1P₄. JTE-013 also inhibited prostanoid-, endothelin 1-, and KCl-induced contraction, the latter suggesting perturbation of L-type calcium channel activity (Salomone et al., 2008). However, vasoconstriction of the basilar artery to prostanoid and KCl is reduced in SK1^{-/-} mice (Salomone et al., 2010). Furthermore, we found that JTE-013 had no significant inhibitory effect on S1P-stimulated calcium mobilization in HTC4 cells over-expressing S1P₃ (Long et al., 2010a) suggesting that JTE-013 ($10 \mu M$) has no activity on S1P₃ or calcium signaling down-stream of S1P receptors.

To highlight the complexity of the pharmacology of S1P receptor ligands, we point to SB649146 (Waters et al., 2006; Pyne and Pyne, 2008). SB649146 is a protean agonist of S1P₁ (see Kenakin, 2001 for review of protean agonism). SB649146 exhibits inverse agonism by reducing S1P₁-mediated G_i activation, competitive antagonism of S1P (displaces dihydro S1P (an S1P₁ agonist) and inhibits S1P-stimulated activation of ERK-1/2 in airway smooth muscle cells) and is a partial agonist when used alone, e.g., SB649146 weakly stimulates the pertussis toxin-sensitive ERK-1/2 pathway in airway smooth muscle cells (Waters et al., 2006). The effect of SB649146 is mediated by binding to S1P₁ in airway smooth muscle cells because treatment of these cells with an antisense oligonucleotide specific for S1P₁ abolished S1P-stimulated activation of ERK-1/2 (Waters et al., 2003). SB649146 also induces endocytosis of a relatively small pool of S1P₁ in airway smooth muscle cells, consistent with a partial agonistic effect on S1P₁, but inhibits S1P-stimulated endocytosis/signaling of S1P₁ (Waters et al., 2006).

The action of VPC23019 (which was initially described as an S1P_{1/3} antagonist, Davis et al., 2005) as an S1P₃ agonist on calcium signaling (Jongsma et al., 2009) might also be explained by complexities in GPCR pharmacology. In this regard, Jongsma et al. (2009) have demonstrated that VPC23019 reduces forskolin-stimulated cAMP accumulation in CHO cells over-expressing S1P₃ suggesting that it might function as an agonist by binding to and stabilizing the S1P₃-G_i conformation. Moreover, VPC23019 stimulated calcium mobilization, a response that was sensitive

to pertussis toxin and thus possibly also mediated by S1P₃/G_i and a G_iβγ-regulated PLCβ2-dependent mechanism (Figure 1C). Crucially, the effect of VPC23019 on S1P-stimulated calcium mobilization (to establish whether it inhibits S1P₃-G_q-mediated calcium signaling) was not tested. Therefore, the potentiating effect of VPC23019 on S1P-induced vasoconstriction described by Salomone and Waeber (2011) might also be explained by biased agonism at S1P₃-G_i and stimulation of PLCβ2/calcium signaling, although this requires formal investigation. This interpretation is not at odds with the data obtained using S1P₃^{-/-} mice, where S1P-stimulated calcium mobilization by both G_i and G_q signaling routes would be ablated.

Davis et al. (2005) demonstrated that VPC23019 competitively inhibited S1P-stimulated GTPγS binding in membranes from HEK 293T cells transiently over-expressing S1P₃ together with mutated C352F mutated G₁₂α, β1, and γ2. However, it should be stated that Davis et al. (2005) did not observe any agonistic effect of VPC23019 in their assay system. In addition, VPC23019 competitively antagonized S1P-stimulated calcium mobilization in T24 cells stably over-expressing S1P₃ (Davis et al., 2005) and displaced radiolabeled S1P binding to S1P₁ or S1P₃ receptors in HEK 293T cells. Therefore, differences found in the two studies appear to be centered around the S1P₃ pharmacology in the different assay systems used. TY-52156, a selective S1P₃ antagonist restores coronary blood flow that was reduced by S1P, and inhibits Rho dependent activation and calcium signaling (Murakami et al., 2010), suggesting that it does not discriminate between S1P₃ receptor conformations. This contrasts with VPC23019 which has no effect on coronary flow (Murakami et al., 2010), possibly because it might not antagonize S1P₃-Rho conformational signaling. In the same study, VPC23019 was shown to inhibit Eu-GTP binding in membranes from cells over-expressing S1P₃. It should also be noted that TY-52156 has been used in only one study to date and is also an S1P₄ antagonist (Murakami et al., 2010). VPC23019 is a phosphorylated lipid and is therefore a possible substrate for lipid phosphate ectophosphatases (LPP, for review see Pyne et al., 2005). Therefore, LPP activity might limit availability of VPC23019 at S1P receptors, and this might account for differences in pharmacology in certain cellular systems dependent on the expression level of LPP. VPC23019 is also a full agonist of S1P₄ and a partial agonist of S1P₅ (Davis et al., 2005) and this may complicate interpretation of its effects in heterogenous tissue preparations.

Salomone and Waeber (2011) also state that “off-target” effects of FTY720 are not likely relevant to the clinical action of FTY720 (via FTY720 phosphate), which occur at nanomolar concentrations in terms of inducing lymphopenia. This is compared with micromolar concentration effects of FTY720 on SK1 (Tonelli et al., 2010), ceramide kinase (Lahiri et al., 2009), 14-3-3 protein (Woodcock et al., 2010), acid sphingomyelinase (Dawson and Qin, 2011), and phospholipase A2 (Payne et al., 2007). There are two important points here. FTY720 has to be taken up into cells, so that it can be phosphorylated by SK2 to form FTY720 phosphate. The K_m of SK2 for FTY720 is $18.2 \mu M$ (Billlich et al., 2003). Thus, we suspect that FTY720 has to be concentrated by the cell to bring its concentration into the micromolar substrate range so that it

can be used by SK2 to form FTY720 phosphate. Notably, the K_i for SK1 inhibition by FTY720 is 2 μM (Lim et al., 2011) and this is therefore close to the concentration of FTY720 that might be expected to accumulate within cells from an extracellular nM concentration. Second, FTY720 and FTY720 phosphate have opposing actions. Thus, the treatment of human cancer cells (MCF-7, MDA-MB-231, and Sk-Br-3 cells) with FTY720 inhibits growth, activates JNK, and transiently inhibits ERK-1/2 activation with no effect on p38 MAPK in MCF-7 cells. In contrast, FTY720 phosphate induces growth of these cells (Nagaoka et al., 2008). FTY720 demonstrates significant anti-cancer activity *in vivo*, and apoptosis of cancer cells, and this is therefore likely to be directly related to inhibition of SK1, an enzyme which is known to play a critical role in promoting cancer cell survival (Pyne and Pyne, 2010). Therefore, one can legitimately question whether SK1, PLA2, etc., are indeed, “off-targets.”

Clearly, screening is required to test the selectivity and specificity of ligands for receptors and enzymes, related targets and “off-targets.” However, in the absence of identified “off-targets” that can explain the observed pharmacology, the alternative possibilities centered around the complexities of GPCR pharmacology must also be considered. Therefore, the purpose of this perspective is to bring these issues to the attention of the research community so that GPCR pharmacology *versus* “off-target” effects can be tested experimentally in the future. In addition, Salomone and Waeber (2011) cite many studies concerning the use of JTE-013,

BML-241, and VPC23019 that have not been validated by genetic deletions or eliminations. However, lack of validation of an effect of a given S1P receptor ligand does not immediately invalidate the conclusion drawn. Moreover, genetic deletions or siRNA elimination are also not without “off-target” effects, especially as GPCRs exist in constitutively active states and/or are regulated by autocrine ligands to induce signaling pathways that regulate gene expression. Some of the genes regulated by genetic deletions or eliminations of GPCRs might induce an effect which is not recapitulated by an acute action of an antagonist. We ascribe to a combined approach using pharmacological ligands and molecular tools to validate specificity (Waters et al., 2003; Long et al., 2010a,b). However, a wider panel of receptor ligands is also required to add power to the validation process and one should be aware of limitations in interpretation of molecular approaches if complexities in the GPCR ligand pharmacology are not fully defined.

In our opinion, multiple assays should be used (e.g., ligand binding, GTP γ S binding, calcium, cAMP, β -arrestin, and ERK-1/2 activation) to characterize competitive antagonism, biased agonism, and protean agonism. The knowledge of which signaling effectors are controlled by the various conformational states of a given S1P receptor should then inform on the appropriate use of defined pharmacological agents to interrogate a biological system. This approach should also enhance information gained from genetic knockout or elimination studies.

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