frontiers in
PHARMACOLOGY

PERSPECTIVE ARTICLE
published: 22 February 2011
doi: 10.3389/fphar.2011.00009

Selectivity and specificity of sphingosine-1-phosphate receptor ligands: caveats and critical thinking in characterizing receptor-mediated effects

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Receptors for sphingosine-1-phosphate (S1P) have been identified only recently. Their medicinal chemistry is therefore still in its infancy, and few selective agonists or antagonists are available. Furthermore, the selectivity of S1P receptor agonists or antagonists is not well established. JTE-013 and BML-241 (also known as CAY10444), used extensively as specific $S1P_2$ and $S1P_3$ receptors antagonists respectively, are cases in point. When analyzing S1P-induced vasoconstriction in mouse basilar artery, we observed that JTE-013 inhibited not only the effect of S1P, but also the effect of U46619, endothelin-1 or high KCl; JTE-013 strongly inhibited responses to S1P in $S1P_2$ receptor knockout mice. Similarly, BML-241 has been shown to inhibit increases in intracellular Ca^{2+} concentration via P_2 receptor or α_{1A}-adrenoceptor stimulation and α_{1A}-adrenoceptor-mediated contraction of rat mesenteric artery, while it did not affect $S1P_3$-mediated decrease of forskolin-induced cyclic AMP accumulation. Another putative $S1P_{1/3}$ receptor antagonist, VPC23019, does not inhibit $S1P_3$-mediated vasoconstriction. With these examples in mind, we discuss caveats about relying on available pharmacological tools to characterize receptor subtypes.

Keywords: JTE-013, BML-241, CAY10444, VPC23019, antagonist

Receptors for sphingosine-1-phosphate (S1P) were discovered and characterized in the late nineties. Ten years ago, there was no selective S1P receptor agonist or antagonist. Suramin, an old anti-protozoal drug, was used as $S1P_3$ receptor antagonist (Ancellin and Hla, 1999; Salomone et al., 2003), but its usefulness was limited because it lacked specificity (Voogd et al., 1993). Recently, a number of ligands for S1P receptors have been screened as agonists and antagonist (Im, 2010). Most of these newly developed agents are commercially available and are increasingly being used to characterize the S1P receptor subtypes involved in specific biological mechanisms and functions. However, they have often not been screened for specificity against a wide array of targets, nor have they been systematically studied *in vivo*, as is required for drugs intended for human use.

In contrast, more detailed pharmacological data is available for compounds that show significant therapeutic potential. Among these, particularly worthy of mention is fingolimod (FTY720), an FDA- and EMA-approved drug, that, once phosphorylated, binds all S1P receptors but $S1P_2$ *in vitro*. *In vivo*, phospho-fingolimod down-modulates lymphocytic $S1P_1$ to inhibit S1P-$S1P_1$-signaling and lymphocyte egress (Matloubian et al., 2004; Martin et al., 2010), which induces remission in patients with multiple sclerosis (Kappos et al., 2010). Fingolimod has been screened against a large number of other targets (including about 100 receptors) to insure S1P receptor specificity (V. Brinkmann, Novartis Institutes for BioMedical Research, personal communication).

At variance with most other available S1P receptor agents, FTY720 has been used in a large number of publications (nearly 1000 PubMed-indexed publications as of January 2011). Possibly because it has been the object of such an intense scrutiny, other, non-S1P receptor-mediated effects of FTY720 have been documented. For instance, FTY720 has been shown to inhibit ceramide synthase (Lahiri et al., 2009), cytosolic phospholipase A2 (Payne et al., 2007), S1P lyase (Bandhuvula et al., 2005), sphingosine kinase (SPK) 1 (Vessey et al., 2007; Tonelli et al., 2010), and acid sphingomyelinase (Dawson and Qin, 2011), stimulate 27-hydroxycholesterol production (Blom et al., 2010), bind to 14-3-3 proteins and inhibit their pro-survival signaling (Woodcock et al., 2010). Because most of these effects were observed at relatively high FTY720 concentration, they may not be relevant to the *in vivo* effects of the drug. But investigators should be cautious when interpreting the results of *in vitro* studies, particularly when these studies use high FTY720 concentrations. Incidentally, the fact that fingolimod does not discriminate between $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ receptors may not matter *in vivo*. Because blood levels of FTY720 are low (<3 nM), the drug does not occupy $S1P_3$ receptors to a significant extent. However, after down-regulation of $S1P_1$ receptors, homeostatic activation of $S1P_2$ and $S1P_3$ by endogenous plasma S1P (>200 nM) becomes predominant, and the S1P signaling balance is shifted from $S1P_1$ to $S1P_2$/$S1P_3$, and thus from G_i to $G_{12/13}$/Rho/Rho kinase activation. The observed functional consequence of FTY720 administration may therefore be a sum of drug- and endogenous S1P-mediated effects, and be very similar with $S1P_1$ selective agents and non-subtype selective S1P modulators.

We chose to focus this *Perspective* on JTE-013 and BML-241 because they are commonly used despite reports of their lack of specificity. We also discuss VPC23019, for which evidence of $S1P_3$ receptor antagonism activity seems weak. JTE-013 was developed by Central Pharmaceutical Research Institute, Japan Tobacco Inc. Its patent (Patent WO 01/98301; December 27, 2001) stated that JTE-013 inhibited the specific binding of radiolabeled S1P to membranes of CHO cells transfected with human and rat $S1P_2$ receptors, with IC_{50} values of 17 ± 6 and 22 ± 9 nM, and did not affect S1P binding to $S1P_3$ and $S1P_1$, at concentrations up to 10 μM (Osada et al., 2002; Ohmori et al., 2003). Based on these data, JTE-013 has been considered since then a specific $S1P_2$ receptor ligand and, following further experimental data, a $S1P_2$ antagonist. Because JTE-013 had been used to characterize the S1P receptor mediating canine coronary artery contraction (Ohmori et al., 2003), we used it in rodent isolated vessels to assess the role of $S1P_2$ receptors in S1P-induced vasoconstriction of basilar arteries (Salomone et al., 2008). We knew that S1P-induced vasoconstriction was abolished in $S1P_3^{-/-}$ mice and were therefore surprised to see that JTE-013 inhibited vasoconstriction to S1P in arteries from wild type mice, because this suggested that $S1P_2$ instead of, or in addition to, $S1P_3$ was mediating S1P effect. Investigating further, however, we realized that JTE-013 inhibited vasoconstriction not only to S1P, but also to the prostanoid analog U46619, endothelin-1 and high KCl (Salomone et al., 2008; of note, vasoconstriction induced by high KCl is not receptor-mediated but related to L-type Ca^{2+} channels). We then performed a critical genetic control experiment, and found that JTE-013 inhibited S1P-induced vasoconstriction in $S1P_2^{-/-}$ mice, demonstrating that this effect was not related to $S1P_2$ receptors. Yet, JTE-013 is still widely used to characterize $S1P_2$-dependent effects (**Table 1**). Of note, most studies that did validate $S1P_2$-blocking effects of JTE-013 used concentrations equal to or lower than 1 μM, while we found evidence of non-specificity at 10 μM. It is therefore possible that JTE-013 retains sufficient selectivity and remains a useful antagonist in the submicromolar range, but $S1P_2$ receptor involvement should be validated by other means.

BML-241 was developed at the same time as JTE-013, by rational drug design using the structure of S1P to interrogate a three-dimensional database. Two novel compounds were identified that showed antagonist activity. When tested at 10 μM, "Compound 2" inhibited by 37% S1P-induced increases in $[Ca^{2+}]_i$ in HeLa cells expressing $S1P_3$ receptors and by about 7% $[Ca^{2+}]_i$ increases in cells expressing $S1P_1$ receptors (Koide et al., 2002). Strikingly, this study was based on measurements with a single BML-241 concentration and the comparison of only two potential targets ($S1P_1$ and $S1P_3$ receptors) in one assay; furthermore, an inhibition by less than 40% by a 10-μM antagonist concentration in cell culture might be considered less than impressive. Nevertheless, at least 20 studies (**Table 2**) were published using BML-241 (also known as CAY10444), most of them after the publication of an article showing that BML-241 inhibits $[Ca^{2+}]_i$ increases via purinergic P_2 receptor or α_{1A}-adrenoceptor stimulation and α_{1A}-adrenoceptor-mediated contraction, while not affecting the $S1P_3$-mediated decrease of forskolin-induced cAMP accumulation (Jongsma et al., 2006). In a third of these studies, BML-241/CAY10444, tested at 1 or 10 μM, had no effect, leading the authors to conclude on a lack of involvement of $S1P_3$ receptors in their paradigm. It is however possible that the BML-241/CAY10444 concentration used in these studies was too low to block $S1P_3$ receptors to a significant extent; indeed, using a β-arrestin recruitment assay, Wetter et al. (2009) showed that their $S1P_3$ cell line response to S1P was inhibited to 78% of the receptor response by 100 μM BML-241/CAY10444, with an IC_{50} of 4.6 μM. A less likely, but possible explanation for negative studies with BML-241 is that a non-specific action of the drug may have counteracted the partial blockade of $S1P_3$ receptors. In light of the low affinity of BML-241/CAY10444 for $S1P_3$ receptors, it does not appear that the few studies that documented an effect of the antagonist at 1 or 5 μM were justified in implicating $S1P_3$ receptors. Two studies that used higher BML-241/CAY10444 concentrations (50 and 100 μM) did observe an antagonist effect. In one of these studies, the $S1P_1$ agonist SEW2871 had no effect, while the $S1P_1$/$S1P_3$ receptor antagonist VPC23019 reproduced the effect of BML-241/CAY10444 (Lichte et al., 2008). In the other study, B-cell migration was promoted by a mixed $S1P_1$/$S1P_3$ receptor agonist (VPC24191), but not by the $S1P_1$ agonist SEW2871. Furthermore, B-cells from $S1P_3^{+/-}$ mice migrated to S1P, whereas cells from $S1P_3$ knockout mice were unable to migrate to S1P at all concentrations of S1P tested (Donovan et al., 2010). Interestingly, in this study B-cell migration was only slightly, and not significantly, inhibited by BML-241/CAY10444, tested at concentration as high as 100 μM.

It is increasingly recognized that S1P, generated intracellularly by SPK, can be released into the extracellular space and thereby stimulate membrane S1P receptors, establishing an autocrine loop (Kim et al., 2009; Takabe et al., 2010). When this occurs, even a specific S1P receptor antagonist might inhibit the response to an agonist other than S1P. Autocrine loops might account for the fact that JTE-013 and BML-241 inhibit responses to U46619, endothelin-1 and high KCl, and to purinergic P_2 receptor or α_{1A}-adrenoceptor stimulation, respectively. However, autocrine loops cannot explain why an agonist such as JTE-013 has any action in mice lacking $S1P_2$ receptors. Potential S1P autocrine loops should be taken into account when screening S1P receptor antagonists in complex systems (cell or organism level), particularly when both SPK inhibitors and S1P receptor blockade (pharmacologically or by preventing receptor expression) attenuate the response (Peter et al., 2008; Schnitzer et al., 2009; Salomone et al., 2010).

VPC23019 was initially described as an $S1P_{1/3}$ receptor antagonist, with pK_B values of 7.5 and 6.0 for the $S1P_1$ and $S1P_3$ receptors, respectively (Davis et al., 2005). Since then it has been mainly used for characterizing $S1P_1$-receptor mediated responses. In a few studies, however, it has been also used as an $S1P_3$ receptor antagonist. For example, as mentioned above, Lichte et al. (2008), reported that S1P-induced calcium signaling in human keratinocytes is mainly mediated by $S1P_3$, because it can be blocked by the putative $S1P_3$ antagonists BML-241, and VPC23019. Recently, Jongsma et al. (2009), using three different assays, have shown that several compounds of the VPC series, including VPC23019, behave as full or partial agonists at $S1P_3$ receptors. Although obtained *in vitro*, in a system expressing high $S1P_3$ receptor density, these data suggest that VPC23019 is a less than ideal tool to characterize $S1P_3$-mediated responses. When studying the effects of S1P on vascular tone, we found that VPC23019 potentiated S1P-induced contractile response in both rat and mouse basilar arteries with intact endothelium, while it failed to do so in preparations without

Table 1 | Some published data obtained with JTE-013 (among more than 50 PubMed-indexed citations).

System	Effect	Concentration	Inferred significance	Validation	Conflicting data/caveat	Reference
Smooth muscle cells/HUVEC	Cell migration	Up to 10 μM	S1P inhibits cell migration via $S1P_2$	None		Osada et al. (2002)
Hepatocytes	DNA synthesis	Up to 10 μM	S1P inhibits hepatocyte proliferation via $S1P_2$	None		Ikeda et al. (2003)
Coronary artery smooth muscle cells	Contraction	Up to 10 μM	S1P contracts coronary muscle via $S1P_2$	None		Ohmori et al. (2003)
Transfected CHO cells	Ca^{2+} mobilization	Up to 1 μM	$S1P_2$ negatively regulates cell motility	$S1P_2$ overexpression		Arikawa et al. (2003)
Melanoma B16 cells	ERK activation Cell migration					
Human coronary smooth muscle cells	cAMP production	Up to 10 μM	$S1P_2$ induces cAMP production through PGI_2	siRNA-mediated $S1P_2$ knockdown		Damirin et al. (2005)
Mouse spiral modiolar artery	Contraction	Up to 3 μM	$S1P_2$ receptors mediate modiolar artery contraction			Kono et al. (2007)
HUVEC	Permeability	0.2 μM	$S1P_2$ increases vascular permeability	$S1P_2$ overexpression		Sanchez et al. (2007)
Rabbit bladder smooth muscle	Contraction	1 μM	S1P contracts bladder smooth muscle via $S1P_2$		Phospho-FTY720 also contracted	Watterson et al. (2007)
U373 glioblastoma cells	uPAR and PAI-1 mRNA expression	1 μM	S1P induces uPAR and PAI-1 mRNA expression via $S1P_2$	siRNA-induced $S1P_2$ knockdown		Bryan et al. (2008)
WiT46 cells	CTGF expression	1 μM	S1P induces CTGF expression via $S1P_2$	$S1P_2$ overexpression		Li et al. (2008a)
Rat vascular smooth muscle cells	Cell migration and Rac activation	1 μM	S1P inhibits cell migration via $S1P_2$	$S1P_2$ overexpression		Takashima et al. (2008)
Rat/mouse basilar artery	**Contraction**	**10 μM**	**$S1P_3$ mediate basilar artery constriction JTE-013 is not specific for $S1P_2$**	**$S1P_2$ and $S1P_3$ knockout mice**		**Salomone et al. (2008)**
Rat vascular smooth muscle cells	Proliferation	1 μM	S1P inhibits cell proliferation via $S1P_2$			Wamhoff et al. (2008)
Hamster resistance arteries	Contraction	1 μM	S1P induces contraction via $S1P_2$	$S1P_2$ knockdown by antisense oligonucleotide	No proof of $S1P_2$ mRNA or protein decrease	Peter et al. (2008)
Mouse neural progenitors	Cell migration	0.25 nmol/h, icv	S1P inhibits cell migration via $S1P_2$	shRNA-mediated $S1P_2$ knockdown		Kimura et al. (2008)
Human mast cells	Cytokine/ chemokine secretion	100 nM	S1P degranulates mast cell and modulates anaphylaxis via $S1P_2$	siRNA-induced $S1P_2$ knockdown		Oskeritzian et al. (2010)

(continued)

Table 1 | Continued

Mouse	Passive anaphylaxis	20 μg/mouse		$S1P_2$ knockout mice	
Hamster resistance artery	Myogenic tone	1 μM	S1P induces myogenic tone via $S1P_2$		Lidington et al. (2009)
Isolated perfused mouse lung	Vasoconstriction	10 μM	S1P modulates pulmonary vascular tone *in vivo* via $S1P_2$	$S1P_2$ knockout mice	Szczepaniak et al. (2010)
Mouse	Proatherosclerotic cytokine release	1.2 mg/kg	$S1P_2$ receptors regulate macrophage retention and inflammatory cytokine secretion	$S1P_2$ knockout mice	Skoura et al. (2011)

endothelium (Salomone et al., 2008). We interpreted this finding as a result of $S1P_1$ antagonism exerted by VPC23019, because $S1P_1$ receptors located on vascular endothelium are known to stimulate nitric oxide release and induce vasodilatation (and may therefore counteract vasoconstriction induced by S1P through $S1P_3$ receptors). However, we were surprised not to observe inhibition of S1P-induced vasoconstriction by VPC23019, at concentrations as high as 10 μM, when solid evidence ($S1P_3$ knockout mice) indicated that S1P-induced vasoconstriction was mediated by $S1P_3$ receptors. Indeed, Murakami et al. (2010) showed that the novel potent and selective $S1P_3$ antagonist TY-52156 restores coronary blood flow reduced by S1P, but VPC23019 was inactive in this system. Interestingly, the same study also showed that in isolated coronary smooth muscle cells, TY-52156 inhibited both Rho activation and Ca^{2+} signal, whereas VPC23019 only inhibited Ca^{2+} signal. Taken together with our data, this study suggests that results obtained with VPC23019 should be interpreted with caution.

While we are emphasizing in this *Perspective* issues related to three particular receptor antagonists, our purpose is to make a broader methodological point on the distinction between selectivity and specificity. The term "selectivity" should refer to the ability of a drug to discriminate between related targets (e.g., receptors or enzymes), showing a higher binding affinity for one subtype or isoform. Selectivity should be assessed by screening in pure systems (e.g., cell lines transfected with one receptor subtype at a time) and eventually in complex systems, including *in vivo* wild type and genetically altered models. The term "specificity" should refer not only to the ability of a drug to identify a receptor of interest, but also to its potential for discriminating between negative and positive interactions; i.e., the drug should bind the receptor with appropriate affinity, the drug should have low/no cross-reactivity with other receptors. In order for "specificity" to be assessed, the drug should therefore be screened for its interaction with the target of interest and for its interaction with as many unrelated biological targets as possible. For receptors, ligand binding studies may be considered adequate to quantitatively assess affinity for the binding sites under examination, but require further assessment of function, in the presence and absence of the reference agonist, to categorize a given ligand as agonist or antagonist.

Until the end of 1980s, pharmacological studies were mainly based on the use of agonists and antagonists (Salomone, 2010). More recently, investigators have also manipulated the expression levels of receptors using molecular techniques and genetic alterations. This approach represents, under critical evaluation, the most specific pharmacologic strategy available today. When using a drug as an agonist or antagonist, one has to consider the probability of off-target effects; instead, when using a receptor knock out animal, one can exclude, with great degree of confidence, that an effect is mediated by the receptor product of the deleted gene. We therefore believe that such a molecular genetic control, whenever available, should be considered the best validation in analytical and experimental pharmacology. Unfortunately, investigational pharmacological agents, agonists or antagonists, are increasingly being used to come to pharmacologic conclusions on receptor function, even though data on their selectivity is seldom sufficient and little information on their specificity is available. As this *Perspective* demonstrates, these conclusions are sometimes drawn despite conflicting evidence (see shaded rows in **Tables 1 and 2**), such as that coming from gene-deletion studies.

In conclusion, experimental ligands not used in humans (for which extensive pre-clinical characterization has uncovered potential off-target actions) and for which only limited information from simple systems (*in vitro*, transfected cell lines) is available should be used with caution and pharmacological data obtained with them should be considered as weak evidence, unless supported by consistent stronger evidence (for example genetic knockdown or knockout data, or concurrent pharmacological evidence obtained with other chemically unrelated ligands). Furthermore, negative and/or conflicting data obtained with experimental ligands should be considered and quoted; in other words, negative and/or conflicting data should be used for retrospective analysis and to interpret data already published with these compounds. Finally, potential off-target effects, including non-receptor-mediated effects, should always be considered and might sometimes be suspected by looking at the reversibility (many receptor-mediated effects are reversible, toxic non-specific effects are often irreversible), concentration–response or dose–response relationship, or at the kinetics of the response (some effects are too slow or too fast to be compatible with a given receptor function).

Table 2 | Published data obtained with BML-241/CAY10444.

System	Effect	Concentration	Inferred significance	Validation	Conflicting data/ caveat	Reference
Transfected HeLa cells	$[Ca^{2+}]i$ increase	10 μM	Blocks $S1P_3$ receptors	No effect on $S1P_1$-Hela		Koide et al. (2002)
Endothelial cells	Expression of adhesion molecules	1–10 μM	$S1P_3$ receptors modulate adhesion molecule expression	Antisense oligonucleotides		Kimura et al. (2006)
Pancreatic Islet β Cells	Cell survival	10 μM	$S1P_3$ receptors mediate protective effects on β-cells against cytokine-induced apoptosis.	None		Laychock et al. (2006)
B lymphocytes	Rap1 activation	10 μM	$S1P_3$ receptors activate Rap1 and might promote B-cell adhesion and migration	None		Durand et al. (2006)
Transfected CHO cells	**$[Ca^{2+}]i$ increase**	**10 μM**	**BML-241 is not selective**			**Jongsma et al. (2006)**
Astrocytes	Cell migration	10 μM	No role of $S1P_3$	none		Sato et al. (2007)
Fibroblast-like synoviocytes	Cell migration Cytokine/ chemokine secretion Cell survival	5 μM	S1P stimulates FLS migration through $S1P_1$ and $S1P_3$, induces cytokine/chemokine secretion through $S1P_2$ and $S1P_3$, and protects from cell apoptosis via $S1P_1$.	None		Zhao et al. (2008)
Multiple myeloma cells	Cell survival	10 μM	$S1P_3$ receptors mediate S1P-induced STAT3 phosphorylation and Mcl-1 upregulation	None		Li et al. (2008b)
Keratinocytes	$[Ca^{2+}]i$ increase	50 μM	$S1P_3$ receptors mediate $[Ca^{2+}]_i$ increase	No effect of SEW2871 ($S1P_1$ agonist), inhibition by VPC23019	Specificity of SEW2871 and VPC23019 non-fully characterized	Lichte et al. (2008)
Transfected HEK-293 cells	$[Ca^{2+}]i$ increase	50 μM	$S1P_3$ receptors mediate $[Ca^{2+}]i$ increase	No effect of SEW2871 ($S1P_1$ agonist), inhibition by VPC23019	Specificity of SEW2871 and VPC23019 non-fully characterized	Lichte et al. (2008)
Mouse cardiomyocytes	Erk activation	0.1–10 μM	No role of $S1P_3$	none		Tao et al. (2009)
B35 neuroblastoma cells	Cell migration	10 μM	No role of $S1P_3$	No effect with other $S1P_3$ antagonists (VPC23019, VPC25239)		Hans et al. (2009)
Embryonic stem cells	Erk activation	1–10 μM	$S1P_5$, not $S1P_3$, activate Erk1/2	None		Rodgers et al. (2009)

(continued)

Table 2 | Continued

Tango EDG3 cell lines	GPCR β-arrestin recruitment and deactivation	1 nM–0.1 mM	The EDG3 response to S1P is inhibited by CAY-10444 with an IC_{50} of 4.6 μM	None		Wetter et al. (2009)
Bronchial smooth muscle	Contractility	10 μM	No role of $S1P_3$	none		Chiba et al. (2010)
Granulosa lutein cells	Cell migration	1 μM	$S1P_3$ receptors are involved in FF-HDL- and S1P-stimulated granulosa cell migration	Effect of suramin	Suramin is non-specific	Becker et al. (2010)
Mouse cardiomyocytes	Cell viability	10 μM	$S1P_3$ receptors play a role in Akt activation and cardiomyocyte viability	none		Tao et al. (2010)
Coronary artery	Vasorelaxation	10 μM	$S1P_3$ receptors mediate endothelium dependent coronary relaxation	No effect of W146 ($S1P_1$ antagonist), but inhibition by VPC23019 (mixed $S1P_1$/$S1P_3$ antagonist)	Specificity of VPC23019 not fully characterized	Mair et al. (2010)
Immature B lymphocytes	Cell migration	100 μM	$S1P_3$ receptors are responsible for immature B-cell chemotaxis to S1P	$S1P_3^{-/-}$ immature B-cells unable to migrate to S1P		Donovan et al. (2010)
Estrogen receptor-positive breast cancer cells	Cell migration	10 μM	$S1P_3$ stimulates the accumulation of phosphor-ERK-1/2 and promotes cell migration	siRNA knockdown		Long et al. (2010)
Ovarian cancer cells	Cell proliferation and survival	1 μM	$S1P_3$ receptors do not play a role in cell proliferation and survival	None		Illuzzi et al. (2010)
Mesenteric venules	Microvessel permeability	10 μM	$S1P_3$ receptors do not play a role in the protection by S1P of PAF-induced permeability	None		Zhang et al. (2010)

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 December 2010; accepted: 09 February 2011; published online: 22 February 2011.

Citation: Salomone S and Waeber C (2011) Selectivity and specificity of sphingosine-1-phosphate receptor ligands: caveats and critical thinking in characterizing receptor-mediated effects. Front. Pharmacol. ***2****:9. doi: 10.3389/fphar.2011.00009*

This article was submitted to Frontiers in Experimental Pharmacology and Drugs Discovery, a specialty of Frontiers in Pharmacology.