

LETTER

Structural basis of allosteric and bitopic ligands binding in sphingosine-1-phosphate receptors 2 and 3

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Dear Editor,

Allosteric ligands of G protein-coupled receptors offer advantages over orthosteric ligand in subtype selectivity, spatiotemporal sensitivity, and potentially biased signaling, yet their discovery remains challenging. Here, we report two crystal structures of human sphingosine-1-phosphate receptor 3 (S1P₃) in complex with the simultaneously binding of bitopic ligand SPM-242 and allosteric ligands Cpd-32 or CYM52581. To further reveal the inhibition mechanism of antagonists and ligand subtype selectivity, two cryo-electron microscopy structures of S1P₂ and S1P₃ in complex with heterotrimeric G_i protein were solved. The S1P₃ complexes reveal an allosteric site that lies outside of the helical bundle in S1P₃ receptor, which is a new site recognized by allosteric modulators in class A GPCR. Structural comparison further explains the selectivity of bitopic antagonist SPM-242 and allosteric antagonists CYM52581 on S1P₃ over S1P₁. These structural studies, together with functional assays, provide structural insights into the bitopic and allosteric antagonism of class A GPCRs, which will further facilitate the design of selective drugs targeting these receptors.

The S1P receptor family (S1P₁–S1P₅) regulates cell survival, cell migration, and inflammation. Endogenous agonist Sphingosine-1-phosphate (S1P), derived from

the sphingomyelin metabolism, is abundant (>200 nmol/L) in plasma (Hammad et al., 2012). Among the S1Ps, S1P₂ suppresses tumor angiogenesis bone loss, while S1P₃ drives metastasis and disrupts the blood-tumor barrier, making it attractive therapeutic target for cardioprotection, fibrosis-related diseases, and breast cancer. However, existing ligands (e.g. JTE-013) (Chew et al., 2016) lack specificity or potency, hindering clinical translation (Fig. 1A). Thus, allosteric agonists, as well as the novel bitopic agonist for S1P₂ and S1P₃ had been screened and CYM-5520 and CYM-5541, which had been used as the therapy for osteoporosis and ischemia-reperfusion injury, were discovered (Jo et al., 2012; Satsu et al., 2013) (Fig. 1A–C). In addition, SPM-242 was screened for treating multiple diseases, including sepsis and asthma (Jo et al., 2012; Kohno et al., 2004) (Fig. 1A and 1D). Meanwhile, Cpd-32 and CYM52581 have been investigated to treat pain and cardiopulmonary diseases, respectively (Fig. 1A, 1E and 1F).

Here, we present two crystal structures S1P₃-SPM-242-Cpd-32 and S1P₃-SPM-242-CYM52581 at 3.0 Å and 3.6 Å resolution, respectively (Fig. 1G; Table S1). To reveal its antagonism, we also report cryo-electron microscopy (cryo-EM) structures of S1P₂ or S1P₃ in complex with G_i protein (Fig. 1H). Among these four structures,

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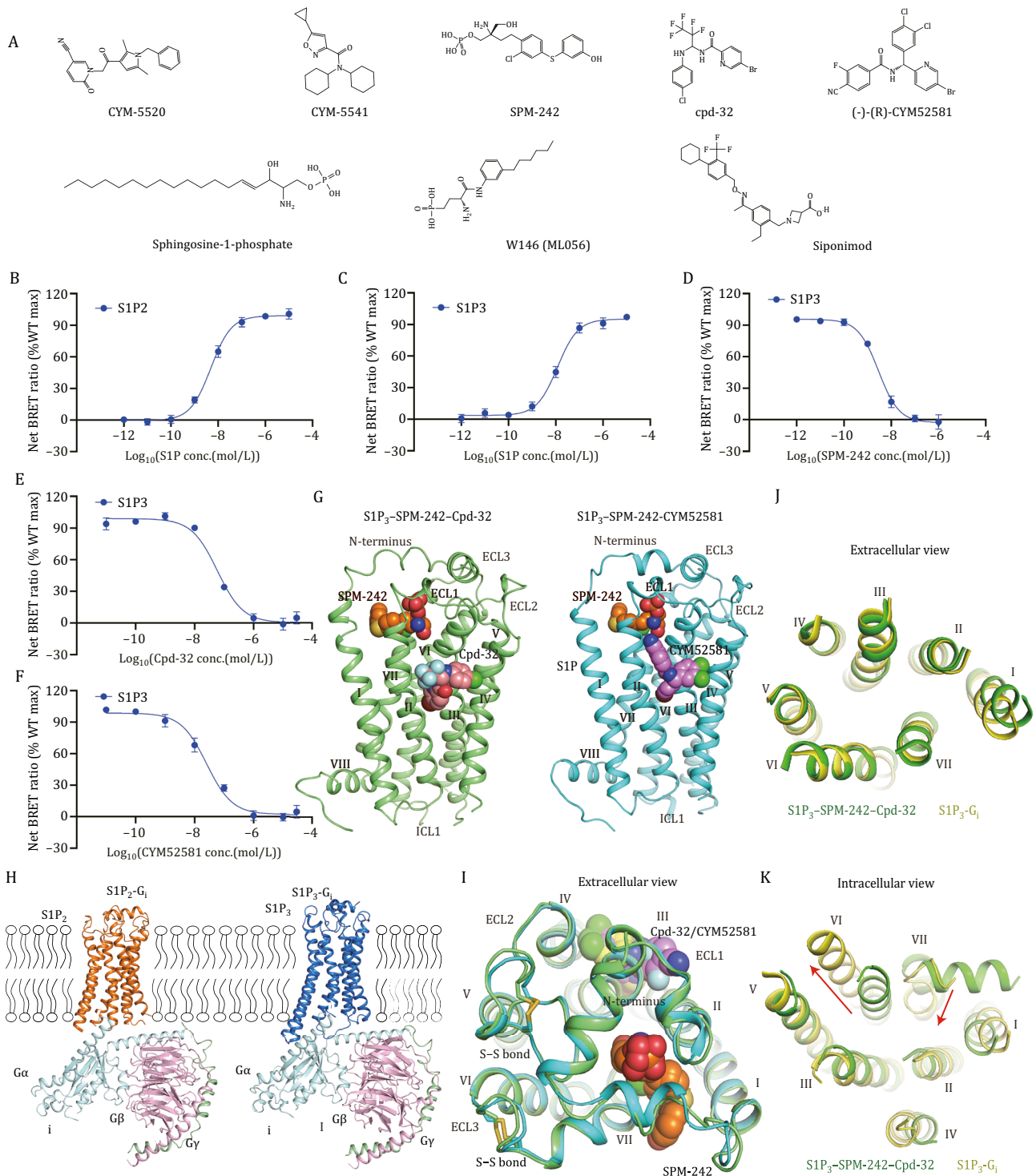


Figure 1. Structural, functional, and pharmacological profiling of S1P receptors modulators. (A) Structure of different ligands: CYM-5520, CYM-5541, (R)-SPM-242, Cpd-32, CYM52581, S1P, W146, and Siponimod. (B and C) TRUPATH assays of agonist S1P induced G_i accumulation of wild-type S1P₂ (B) and S1P₃ (C). (D–F) Trupath assay of antagonist SPM-242 (D), Cpd-32 (E), and (CYM52581) (F) induced G_i accumulation of wild-type S1P₃. At least three independent experiments were performed in duplicate, and data are shown as mean ± SEM. [Tables S3](#) and [S4](#) for detailed statistical evaluation. (G) Crystal structures of S1P₃ with SPM-242 and Cpd-32, and S1P₃ with SPM-242 and CYM52581. The structures are shown in cartoon representation, with the receptors colored lime and cyan, respectively. The ligands SPM-242, Cpd-32 and CYM52581 are shown in spheres representation and colored orange, yellow, and violet, respectively. (H) Cryo-EM structures of S1P₂-G_i and S1P₃-G_i complexes. S1P₂ and S1P₃ are shown in orange and marine cartoon representation, respectively. G_α, G_β, and G_γ are colored light-cyan, light-pink, and light-green, respectively. (I) Extracellular view of crystal structures. The disulfide bonds are shown as yellow sticks. (J and K) Extracellular view (J) and Intracellular view (K) of inactive and active S1P₃ structures. The red arrows indicate the large conformation change of helix VI and VII.

S1P₃-SPM-242-Cpd-32 and S1P₃-SPM-242-CYM52581 exemplify the inactive states, while S1P₂-G_i and S1P₃-G_i complexes represent the active state of S1P₂/S1P₃ receptor (Table S1). These engineered receptors retain native like binding of S1P, SPM-242, Cpd-32, and CYM52581 as well as synergistic stabilization of orthosteric and allosteric ligands (Fig. S1A-D). Together with extensive functional assays, these structures provide a structural framework for bitopic and allosteric ligand-binding mode and selectivity as well as synergistic inhibition mechanisms of different types of antagonists (Fig. S2A and S2B). Strong and unambiguous electron densities are observed for all ligands and their interacting residues in the S1P₂ and S1P₃ structures (Fig. S3-5).

The S1P₃-SPM-242-Cpd-32 and S1P₃-SPM-242-CYM52581 complexes share similar the seven-transmembrane (7TM) helical bundle with a α atom root-mean square-deviation (r.m.s.d.) of 0.61 Å (Fig. 1I). Thus, we will primarily focus on the S1P₃-SPM-242-Cpd-32 complex due to its higher resolution. S1P₃ adopts a canonical seven-transmembrane architecture, with its N-terminal α -helical occluding the extracellular ligand-binding pocket. Two disulfide bonds are observed within extracellular loop 2 (ECL2) and extracellular loop 3 (ECL3) of S1P₃, which are coincident with S1P₁, S1P₂, and S1P₅ structures (Chen et al., 2022; Yuan et al., 2021; Zhao et al., 2022) (Fig. 1I). The conserved disulfide bonds constrain the conformation of ECL2 and ECL3, further stabilizing the conformations of ECL1 and N terminus and enclosing the hydrophobic ligands (Fig. 1I). A structural comparison of the S1P₃-SPM-242-Cpd-32 complex with the S1P₃-G_i structure reveals similarities on the extracellular side (Fig. 1J). However, a significant conformational difference emerges in the transmembrane region: helix VI exhibits a pronounced outward displacement of 8.5 Å (measured at the α of R237^{6.29}), while helix VII shifts inward by 6.5 Å (measured by the α of T299^{7.54}) (Fig. 1K). These movements highlight distinct rearrangement associated with S1P₃ receptor activation.

SPM-242 contains a polar, zwitterionic head group and a hydrophobic tail (Jo et al., 2012) (Fig. 1A). It occupies both the orthosteric pocket shaped by N-terminus, helices II-III, ECL1, and an allosteric pocket formed by helices I, II, and VII, which is different from W146 (ML056), S1P and Siponimod in all solved S1P receptors (Chen et al., 2022; Liu et al., 2013; Hanson et al., 2012; Sanna et al., 2006; Yuan et al., 2021) (Figs. 1I, S6A and S6B). To accommodate SPM-242, the extracellular tip of helix I in S1P₃ strikes outwardly away from helix VII by 2 Å (referenced by α of T42) in comparison with the S1P₁-W146 complex (Fig. S6C and S6D). As a result, the binding site of SPM-242 is much closer to the extracellular surface when compared with W146, as it inserted in the gap between helices I and VII instead of occupied in the helical bundle (Fig. S6E).

SPM-242's zwitterionic head mimics S1P's phosphate/amine groups, mediating high-affinity ligand binding via polar interactions with Y22, N95^{2.60}, R114^{3.28}, E115^{3.29}, S99^{ECL1}, and T103^{ECL1} (superscript indicates residue number according to the Ballesteros-Weinstein nomenclature) (Ballesteros and Weinstein, 1995) (Fig. 2A and 2B). Alanine substitutions of Y22, N95^{2.60}, R114^{3.28} or E115^{3.29} impaired S1P-induced G_q activation, while S99^{ECL1}A / T103^{ECL1}A mutations reduced SPM-242 inhibition by 10- and 22-fold, respectively (Fig. S7A-C and Table S2).

Unlike ligands such as S1P-bound W146 (Hanson et al., 2012), the hydrophobic moiety of SPM-242 extends to a unique allosteric sites (helices I, II and VII) (Fig. S6C and S6D). Non-conserved residue I284^{7.39} forms hydrophobic interactions with SPM-242's chlorobenzene group, and its alanine mutation (7-fold binding loss) or leucine (complete inhibition loss) highlights the importance of steric constraints (Figs. 2A, 2B, S7D, S7E and Table S2). Upon binding of SPM-242, the large side chain of F46^{1.39} rotates about 70°C and forms hydrophobic interactions with I96^{2.61} and Y92^{2.57} in the helix II (Fig. S6D). The side chain rotation pushes helix I outwardly to facilitate SPM-242 binding (Fig. S6D). Replacement F46^{1.39} with alanine antagonistic effect by 2-fold, implying the importance of the interactions between F46^{1.39} (Fig. S7F and Table S2). Further, mutating T42^{1.35} and V285^{7.40} or W282^{7.37} moderately decreased the antagonistic effects of SPM-242 (Fig. S7G-I and Table S2). While Q281^{7.36} enhances the antagonist by disrupting unfavorable hydrophilic interactions (Fig. S7J and Table S2).

Cpd-32 and CYM52581, two structurally distinct S1P₃ allosteric antagonists, share a lipid-facing pocket shaped by helices II-IV (Fig. 1A and S8A-C). Unlike PAR2 (AZ3451) and CB1 (ORG27569), which utilize helices II-IV as binding sites, the binding sites of Cpd-32 and CYM52581 are much closer towards the extracellular membrane surface (Fig. S8A-H).

Cpd-32 is composed of a bromopyridine-carboxamide, a pentafluoropropyl and a chloroaniline moiety forming an acylaminal scaffold (Figs. 1A, 2C, and 2D). The nitrogen atoms of the acylaminal form a hydrogen bond with the side chain of S117^{3.31}, which helps the recognition of Cpd-32 to S1P₃ (Fig. 2C and 2D). Mutating this residue to alanine substantially reduced the inhibitory potency of Cpd-32 by ~33-fold, which almost eliminates the inhibition by this antagonist (Fig. 2E and Table S2).

The bromopyridine of Cpd-32 moiety binds a hydrophobic pocket (helices II-IV) involving L87^{2.52}, A91^{2.56}, V120^{3.34}, W162^{4.50}, and F166^{4.54} (Fig. 2C-D), and mutating these residues appeared 8-30-fold of ligand binding (Fig. 2E, 2F and Table S2). The pentafluoropropyl moiety of Cpd-32 fits into a sub-pocket shaped by helices II and III (A91^{2.56}, V94^{2.59}, and L113^{3.27}) (Fig. 2C and 2D). SAR data suggested that replacement of the pentafluoropropyl

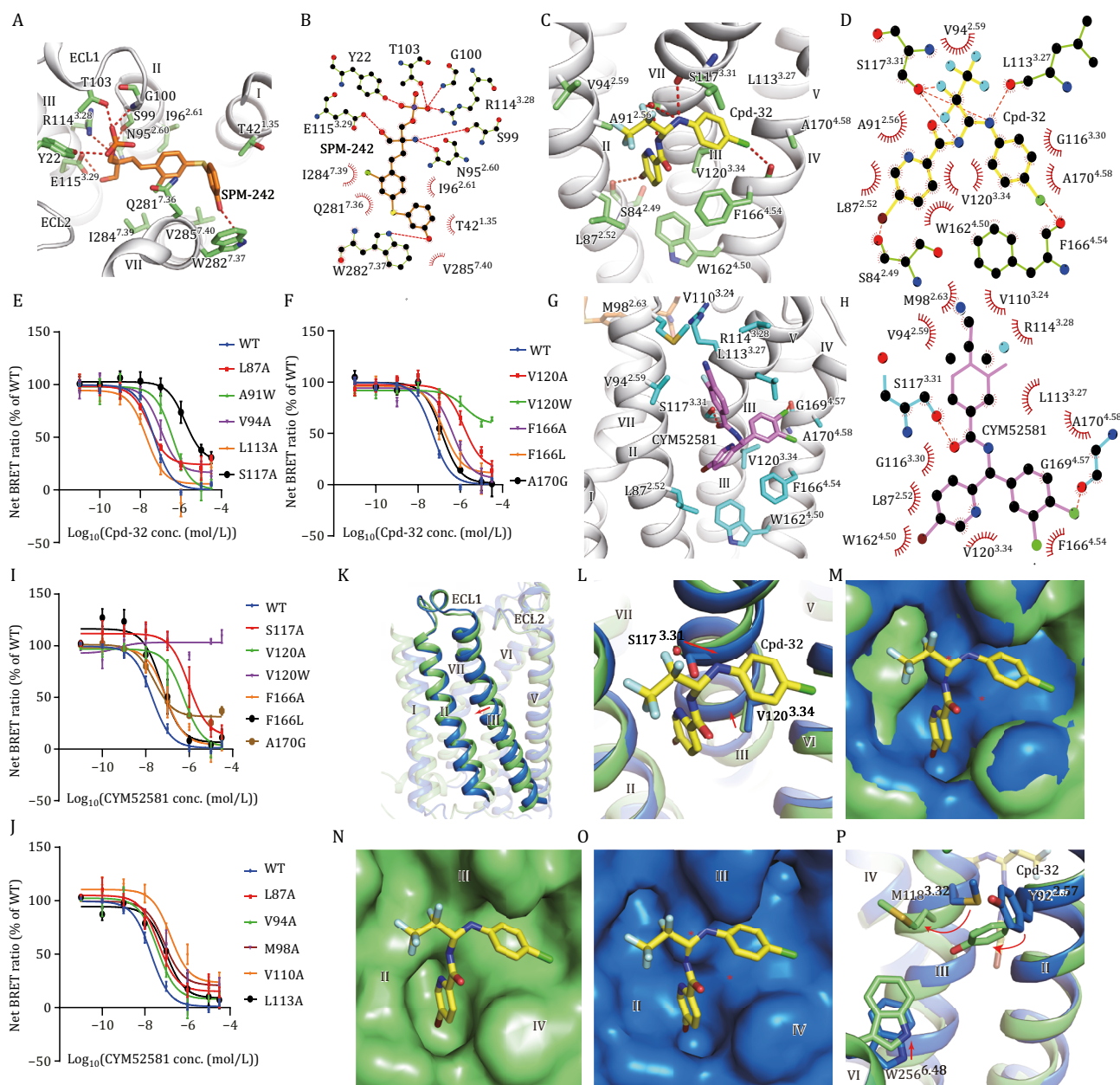


Figure 2. Ligand-binding modes and interaction networks of S1P₃ receptor. (A) Ligand-binding pocket of SPM-242. S1P₃ is shown in gray cartoon representation. The ligand SPM-242 (orange carbons) and S1P₃ residues (lime carbons) are shown in sticks representation. Hydrogen bonds are red dashed lines. (B) LigPlot+ diagram indicates the S1P₃ residues (lime, labeled according to Ballesteros-Weinstein numbering) interacting with SPM-242 (orange carbons). (C) Ligand-binding pocket of Cpd-32. S1P₃ is shown in gray cartoon representation. Cpd-32 (yellow carbons) and S1P₃ residues (lime carbons) are shown as sticks. Hydrogen and halogen bonds are shown as red dashed lines. (D) LigPlot+ diagram indicates the S1P₃ residues interact with Cpd-32 (yellow carbons). (E and F) Trupath assays of antagonist Cpd-32. At least three independent experiments were performed in duplicate, and data are shown as mean $\pm$ SEM. **Table S2** for detailed statistical evaluation. (G) Ligand-binding pocket of CYM52581. S1P₃ is shown in gray cartoon representation. CYM52581 (violet carbons), SPM-242 (orange carbons), and S1P₃ residues (cyan carbons) are shown as sticks. Hydrogen and halogen bonds are shown as red dashed lines. (H) Schematic representation of the interactions between S1P₃ and CYM52581 analyzed using the LigPlot+ program. (I and J) Trupath assays of antagonist CYM52581. At least three independent experiments were performed in duplicate, and data are shown as mean $\pm$ SEM. **Table S2** for detailed statistical evaluation. (K) Structural superimposition of S1P₃ in the inactive and active states. The inactive and active S1P₃ structures are shown in cartoon representation, with the receptors colored lime and marine, respectively. Structural superimposition of inactive and active S1P₃ shows relative movements in helix III0020 of those two structures, which are highlighted by a red arrow. (L) The conformational changes of allosteric ligand-binding site during receptor activation. S117^{3.31} and V120^{3.34} are shown as lime and marine sticks in the inactive and active S1P₃, respectively. The red arrows indicate the conformational changes of those residues. Cpd-32 are shown as yellow sticks. (M–O) The structures of inactive

group of Cpd-32 with a longer dodecafluoropropyl group or a rigid chlorobenzene substituent results in severe loss of antagonistic potency by 45- or 88-fold (Nguyen et al., 2012), probably due to steric clashes with the side chains of A91^{2.56} or V94^{2.59} (Fig. 2E and Table S2).

Cpd-32's chloroaniline group engages helices III and IV via hydrophobic interactions with L113^{3.27}, V120^{3.34}, F166^{4.54}, and A170^{4.58} (Fig. 2C and 2D). A halogen bond between its chlorobenzene and F166^{4.54}'s backbone carbonyl further stabilizes binding. SAR data showed that replacement of the chloroaniline by dimethylcyclohexane results in a dramatic drop (516-fold) in ligand potency (Nguyen et al., 2012). In addition, TRUPATH assay suggests the replacement of the side chain of V120^{3.34} with a bulky side chain of tryptophan resulted in a complete loss of inhibitory potency of Cpd-32, which might be caused by the introduction of the steric hindrance with this ligand (Fig. 2F and Table S2). Different from Cpd-32, CYM52581 features a bromopyridine, a dichlorobenzene, and a fluorobenzonitrile residue connected by a central carboxamide moiety (Figs. 1A, 2G, and 2H). Unlike Cpd-32, its carboxamide orients closer to helix III (Fig. S8G and S8H), enabling its carbonyl oxygen atom of the carboxamide core of CYM52581, instead of a nitrogen atom in the acylaminal scaffold of Cpd-32, forms a hydrogen bond with S117^{3.31} (Fig. 2G and 2H).

The bromopyridine moiety of CYM52581 also binds to the hydrophobic pocket formed by helices II-IV (Fig. 2G). Replacement of S117^{3.31}, V120^{3.34}, and F166^{4.54} in the pocket to alanine reduced the affinity of CYM52581, mirroring Cpd-32 effects (Fig. 2I and Table S2). However, its bromopyridine shifts closer to helix II, weakening F166^{4.54}'s interaction (Fig. 2G). Indeed, mutating F166^{4.54} to alanine showed less impact on ligand potencies (9-fold vs. 4-fold), highlighting the importance of the hydrophobic interactions in ligand binding (Fig. 2I and Table S2). Its *para*-chlorine rotates ~60° and points to upper helix IV compared with Cpd-32 (Fig. S8G and S8H), forming a halogen bond interaction with the backbone carbonyl of G169^{4.57} (Fig. 2G and 2H). SAR data showed a 7-fold loss of antagonistic effect when the dichlorobenzene is replaced with a benzyl substituent (Roberts et al., 2019). In addition, the *meta*-chlorine atom forms a hydrophobic interaction with F166^{4.54}, and alanine mutation of this residue exhibited a 4-fold potency loss according to the TRUPATH assay (Fig. 2G-I and Table S2). This aligns well with previous SAR studies which showed that substitution of

dichlorobenzene with chlorobenzene decreased the inhibition by 1.6-fold (Roberts et al., 2019).

The bitopic antagonist SPM-242 and allosteric antagonists Cpd-32 or CYM52581 are preventing the receptor activation through different mechanisms. The bitopic antagonist SPM-242 is most likely to inhibit the receptor activation by occupying the endogenous ligand pocket and preventing the access of the agonist (Fig. S6A and S6B). As described above, the zwitterionic head of SPM-242 binds to the orthosteric pocket formed by N terminus, helices II-III, and ECL1, which forms direct competition with the phosphonate and amine groups of the endogenous ligand S1P (Fig. S6B). In addition, the hydrophobic tail of SPM-242 occupies the tunnel formed by helices I, II, and VII (Fig. S6C and S6D). Unlike allosteric antagonists (Cpd-32/CYM52581), SPM-242 likely obstructs ligand access via membrane pathways, analogous to S1P ligand binding (Fig. S6E).

On the contrary, the allosteric ligands Cpd-32 or CYM52581 bind helix III, restricting the inter-helical rearrangement critical for S1P₃ activation. Structural superimposition of S1P₃ in the active and inactive states showed moderate structural shifts (Cα r.m.s.d. 1.62 Å), including helix III shifts to the helix II in the active (Fig. 2K). This displaces S117^{3.31} (2.6 Å outward) and V120^{3.34}, collapsing the allosteric ligand-binding cavity (Fig. 2L-O). Vice versa, ligand binding locks helix III in inactive state and antagonizes the receptor by preventing one of the key conformational changes during activation (Zhao et al., 2022). In addition, the side chain of M118^{3.32} flips away from helix II and points to the center of the helical bundle upon receptor inhibition, while the side chain of Y92^{2.57} also undergoes the notable rotation towards the center of the helical bundle from helix VII (Fig. 2P). As a result, the side chains of Y92^{2.57} and M118^{3.32} form hydrophobic interactions with transmission microswitch W256^{6.48}, which mimic the antagonist and further prevent the receptor activation, stabilizing the receptor in the inactive state (Fig. 2P).

As the bitopic and allosteric ligands bind to two completely different pockets of S1P₃ and inhibit the receptor in distinct mechanisms, the simultaneous binding of these ligands is synergistic. The binding of allosteric ligands, which stabilize the receptor in the inactive state, further facilitates the binding of bitopic ligand SPM-242. Thermo-stability assays show SPM-242 alone minimally effects stability, while Cpd-32/ CYM52581 increase stability by ~8°C. Co-binding both ligands further boosts stability by ~12°C (Fig. S2A and S2B).

and active S1P₃ are shown in lime and marine surface representation, respectively. And allosteric ligand Cpd-32 are shown as yellow sticks. Cpd-32 binding surface on the inactive S1P₃ (N) and the corresponding surface of active S1P₃ with Cpd-32 (O). A red asterisk indicates the steric hinderance between Cpd-32 and the surface of active S1P₃. (P) M118^{3.32}, L122^{3.36} and W256^{6.48} in inactive and active S1P₃ are shown as lime and marine sticks, respectively. Cpd-32 is shown in yellow sticks representation. The red arrows indicate the conformational changes of Y92^{2.57}, M118^{3.32} and W256^{6.48} in inactive S1P₃ relative to those residues in the active S1P₃.

S1P₃ and S1P₁ share high sequence homology (49% identity; C α r.m.s.d. 0.89 Å), with only 3 orthosteric pocket residue differences (Hanson et al., 2012). However, the bitopic binding site of SPM-242 in S1P₃ provides the second binding site, which is relatively divergent, and there are 4 residue differences within the hydrophobic pocket composed of a total of 6 amino acids (Figs. S6A and S9). The high sequence variety might serve for the high selective ligand design, and indeed SPM-242 exhibits a high selectivity of S1P₃ over S1P₁ (Jo et al., 2012). The four residues, I96^{2.61}, Q281^{7.36}, W282^{7.37}, and I284^{7.39}, might be the determinants of the selectivity for SPM-242. Site-directed mutagenesis showed that the replacement of residues such as I284^{7.39} with corresponding residue leucine in S1P₁ decreased the antagonistic effect by 11-fold in the presence of SPM-242 (Table S2).

Besides the bitopic site, the allosteric site of S1P₃, though conserved across S1P receptors, exhibits greater sequence divergence (5/13 residue differences) enabling selective ligand design. CYM52581 achieves 140-fold selectivity toward S1P₃ over S1P₁. Among the 5 residues, V94^{2.59}, M98^{2.63}, and V110^{3.34} formed a hydrophobic environment to accommodate the fluorobenzonitrile moiety of CYM52581, and mutating them to the counterpart residues greatly impaired the binding of allosteric ligands (Fig. 2G, 2H, 2J and Table S2). For instance, mutating V110^{3.34} to glutamine or leucine of other S1P receptors dramatically decreases the antagonistic effect of CYM52581 by 75- or 56-fold, potentially due to sterically hindering the CYM52581 binding of larger side chains (Table S2). In addition, V94^{2.59}A or M98^{2.63}L mutants, which possibly weaken the hydrophobic interactions, also decreased the antagonistic effect of CYM52581 by 2- or 7-fold (Fig. 2J and Table S2). The other two different residues in the allosteric site, F166^{4.45} and A170^{4.58}, form hydrophobic interactions with the chlorine atoms of chlorobenzene and serve for the ligand selectivity of S1P₃ (Fig. 2G and 2H). Mutating these residues to corresponding residues as in other S1P receptors showed ~2-fold decrease of the CYM52581 binding (Fig. 2I and Table S2). Thus, the high sequence variation within the allosteric pocket might serve for the specific ligand design targeting each of the S1P receptors.

In this study, we have determined two distinct crystal structures of inactive S1P₃ receptor in complex with the bitopic antagonist SPM-242 as well as the allosteric antagonist Cpd-32 or CYM52581. Additionally, we also solved the cryo-EM structures of S1P₂ and S1P₃ in complex with G_i proteins. These structures offer a comprehensive understanding of the unique binding mechanism of the bitopic ligand SPM-242 and the process of receptor activation. Unlike previous S1P receptor structures, SPM-242 simultaneously occupies the orthosteric site and blocks the ligand access entrance.

Supplementary data

Supplementary data is available at *Protein & Cell* online <https://doi.org/10.1093/procel/pwaf068>.

Footnotes

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Y.W. optimized the construct, developed the purification procedure, purified the S1P₃ receptors for crystallization and performed crystallization trials and designed the functional assays, and prepared the manuscript. Q.C. prepared the protein sample for cry-EM studies and performed TRUPATH assay. H.W., K. L., and J. W. helped with the functional assay. M.W. and K.C. collected X-ray diffraction data. M.W. and S.H. collected cryo-EM data, performed cryo-EM data processing. Y.Z. helped with designing functional assay. C.Y. and L.M. expressed the proteins. C.G., G.S., M.G., and A.P. supported the identification of the tool compounds (SPM-242, Cpd-32 and CYM52581) and commented on the manuscript. E.S. determined potencies of S1P₃ antagonists. Q.T. helped with functional assay and performed cryo-EM data processing, solved cryo-EM structure and wrote the first draft. Q.Z. and B.W. initiated the project, planned and analyzed experiments, supervise the research, and wrote the paper. No AI-assisted technologies were used in this work.

The authors declare no competing interests. All subjects enrolled in this study were informed and consent was acquired. The authors declare their agreement to publish.

Atomic coordinates for the structures of S1P₃-SPM-242-Cpd-32, and S1P₃-SPM-242-CYM52581 have been deposited in the RCSB Protein Data Bank (PDB) under accession codes 9W0H and 9W0L. Atomic coordinates

and cryo-EM density maps for the structures of inactive S1P₂-G_i and S1P₃-G_i have been deposited in the RCSB Protein Data Bank (PDB) under accession codes 9WOM and 9W0O, and the Electron Microscopy Data Bank (EMDB) under accession codes EMD-65508 and EMD-65510.

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