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Demystifying P2Y₁ Receptor Ligand Recognition through Docking and Molecular Dynamics Analyses

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Abstract

We performed a molecular modeling analysis of 100 nucleotide-like bisphosphates and 46 non-nucleotide arylurea derivatives previously reported as P2Y₁R binders using the recently solved hP2Y₁R structures. We initially docked the compounds at the X-ray structures and identified the binding modes of representative compounds highlighting key patterns in the structure–activity relationship (SAR). We subsequently subjected receptor complexes with selected key agonists

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Author Contributions

S.P. collected binding data and performed molecular docking calculations. R.D.O. performed molecular dynamics simulations. A.C. oversaw molecular dynamics simulations, analyzed the data, and wrote the manuscript. K.A.J. oversaw the whole project and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jcim.7b00528. Supplementary data analysis (detailed description of SAR and initial X-ray complexes), Figures S1–S9 (P2Y₁-like receptors sequence alignment, additional docking figures of nucleotide and non-nucleotide ligands, comparison between MRS2500 initial and final position during MD simulation of the different species considered, final state of MD simulation runs not considered, superimposition of protein structures extracted from selected trajectories) and Tables S1–S6 (structures of initially docked compounds, salt-bridges persistency during MD simulation of selected complexes, and TM movements during MD simulations) (PDF)

HTMD computational protocol (PDF)

Video of MD simulations of the MRS2500(–3a)-hP2Y₁R X-ray complex (AVI)

Video of MD simulations of the modeled 2MeSADPhP2Y₁R complex (AVI)

Video of MD simulations of the modeled MRS2268(–3a)-hP2Y₁R complex (AVI)

Video of MD simulations of the modeled MRS2268(–3b)-hP2Y₁R complex (AVI)

Video of MD simulations of the BPTU-hP2Y₁R X-ray complex (AVI)

Final 3D coordinates of the MRS2500(–3a)-hP2Y₁R complex extracted from run s2 (PDB)

Initial 3D coordinates, topology, and parameters of the 2MeSADP-hP2Y₁R system (ZIP)

Final 3D coordinates of the 2MeSADP-hP2Y₁R complex extracted from run s1 (PDB)

Initial 3D coordinates, topology, and parameters of the apo hP2Y₁R system (ZIP)

Final 3D coordinates of the MRS268(–3a)-hP2Y₁R complex extracted from run s3 (PDB)

Initial 3D coordinates, topology, and parameters of the BPTU-hP2Y₁R system (ZIP)

Final 3D coordinates of the MRS268(–3b)-hP2Y₁R complex extracted from run s2 (PDB)

Final 3D coordinates of the BPTU-hP2Y₁R complex extracted from run s3 (PDB)

Initial 3D coordinates, topology, and parameters of the MRS2268(–3a)-hP2Y₁R system (ZIP)

Initial 3D coordinates, topology, and parameters of the MRS2268(–3b)-hP2Y₁R system (ZIP)

Final 3D coordinates of apo hP2Y₁R structure extracted from run s3 (PDB)

Initial 3D coordinates, topology, and parameters of the MRS2500(–3a)-hP2Y₁R system (ZIP)

(2MeSADP and MRS2268) and antagonists (MRS2500 and BPTU) to membrane molecular dynamics (MD) simulations (at least 200 ns run in triplicate, simulation time 0.6–1.6 μ s per ligand system) while considering alternative protonation states of nucleotides. Comparing the temporal evolution of the ligand–protein interaction patterns with available site-directed mutagenesis (SDM) data and P2Y₁R apo state simulation provided further SAR insights and suggested reasonable explanations for loss/gain of binding affinity as well as the most relevant charged species for nucleotide ligands. The MD analysis also predicted local conformational changes required for the receptor inactive state to accommodate nucleotide agonists.

Graphical Abstract

1. INTRODUCTION

The P2Y receptors (P2YRs) are G protein-coupled receptors (GPCRs) activated by extracellular nucleotides.¹ To date, eight distinct mammalian P2YR subtypes have been cloned and characterized. Based on G protein-signaling selectivity and structural similarity, P2YRs are divided into two subfamilies: P2Y₁₂R-like G_i coupled receptors (P2Y₁₂, P2Y₁₃, and P2Y₁₄) and P2Y₁R-like G_q coupled receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁). The P2Y₁R is activated by endogenous ADP (Chart 1) and is expressed at high levels in the human prostate, ovary, small intestine, colon, and placenta.² This receptor is involved in several disease states such as cancer, pain, and traumatic injury in the brain.^{3–6} The P2Y₁R was also reported to mediate the positive autocrine signal of ATP in human pancreatic β -cells, thus playing a role in the regulation of electrical activity, intracellular Ca²⁺ levels, and insulin secretion.⁷ However, the most studied role of P2Y₁R in physiological processes is the initiation of platelet activation and aggregation, as it entails potential pharmaceutical applications.¹ Indeed, P2Y₁R antagonists have been developed as experimental antithrombotic drugs.⁸

The human (h) P2Y₁R X-ray structures (Figure 1) in complex with a nucleotide (orthosteric) antagonist (1'*R*, 2'*S*, 4'*S*, 5'*S*)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane (MRS2500, Chart 1) and with a non-nucleotide (allosteric) antagonist 1-(2-(2-(*tert*-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)-phenyl)urea (BPTU, Chart 1) were recently reported.⁹ The structures confirmed the overall GPCR architecture characterized by an extracellular amino terminus (N-Term), an intracellular carboxy terminus, and seven transmembrane-spanning helices (TMs) connected by three extracellular and three intracellular loops (ELs and ILs, respectively). In the

extracellular domain, four cysteine residues connect the N-terminal domain to TM7 and EL1 to EL2 through two disulfide bridges. The two protein structures are nearly identical in conformation, but the two ligand-binding sites are disparate, i.e., they do not overlap and are located in completely different receptor regions (Figure 1C). The binding site for MRS2500 involves mainly distal extracellular regions around the opening to the canonical orthosteric binding site, while BPTU binds at an allosteric site located on the outer hydrophobic surface of the TM bundle and is in contact with the phospholipid bilayer. It is hypothesized that the charged MRS2500 approaches from the extracellular aqueous medium, while the uncharged BPTU approaches through the phospholipid bilayer.⁹ Although cholesterol binding sites were previously noted at the phospholipid interface of many GPCRs,¹⁰ the BPTU binding site is unique and does not correspond to a P2Y₁R cholesterol binding site (Figure 1C). The unusual location of BPTU on the outer perimeter of the receptor protein, therefore, expanded the range of sites considered for binding of chemically diverse allosteric GPCR modulators.

From a pharmacological perspective, MRS2500 is a high affinity ($K_i = 0.8$ nM) P2Y₁R-selective antagonist that was shown to inhibit ADP-induced platelet aggregation and thrombus formation in rodents and the cynomolgus monkey.^{11,12} From the structural standpoint, MRS2500 is a nucleoside 3',5'-bisphosphate containing a sterically constrained bicyclo[3.1.0]hexane (methanocarba) ring system in a North (N) conformation in place of the ribose tetrahydrofuryl ring.¹³ Nucleotide-like ligands are well-known P2Y₁R binders, and in the last two decades extensive synthetic efforts have been devoted to elucidating their SAR.^{14–23} BPTU is a non-nucleotide antagonist ($K_i = 6.0$ nM) belonging to a series of arylurea derivatives that require a hydrophobic character for P2Y₁R affinity, which has also been explored for antithrombotic activity.^{24–30}

Molecular recognition at the P2Y₁R has been studied using site-directed mutagenesis (SDM)^{9,31,37–41} (Table 1), and SAR analysis of both nucleotide^{14–23} and non-nucleotide^{24–30,34–36} ligands (mainly antagonists). Unlike for other Class A GPCRs, the lack of a suitable receptor template severely limited the success of structure-based approaches, which relied upon homology models to elucidate the ligand recognition at the P2YRs.^{31,32} The availability of P2YR crystallographic structures^{9,33,42} now offers the opportunity to decipher at the molecular level the SAR and SDM data at the P2Y₁R by analyzing ligand–receptor interaction patterns with high resolution. In this study, we analyzed the SAR of 146 previously published P2Y₁R binders (Table S1) by docking them to the inactive crystallographic hP2Y₁R structure with the aim of deciphering the role of specific protein residues surrounding the cocrystallized ligands.

We also applied molecular dynamics (MD) to hP2Y₁R homology models in complex with the two cocrystallized antagonists, MRS2500 and BPTU, and a nucleotide agonist MRS2268 (Chart 1), closely related to MRS2500, to shed light on the differences between the orthosteric and allosteric antagonists⁴³ and on the effect of chemical modifications that toggle between agonist and antagonist functional behavior. We also complemented our analysis by running MD simulations of the apo receptor (by removing the ligand from the MRS2500-hP2Y₁R X-ray complex) and the modeled complex with the synthetic agonist most commonly used in SDM experiments, 2-methylthioadenosine 5'-diphosphate (2MeSADP, Chart 1). This analysis overcomes previous limitations and opens new horizons

for the future derivatization of known ligands to enhance pharmacological properties and the discovery of novel chemotypes by in silico methods^{44,45} for the P2Y₁R and other closely related receptors.

2. MATERIALS AND METHODS

2.1. Protein Preparation

We based our molecular modeling analysis on the two recently reported P2Y₁R crystallographic structures in complex with the nucleotide antagonist MRS2500 (Protein Data Bank (PDB)⁴⁶ ID: 4XNW) and the non-nucleotide antagonist BPTU (PDB ID: 4XNV).⁹ The structures were prepared using the Protein Preparation Wizard tool implemented in the Schrödinger suite⁴⁷ by removing cocrystallized hetero groups and water molecules. Hydrogen atoms and missing side chains of residues whose backbone coordinates were observed in the X-ray structure were added. The orientations of polar hydrogens were optimized, and the protonation states of titrable residues were determined according to H-bond patterns with surrounding residues. At the end of the preparation procedure, the overall structure was minimized with harmonic restraints on the heavy atoms (RMSD < 0.30 Å, default convergence criteria) to remove strain.

For the docking analysis, the fusion protein replacing IL3 in the X-ray construct was retained, whereas for the MD simulation homology models restoring the hP2Y₁R native sequence (UniProt ID: P47900) based on the X-ray structures were built with Prime⁴⁸ (knowledge-based method, ClustalW alignment). The models were then prepared with the Protein Preparation Wizard tool implemented in the Schrödinger suite⁴⁷ as described above, except for the protomeric state of histidine residues that were all considered protonated on the delta nitrogen.

2.2. Docking

Structures of P2Y₁R ligands, collected from the literature, were built and prepared for docking using the Builder and the LigPrep tools implemented in the Schrödinger suite.⁴⁹ Specifically, possible ionization states at pH 7 ± 1 and tautomers were generated using Epik,⁵⁰ and geometries were optimized using the OPLS_2005 force field. Molecular docking of P2Y₁R ligands at the crystal structures was performed with the Glide package from the Schrödinger suite:⁵¹ nucleotide-like ligands (both agonists and antagonists) were docked at the MRS2500-bound P2Y₁R structure, whereas non-nucleotide antagonists were docked at the BPTU-bound P2Y₁R structure. In both cases, a Glide Grid was centered on the centroid of the cocrystallized ligand, by using an inner box (ligand diameter midpoint box) of 14 Å × 14 Å × 14 Å and an outer box (box within which all the ligand atoms must be contained) that extended 20 Å in each direction from the inner one. Docking was performed considering the protein residues rigid while using the standard precision (SP) scoring function for all the compounds and the extra precision (XP) scoring function for selected representative ligands listed in Tables 3 and 4, respectively. Nucleoside ligands did not display significant differences in the poses returned by the two scoring functions. However, the XP scoring function discerned better between active and inactive non-nucleotide ligands, probably due to the more hydrophobic nature of the ligand–protein interactions. The top

scoring docking conformations for each ligand were subjected to visual inspection and final binding poses were selected on the basis of the protein–ligand interactions that were established rather than the calculated docking score. To this aim, we used the interaction pattern established by the MRS2500 and BPTU X-ray complexes (Figure 1A and B) as reference. Specifically, for nucleotide ligands poses correctly placing the phosphate groups were selected, whereas for non-nucleotide ligands poses featuring at least one H-bond with L102^{2,55} and correctly placing ring A in the hydrophobic cavity between TM2 and TM3 were preferred. In most cases, the selected docking poses ranked second or third.

2.3. Molecular Dynamics

MD simulations were performed on hP2Y₁R homology models in the apo form and in complex with the nucleotide 3',5'-bisphosphate ligands MRS2500 and MRS2268, the nucleotide diphosphate agonist 2MeSADP, and the non-nucleotide urea derivative BPTU. The apo receptor was modeled by removing the ligand from the MRS2500-hP2Y₁R complex. Bisphosphate ligands (MRS2500 and MRS2268, Chart 1) were modeled by considering the following charged species: the –4 species with both phosphate groups deprotonated, two –3 species with either the 3'-phosphate (“–3a”) or the 5'-phosphate (“–3b”) completely deprotonated, and the –2 species with both the 3'-phosphate and the 5'-phosphate half deprotonated (see Figure S1). The hP2Y₁R complexes with MRS2268 and 2MeSADP were built “in situ” starting from the MRS2500-bound complex and virtually modifying the ligand to the desired structure. A computational pipeline (Supporting Information) exploiting HTMD⁵² (Acellera, Barcelona Spain, version 1.5.4) was used to perform MD system setup, equilibration, and production. The ligand–protein complexes were embedded into an 80 Å × 80 Å 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) membrane leaflet generated through the VMD Membrane Plugin tool.⁵³ Overlapping lipids (within 0.6 Å) were removed upon protein insertion, and the systems were solvated with TIP3P⁵⁴ water and neutralized by Na⁺/Cl[–] counterions (final concentration 0.154 M). MD simulations with periodic boundary conditions were carried out with the ACEMD engine (Acellera, version 2016.10.27)⁵⁵ using the CHARMM36^{56,57}/CGenFF(3.0.1)^{58,59} force fields for lipid, protein, and ligand atoms, respectively. Ligand parameters were obtained by analogy through the ParamChem service (<https://cgenff.paramchem.org>, accessed 08/2017, version 1.0.0) without modification.

The systems were equilibrated by performing a 5000-step minimization followed by 40 ns of MD simulation in the NPT ensemble by applying initial constraints that were linearly reduced after 20 ns using the following scaling factors: 0.7 for the ligand atoms, 0.8 for α carbon atoms, and 0.4 for the other protein atoms. During the equilibration procedure, the temperature was maintained at 310 K using a Langevin thermostat with a low damping constant of 1 ps^{–1}, and the pressure was maintained at 1 atm using a Berendensen barostat. Bond lengths involving hydrogen atoms were constrained using the M-SHAKE⁶⁰ algorithm with an integration time step of 2 fs. The equilibrated systems were then subjected to 200 ns of unrestrained MD simulations (NVT ensemble, damping constant of 0.1 ps^{–1}). Long-range Coulombic interactions were handled using the particle mesh Ewald summation method (PME)⁶¹ with grid size rounded to the approximate integer value of cell wall dimensions. A nonbonded cutoff distance of 9 Å with a switching distance of 7.5 Å was used. The

simulations were run in part on an in-house built cluster equipped with three 970, two 980Ti, and one 1080 NVIDIA GPUs (Nvidia, Santa Clara, CA) and in part by exploiting the high-performance computational capabilities of the Biowulf Linux GPU cluster at the National Institutes of Health, Bethesda, MD (<http://biowulf.nih.gov>). As detailed in Table 2, all simulations were run at least for 200 ns in triplicate.

2.4. Trajectory Analysis

MD trajectory analysis was performed with customized tcl and bash scripts exploiting the NAMD 2.10⁶² *mdenergy* function and the root mean square deviation (RMSD) trajectory tool (RMSD-TT) implemented in VMD.⁵³ Selection of representative trajectories was based upon the agreement of the ligand–protein interaction pattern and SDM data. In general, among the replicas, the trajectory returning the lowest RMSD value for the ligand was preferred. Analysis of ribose pseudorotation phase angle (P)⁶³ was performed with an in house tcl script, by using the following equation and the standard definition of ribose dihedral angles ν_0 – ν_4 :

$$P = \tan^{-1} \frac{(\nu_4 + \nu_1) - (\nu_3 + \nu_0)}{2\nu_2(\sin 36^\circ + \sin 72^\circ)}$$

Salt-bridge interactions and their “persistency”, i.e. percentage of simulation time with N–O distance < 4.0 Å for each identified pair of oppositely charged residues, were computed with the Salt Bridges Plugin implemented in VMD⁵³ and a bash script, respectively.

3. RESULTS

We performed a molecular modeling analysis of previously reported P2Y₁R ligands using the recently solved hP2Y₁R X-ray structures to rationalize SAR and SDM data. We collected 100 nucleotide-like bisphosphates and 46 non-nucleotide aryl urea derivatives (full list of compounds in Table S1) structurally related to MRS2500 and BPTU, respectively, that were previously pharmacologically characterized as P2Y₁R binders (mainly antagonists). The corresponding structures and pharmacological data are reported in Tables 3 and 4. For both classes of compounds, we followed a stepwise procedure. We initially performed a docking analysis of all the ligands in Table S1 at the corresponding hP2Y₁R X-ray structure (Glide, standard precision (SP) scoring function). We then inspected all the binding poses and selected 55 representative compounds (40 nucleotide and 15 non-nucleotide ligands), whose binding modes highlighted key patterns in the SAR. We thereafter repeated the docking calculations for these compounds by using the more accurate extra precision (XP) scoring function (see Materials and Methods). We subsequently complemented the docking analysis with membrane MD simulations (at least 200 ns run in triplicate) of the X-ray structures and a few key modeled complexes to evaluate the temporal evolution of the ligand–protein interactions and compare them with the available SAR and SDM data (Table 1). It should be noted that the pharmacological data collected for some nucleotide compounds in Table 3 were determined at P2Y₁R in turkey (t) erythrocytes^{15–17,22,23} or rat (r) platelets,¹⁸ whereas for newer compounds, the data at the hP2Y₁R were reported.¹³ Nonetheless, we docked all the compounds at the hP2Y₁R, because we expected a good

interspecies correspondence in the binding mode and ligand interactions at the P2Y₁R. This assumption is based on the 7TM domain sequence identities between hP2Y₁R/rP2Y₁R and hP2Y₁R/tP2Y₁R of 96% and 89%, respectively, and the observation that all the residues with their side chains within 6 Å of MRS2500 and BPTU in the corresponding X-ray structures are conserved in the three subtypes (Figure S2). However, because of the heterogeneity of the reported data, we did not attempt to explain subtle differences in the binding affinities. Here, we focus instead on understanding the role of (i) specific chemical modifications of ligands and of (ii) residues in the receptor binding sites in determining and modulating the ligand binding affinity. A detailed analysis of the starting X-ray structures as well as of the SAR of compounds listed in Tables 3 and 4 is reported in the Supporting Information. A graphic summary of the discussed SAR data is depicted in Chart 2.

3.1. SAR and Docking of Nucleotide Ligands

3.1.1. Adenine Ring Functionalization—The substitution requirements for the adenine ring that emerged from the SAR analysis (see Supporting Information and Chart 2A) can be summarized as follows: (i) the optimal *N*⁶-alkyl substituent is a methyl group (**8**; *K_i* = 0.330 μM); (ii) there is a steric limit for C2-alkyl substitution; (iii) a two-methylene spacer is optimal to functionalize C2-alkynyl derivatives with H-bond capable moieties (**28** and **29**; *K_i* = 0.023 and 0.132 μM, respectively); and (iv) C8-substitution is not tolerated.

The docking poses of selected derivatives bearing different *N*⁶ (Figure 2A), C2-alkyl (Figure 2B), C2-alkynyl (Figure 2C), and C8 (Figure 2D) substituents help rationalize these observations. The *N*⁶-group (Figure 2A) is hosted in a small hydrophobic pocket at the interface between TM6 and TM7. As the length and the steric hindrance of the *N*⁶-alkyl group increases, the corresponding docking poses show a shift of the adenine scaffold with respect to the reference X-ray complex toward the solvent environment (see for example the docking pose of the *N*⁶-propyl derivative **10**, green carbon sticks in Figure 2A), which disrupts the H-bond network with N283^{6,58} (according to conventional TM numbering⁶⁴). Small C2 substituents, such as the 2-thioether of **14** (ribose derivative, Figure 2B) and the ethynyl moiety of **27** (methanocarba derivative, Figure 2C), fit into a small hydrophobic cavity delimited by C42 (N-term), A43 (N-term), and Q291 (EL3). The introduction of an iodine atom (MRS2500, **23**) enables a halogen bond⁶⁵ with the backbone carbonyl of C42 (N-term) (Figure 2B). As this cavity expands toward the EC environment and the flexible EL region, the larger C2 group of **26** (C2-*n*-pentyl methanocarba, Figure 2B) can be accommodated without disrupting the bidentate H-bond between the adenine core and N283^{6,58}. However, the hydrophobic chain of **26** lies almost completely solvent exposed, which might account for its 9-fold weaker binding affinity with respect to **25** (**26** vs **25**; *K_i* = 0.452 and 0.049 μM, respectively). Consistent with this hypothesis, in the docking poses of **28** and **29** (Figure 2C), the amide and the carboxylic acid moieties, respectively, establish additional H-bond interactions with the side chain of K196 (EL2) and the backbone of K41 (N-term). We speculate that these additional interactions mitigate the solvent-exposure of the C2 substituent and account for the recovered activity of the functionalized C2-alkynyl derivatives (**28** and **29**; *K_i* = 0.023 and 0.132 μM, respectively). The introduction of a C8 substituent (Figure 2D) disfavors the *anti*-glycosidic bond conformation that was observed in the hP2Y₁-MRS2500 X-ray complex, due to the steric hindrance caused by the C8-group's

proximity to the 5'-phosphate group. Although the ligand placement of **3** (C8-Br) and **6** (C8-CH₃) in the binding site with an *anti*-conformation is still possible (Figure 2D), there is a steric clash between the N283^{6,58} side chain and the C8 substituent.

3.1.2. Phosphate Ester Substitution—SAR analysis of phosphate ester regioisomers clearly identified the 5'-phosphate group as fundamental for the binding affinity (5'-bisphosphate derivatives **8** and **12** are active; 5'-chloro and 5'-H derivatives **4** and **7**, respectively, are inactive). In the docking poses of **12** (2',5'-bisphosphate) and **8** (3',5'-bisphosphate) (Figure 3A), the phosphate groups of both ligands superimpose well with the phosphate groups of MRS2500 (**23**) and establish the same electrostatic interactions with polar and positively charged residues in the EL2. Those interactions were not detected in the docking pose of the inactive 2',3'-bisphosphate derivative **7** (Figure 3B), thus suggesting that the interactions anchoring the 5'-phosphate group to the EL2 play a role in determining the binding affinity.

3.1.3. Ribose Ring Substitution—Binding data of ring-constrained nucleosides identified the receptor preference for a North envelope conformation in the ribose ring (cf. **17** vs **18**).¹⁷ Ribose substitution with other aliphatic rings (**30–32**) revealed a linear relationship in binding affinity with the ring size, whereas for acyclic derivatives (**33–40**), no clear trend emerged (see the Supporting Information). In the docking poses of methanocarba (Figure 4A), cyclic (Figures 4B and S3), and acyclic (Figure 4C) nucleotides, the ligands adopted binding modes nearly identical to those of MRS2500 (**23**) with the phosphate groups superimposing well on those of the cocrystallized ligand. We therefore hypothesize that the affinity modulation arises from the different flexibility of the ribose ring substitutes, which might affect the stability of the network of interactions anchoring the phosphate groups.

3.1.4. Nucleotide Agonists—The data collected from literature (Table 3 and Supporting Information) identified both the N⁶ and the 5' substituents as key modulators of the functional P2Y₁R activity of 3',5'-bisphosphate nucleotides. In particular, the presence of both a 6-NH₂ group and a 5'-phosphate ester seemed indispensable for receptor activation: N⁶-alkyl substitution shifts the activity from agonist (MRS2268 (**17**)) or partial agonist (**1**, **5**) to antagonist (MRS2179 (**8**), **19**), and the nucleoside 2',3'-bisphosphate derivative **7** bearing 6-NH₂ is an antagonist. On the other hand, the introduction of a C2 substituent was detrimental and shifted the activity from partial agonism to antagonism (**1** vs **2**). However, the presence of N⁶-alkyl and C2 substituents, which causes the loss of agonist activity in the 3',5'-bisphosphate nucleotide series, is compatible with receptor activation by ADP analogues, such as N⁶-methyl-(N)-methanocarba-ATP and the prototype hP2Y₁R agonist 2MeSADP.^{66,67}

To date, an agonist-bound P2Y₁R X-ray structure is not yet available. We therefore docked nucleotide-like agonists (**1**, **5**, and MRS2268 (**17**)) at the P2Y₁R X-ray structure in complex with MRS2500 (**23**). The resulting docking pose analysis (data not shown), however, was not able to rationalize the differences in the functional efficacy of structurally related analogues, as the scaffolds of agonists and antagonists (e.g., MRS2500 (**23**) and MRS2268

(17)) were perfectly superimposed, thus making the compounds indistinguishable. We therefore investigated the role of the N^6 and the 5' substituents in agonist recognition through MD simulation by modeling receptor complexes with key agonists (see sections 3.3.2 and 3.3.3).

3.2. SAR and Docking of Non-nucleotide Antagonists

The SAR of non-nucleotide antagonists (see Supporting Information and Chart 2B) highlighted (i) the substitution pattern of ring A and (ii) the geometry and nature of the linkers connecting the aromatic rings as key binding affinity determinants. Although the arylureas bind in a shallow site that contacts less than a half of the ligand surface, the docking poses of selected derivatives (Figures 5 and S4) were consistent with the SAR reported in Table 4.

3.2.1. Effect of Ring A Substitutions—Introduction of hydrophobic substituents in ring A is tolerated only in the ortho (BPTU, **41**; $K_i = 6$ nM) and meta (**42**, $K_i = 69$ nM) position. According to our docking analysis, a *m*-trifluoromethyl moiety (**42**, Figure 5A) enables ring A to fit in the hydrophobic cavity between TM2 and TM3, whereas a para substituent (**44**, Figure S4A) does not. Moreover, the position of ring A substituents also orients the entire scaffold, which in turn affects the interactions established by rings B and C. As reported in Table 4, the affinity of *m*-trifluoromethyl derivatives depends upon the substitution pattern of ring C (**42**, $K_i = 69$ nM vs **43** and **44**, $K_i > 5000$ nM).²⁴ In the docking pose of **42** (Figure 5A), the scaffold is slightly turned with respect to the placement of BPTU (**41**) in the X-ray complex. The different orientation causes less favorable π - π stacking interactions between ring B and F119 (EL1) and between ring C and F62^{1.43} and F66^{1.47}, which likely accounts for the lower affinity of **42** with respect to BPTU (**41**). In the docking poses of **43** and **44** (data not shown), both π - π stacking interactions were lost.

3.2.2. Effect of Ether Linker Replacement—The ether linker connecting rings A and B can be replaced by amine functions enclosed in bicyclic systems such as indoline or tetrahydroquinoline moieties, with the introduction of a 7-hydroxyl group in the indoline ring improving the binding affinity. The super-imposition of the docking poses of **47** and **50** (Figure 5B, green and purple carbon sticks, respectively), show that the ligands share the same interaction pattern of the cocrystallized BPTU (**41**), with the hydroxyl moiety of **50** establishing an additional H-bond interaction with Q127^{3.28}, which might account for its higher binding affinity (**50**, $IC_{50} = 0.14$ nM). The amine linker geometry is fundamental to ensure the binding of the compounds. As shown in Figure S3, the steric hindrance brought by a methyl substituent on the amine linker (**45**, Figure S4B) or by a fused seven-membered ring (**48**, Figure S4C) does not allow ring A to fit in the cavity delimited by TM1 and TM2.

3.2.3. Effect of Urea Replacement—The urea moiety connecting rings B and C can be replaced by an amine group contained within five-membered heterocyclic rings (**52–54**), thus suggesting that only one H-bond with the backbone of L102^{2.55} is needed to bind at the P2Y₁R. In the docking poses of compounds **52** and **54** (Figure 5C, blue and yellow carbon sticks, respectively), the loss of the bidentate H-bond with L102^{2.55} is compensated by an additional π - π stacking interaction with F62^{1.43} in TM1, while all the other key interactions

are preserved. As with the linker connecting rings A and B, the linker replacing the urea moiety also needs to satisfy specific geometric requirements. As depicted in Figure S3, the introduction of a nonplanar heterocyclic ring (**53**, Figure S4D) or a sterically hindered substituted heterocyclic ring (**55**, Figure S4E) pushes ring C toward the membrane environment and away from TM1, thus disrupting the two π - π stacking interactions with F62^{1.43} and F66^{1.47}.

3.3. Molecular Dynamics

3.3.1. MRS2500-hP2Y₁R Complex—Initially, we analyzed the stability of the interaction pattern as detected in the X-ray complex (Figure 1A, detailed description in the Supporting Information). For this purpose, we simulated MRS2500 (**23**) (as well as MRS2268 (**17**)) in different protonation states (see Table 1 and Figure S1) bearing a total net charge of -4 (both phosphates fully deprotonated), -3 (one phosphate fully deprotonated, and one-half protonated), and -2 (both phosphates half deprotonated). For the -3 species, we considered both the case in which the 3'- and the 5'-phosphate are fully deprotonated (hereby denoted as "-3a" and "-3b" species, respectively, Figure S1).

To evaluate the ligand stability, we computed the RMSD value during the production runs for the protein α carbon atoms ($C\alpha$), the ligand barycenter, and the barycenter of the different functional units composing the ligand, i.e. the adenine core, the 3'- and the 5'-phosphate groups, and the (*N*)-methanocarba ring. As reported in Table 5, the -3a species returned the lowest average RMSD values for the protein $C\alpha$, and two out of three trajectories returned the lowest RMSD values for the ligand barycenter. Breaking down the RMSD values per functional group revealed that for the other species, the higher ligand fluctuation was due to a position shift of the phosphate esters. A graphical comparison between the initial X-ray binding mode and the final ligand position in each trajectory and for each charged species is shown in Figure S5. A careful analysis of all the trajectories highlighted that the phosphate group protonation state considerably affected the ligand-protein interaction pattern. In particular, the ligand retained the binding mode observed in the X-ray complex only when the 5'-phosphate group was half protonated and the 3'-phosphate was fully deprotonated (-3a species). In the simulations with the -4 species, the 5'-phosphate group lost the H-bonds with T205 (side chain) and D204 (backbone) in EL2 and moved toward TM1 to establish salt bridge and H-bond interactions with residues located in TM7 (Figure S6A). Protonating the 3'-phosphate (-3b species), instead, resulted in this group moving higher in the binding site by maintaining the interaction with R195 (EL2), while disengaging K46 (N-Term) and Y303^{7.32}, and the 5'-phosphate moving toward TM7 to establish salt-bridge interactions with R310^{7.39} (Figure S6B). When considering the -2 species, although the ligand retained its initial placement for most of the trajectory, the salt-bridge between the 3'-phosphate and K46 (N-Term) was lost and the K46 side chain moved away from the binding site (Figure S6C).

We note that the X-ray structure was determined in a pH range between 7.0 and 8.0,⁹ and the ligand protonation state in the crystallographic complex might not reflect its protonation state in solution (during binding affinity data determination) or at physiological conditions in the body. We therefore compared the outcome of the MD simulations of the different species

with available SDM data (Table 1) as well. The mutation of residues surrounding the 5'-phosphate of MRS2500 (**23**) in the X-ray complex, namely T205 (EL2), Y306^{7,35}, and R310^{7,39}, to alanine impairs the binding of both bisphosphate antagonist and diphosphate agonist ligands (Table 1). Interestingly, the R310K mutant recovered the affinity for the diphosphate agonist 2MeSADP (and did affect the binding of bisphosphate antagonists), while the Y306F mutation reduced affinity for both ligand types. These data suggest that two H-bond donors (T205 and Y306) and a positively charged residue (R310) are required in the binding site region surrounding the 5'-phosphate of MRS2500 (**23**). The ratio of H-bond donors and positively charged residues, in turn, supports the hypothesis that the 5'-phosphate carries a -1 charge to mimic the α -phosphate group of the endogenous hP2Y₁R agonist ADP. On the other hand, the binding effects of mutating residues surrounding the 3'-phosphate of MRS2500 (**23**) in the X-ray complex, such as Y303^{7,32}, K46 (N-term), and R195^{7,39}, suggest that a H-bond donor (Y303F mutant strongly reduces MRS2500 binding) and two positively charged residues are required to host the 3'-phosphate. Again, the ratio of H-bond donors and positively charged residues required in this region supports the hypothesis that the 3'-phosphate of MRS2500 (**23**) group carries a -2 charge. Therefore, our analysis envisaging the -3a species as the most relevant species for MRS2500 (**23**) is consistent with both the SDM data and the role conceived for the 5'-phosphate group of nucleotide-like ligands, i.e. to mimic the α -phosphate of the endogenous hP2Y₁R agonist ADP, which carries a -1 and a -2 charge under physiological conditions on the α - and β -phosphate group, respectively.

Following this preliminary analysis, we analyzed the simulations obtained for the -3a species in more detail and selected a representative replica (highlighted in bold in Table 5) for trajectory visualization (Video S1). With respect to the starting initial X-ray complex, the interaction pattern established by MRS2500 (**23**) was stable over time. The only observable difference was the loss of the bidentate H-bond interaction between the ligand's adenine core and the N283^{6,58} side chain. Although this result was quite surprising, in all the simulations we performed (accounting for a total simulation time of 2.4 μ s), the N⁶-methyl group pointed toward the TM bundle, thereby disrupting the bidentate interaction with N283^{6,58}. This behavior is consistent with the electrostatic potential of the small cavity at the interface between TM6 and TM7 featuring polar residues such as Q291 (EL3) and N299^{7,28} in the region facing the EC side and hydrophobic residues such as V302^{7,31} and A286^{6,61} in the region facing the TM bundle (Figure 6A and B).

3.3.2. 2MeSADP-hP2Y₁R Complex—Mutation of the residues surrounding the 3'-phosphate group of MRS2500 (**23**) in the X-ray structure does not affect the binding of 2MeSADP (Table 1). This observation suggests that the region hosting the β -phosphate of ADP might not coincide with the one hosting the 3'-phosphate group of MRS2500 (**23**) in the X-ray complex. To validate this hypothesis, we carried out 200 ns of MD simulation (run in triplicate) of the modeled hP2Y₁R complex with 2MeSADP and compared the results with the simulation of the complex with MRS2500 (**23**). To this end, we assumed that 2MeSADP binds in the same nucleotide binding site and built the ligand in place by virtually modifying MRS2500 (**23**) in the corresponding X-ray complex. All the trajectories were visually inspected and selection of the most representative run was based upon the

comparison of the ligand-interaction pattern observed and the available SDM data (Table 1). As expected, in two out of three trajectories, the highest RMSD values were obtained for the coordinates of the agonist β -phosphate atoms. The trajectory returning the highest RMSD values (run s2) featured the ligand moving higher in the binding site, the α -phosphate group disengaging from the H-bond network with the T205 (EL2) side chain and the D204 (EL2) backbone, and the β -phosphate group establishing H-bond and salt-bridges interaction with the side chains of Y303^{7,32}, R195 (EL2), and K46 (N-Term). As this interaction pattern disagreed with the available SDM data (Table 1), this trajectory was no longer considered. In the other two trajectories (see visualization of run s1 in Video S2), the α -phosphate group established persistent H-bond interactions with the side chains of T205 (EL2) and Y306^{7,35} and the backbone of D204 (EL2), while the β -phosphate group established a salt-bridge with R310^{7,39}. R195 (EL2) interacted intermittently with the β -phosphate group and the ribose C2' and C3' hydroxyl moieties, whereas K46 (N-term) moved away from the binding site. This is consistent with mutagenesis data showing that both the K46A and the R195A mutations do not affect 2MeSADP binding.⁹ The loss of persistent salt-bridges with R195 (EL2) and K46 (N-Term) predicted in the MRS2500-hP2Y₁R complex, allowed water molecules to diffuse into the binding region surrounding the ribose hydroxyl and β -phosphate group moieties (Figure 6C). During the simulation, the adenine core retained its initial position and established a stable H-bond with N283^{6,58}, although not persistently bidentate. The ribose ring maintained a North pucker conformation for 96.5% of the trajectory by adopting mostly (~84.8% of the simulated time) the C3'-endo conformation ($0 < P < 36^\circ$) with the C3' hydroxyl moiety establishing an intramolecular H-bond with the β -phosphate group. This interaction pattern was in better agreement with the experimental SDM data and served as criteria for comparing the MD simulations of the (N)-methanocarpa 3',5'-bisphosphate agonist MRS2268 (**17**).

3.3.3. MRS2268-hP2Y₁R Complex—The selection of both the most relevant protonation state and a representative trajectory to visualize for MRS2268 (**17**) was not straightforward. As mentioned above, to date, an agonist-hP2Y₁R complex X-ray structure has not been solved, thus limiting our knowledge of the receptor conformational changes required to switch from the inactive to active state. X-ray evidence available for another P2YR family member, i.e. the P2Y₁₂R, in complex with an agonist³³ and an antagonist,⁴¹ revealed an unpredicted EC domain plasticity, suggesting that a nucleotide-like agonist binding at the receptor active state might adopt a slightly different binding mode with respect to the one observed in the MRS2500-hP2Y₁R X-ray complex. Moreover, a detailed knowledge of the factors affecting the functional activity of nucleotide 3',5'-bisphosphate agonists like MRS2268 at the hP2Y₁R is still lacking, as SDM data available thus far have assessed the role of specific site mutations in determining (i) the binding affinity of nucleotide 3',5'-bisphosphate antagonists (MRS2500 (**23**) and MRS2179 (**8**)) and (ii) the binding affinity and functional selectivity of nucleotide 5'-diphosphate agonists (2MeSADP). Considering these limitations, the selection of the trajectories for the MRS2268-hP2Y₁R complex was guided by the knowledge gained from the SAR described in section 3.1.4, which identified the 6-NH₂ and the 5'-phosphate groups as fundamental to confer agonist activity to 3',5'-bisphosphate ligands. We therefore focused on the interaction patterns involving those two key substitutions when analyzing the MRS2268-hP2Y₁R complex simulations and

compared the results with the analysis of the hP2Y₁R in complex with MRS2500 (**23**) and 2MeSADP described in the previous sections. We also focused on the species carrying a total net charge of -3 , i.e. $-3a$ and $-3b$ and describe here the differences between them. A depiction of the final state of a selected trajectory for each of the other two charged species initially considered, along with a brief description of the main features of all the trajectories, is reported in Figure S7.

At variance with the MD simulation in complex with MRS2500, in all the simulations in complex with MRS2268 (**17**), the ligand moved deeper in the binding site following conformational rearrangements of the EC region and established persistent H-bond interactions with the side chains of N283^{6,58} and N299^{7,28} through the 6-NH₂ group and adenine N7.

In the simulations of the $-3a$ species of MRS2268 (Video S3 representing the visualization of run s3), the agonist 5'-phosphate group established an additional H-bond with the side chain of Y306^{7,35}. In the other two trajectories (data not shown), the H-bond interactions with the side chain of T205 (EL2) and the backbone of D204 (EL2) were less persistent, and the 5'-phosphate established an alternative network of H-bonds and salt-bridges involving the side chains of N283^{6,58} and R287^{6,62}, respectively. This alternative H-bond network was also detected at the end of the selected trajectory visualized in Video S3. On the other hand, the interaction pattern around the 3'-phosphate was stable and persistent in all the trajectories.

In the simulations of the $-3b$ species of MRS2268 (Video S4 representing the visualization of run s2), a slightly different interaction pattern around the 5'-phosphate was observed. At the beginning of the simulation, the 5'-phosphate established an additional H-bond with Y306^{7,35}, while maintaining the network of H-bonds with residues in the EL2 and the salt-bridge with R310^{7,39}. After ~ 30 ns, the ligand moved deeper in the binding site and the 5'-phosphate established an additional salt-bridge with R128^{3,29}. The interaction around the 3'-phosphate was stably maintained and also involved a H-bond interaction with Y110^{2,63}. In the other two trajectories, the side chain of R195 (EL2) moved away from the 3'-phosphate group and allowed water molecules to diffuse into the binding site, analogously to that observed in the simulations with 2MeSADP (e.g., the final snapshot of run s3 in Figure 6D).

3.3.4. BPTU-hP2Y₁R Complex—In the MD simulations of the BPTU-hP2Y₁R complex (accounting for a total simulation time of $1.6 \mu\text{s}$), the ligand position remained stable. With respect to the initial ligand-protein interaction pattern, the H-bond with L102^{2,55} involved only one urea nitrogen atom, as anticipated from the SAR analysis (see Video S4 representing the visualization of run s3). The three aromatic moieties maintained stable π – π stacking interactions, in particular between ring C and F62^{1,43} and F66^{1,47}, and between ring B and F119 (EL1). In the trajectory visualization, M123^{3,24} strongly contributed to maintaining the hydrophobic cavity hosting ring A. In another trajectory (s2) TM7 was bent outward at the end of the simulation (Figure S8A), whereas in run s4, after approximately 389 ns, the ligand escaped the binding site and moved toward the EC environment. During the ligand egress pathway (Figure S8B), a π – π stacking interaction anchored ring C to F109^{2,62}, ring B maintained a π – π stacking interaction with F119 (EL1), and ring A was

still partially occupying the hydrophobic cavity at the interface between TM1 and TM2. We therefore hypothesize that the placement of ring A in the hydrophobic spot might represent an initial stage of ligand binding to facilitate the scaffold anchoring at the TMs.

3.3.5. Comparison with the Apo hP2Y₁R—We analyzed (i) the formation of salt-bridges, (ii) distribution of water molecules, and (iii) TM movement during the MD simulation as a function of the presence or absence of a ligand. To this end, we compared the MD simulations of ligand-bound complexes with the apo receptor, to rule out aspects presumably representing intrinsic features of the receptor.

Persistency of Salt-Bridges: The analysis of the cumulative persistency of salt-bridges among the replicas is reported in Table 6, whereas the detailed per-replica analyses of ligand–protein complexes are reported in Tables S2–S5. In the tables, only salt-bridges having a persistency higher than 20% of the simulation time (i.e., being present for at least 120 ns) for at least one system (Table 6) or one replica (Tables S2–S5) are reported. The salt-bridge connecting the EC tip of TM5 and TM6 (D289^{6.64}-R212^{5.33}) was found in all the systems and likely represents an intrinsic feature of the hP2Y₁R. The salt-bridge between the side chains of D208 (EL2) and K41 (N-Term) was persistent in the MD simulations with the nucleotide agonist MRS2268 and present for about only 30% of the simulation time in the apo and BPTU-bound receptors. In those systems, the salt-bridge creates a pocket cap that shields the adenine core of MRS2268 from the EC medium. Consistent with this hypothesis, the salt-bridge was not formed in the simulation of nucleotide ligands bearing a C2-substituent (MRS2500 and 2MeSADP). More detailed analysis of the corresponding trajectories revealed that the C2 substituent prevented K41 (N-Term) from approaching D208 (EL2) by establishing van der Waals interactions with the K41 side chain carbon atoms.

In our simulations, the D204(EL2)-K280^{6.55} salt-bridge is a characteristic of 3',5'-bisphosphate ligands. Indeed, in the simulation with 2MeSADP, K280^{6.55} moved away from the binding site and established H-bond interactions with the Y214^{5.35}, H132^{3.33}, and Y136^{3.37} side chains and was therefore not available to interact with D204. SDM data^{37,41} showed that the K280A mutant affected the 2MeSADP affinity and efficacy, while reducing the binding of MRS2179. Moreover, SDM data also confirmed that both the H132A and Y136A mutations slightly reduced 2MeSADP potency.³⁷ Our MD analysis agrees with these observations and suggests that the effects of the K280A mutation on the binding of diphosphate and 3',5'-bisphosphate nucleotides arises from two different phenomena, i.e. (i) the lack of an interaction network involving residues in TM3 putatively participating in receptor activation and (ii) the loss of the D204(EL2)-K280^{6.55} salt-bridge contributing to delimit the lower portion of the site hosting the 3',5'-bisphosphate nucleotides. Interestingly, in the BPTU-bound complex, D240 (EL2) is involved in salt-bridges with R310^{7.39} that were detected in previously published MD simulation of the hP2Y₁R⁶⁸. In the simulation with the nucleotide ligands this salt-bridge was not feasible, because R310^{7.39} established ionic interaction with the ligands' phosphate moieties. In the simulation of the apo receptor, R287^{6.62} underwent conformational changes and established salt-bridges with D208 (EL2) and to a minor extent D204 (EL2). Overall, these results suggest that, when the

orthosteric site is not occupied, changes in the orientation of the D204 (EL2) and R287^{6,62} side chains occur and contribute to reshape the binding cavity. This hypothesis agrees with SDM data envisaging a role for these two residues in receptor activation.³⁸

The different set of salt-bridges involving D329 (IC tip of TM7/H8) suggests a role for this residue in establishing ionic locks that might stabilize the receptor in different conformational states. However, it was difficult to identify a clear pattern among different types of ligands and correlate them with the presence/absence of a specific salt-bridge. A few salt-bridges were also observed only in the MRS2268-hP2Y₁R complex and reflected the conformational changes occurring at the EC tip of TM6/7 (D289^{6,64}-R285^{6,60} and D300^{7,29}-R301^{7,30}), presumably to accommodate the 6-NH₂ moiety and the outward bending of TM5 (D250(IL3)-R256^{6,31}).

Analysis of Water Molecule Distribution and TM Movements: We compared graphically the number of water molecules present within the TM bundle at the end of the selected trajectories previously described. As depicted in Figure 7, with respect to the complex with MRS2500 (Figure 7A), orthosteric agonists (mostly 2MeSADP and, to a minor extent, MRS2268) enabled a higher number of water molecules to diffuse into the TM bundle (Figure 7C–E). We ascribe this phenomenon to a side chain rotation of R195 (EL2), which during the MD simulation was disengaged from ionic interactions with the nucleotide agonists, while remaining tightly bound to the 3'-phosphate of MRS2500. However, the comparison with the apo and BPTU-bound receptor structures (Figure 7B and C) clearly shows that the diffusion of water molecules also occurred when the orthosteric binding site was unoccupied. Therefore, the diffusion of water molecules cannot be considered, at least in the context of our simulations, as an activation signature of the receptor. Interestingly, in the final structure extracted from the MD trajectory of the 2MeSADP-hP2Y₁R complex (Figure 7C), the water molecules diffused into a specific area of the cavity lying under the orthosteric binding site. By visualizing the residues surrounding this accessory binding site, we identified a set of hydrophobic residues (Figure 7G and H) whose mutation affected 2MeSADP efficacy in SDM experiments.^{31,37,38,41} Although their role in receptor activation is unclear, we hypothesize that they might constitute an accessory binding site hosting the adenine core of dinucleotides.

Analysis of TM movements, measured by calculating the RMSD value of each TM centroid with respect to the initial conformation after the equilibration phase (Table S6), and superimposition of the final structures extracted from the selected MD trajectories (Figure S9) confirmed that, during the MD simulation, the receptor persisted in an inactive-like conformation. Nonetheless, specific conformational rearrangements were observed as a function of the presence or absence of a bound ligand, as well as of the ligand type (agonist, antagonist, allosteric modulator). In particular, (i) EL2 moved away from the orthosteric binding site in the agonist-bound complexes (see Figure S9A and C); (ii) in the simulations of MRS2268, the –3a species resembled the MRS2500-hP2Y₁R complex (data not shown), whereas the –3b species resembled the 2MeSADP-hP2Y₁R complex (Figure S9C); (iii) BPTU conformationally locked TM1 and TM2 by impeding the outward movement of their IC tips (Figure S9A); and (iv) in the apo and BPTU-bound systems conformational

rearrangements occurred that reshaped the orthosteric binding site with part of the N-Term creating a pocket cap (already described in the Persistency of Salt-Bridges section).

4. DISCUSSION

The P2Y₁R is a current target for antithrombotic therapy and a potential target for other conditions such as cancer, diabetes, CNS disorders, and pain. In this study, we used the recently solved X-ray structures of the hP2Y₁R complexes with an orthosteric nucleotide antagonist, (*N*)-methanocarba 3',5'-bisphosphate MRS2500 (**23**), and a non-nucleotide allosteric antagonist, diarylurea BPTU (**41**), to perform a molecular modeling analysis aimed at rationalizing the SAR of 146 known P2Y₁R binders collected from literature. We initially docked nucleotide-like and non-nucleotide ligands at the corresponding X-ray structures based on their structural similarity with the cocrystallized ligand. The obtained docking poses served as a basis to rationalize binding data collected from literature. We subsequently validated some of the hypotheses suggested by the docking analysis with MD simulation of selected key ligand-protein complexes complemented with a reflection on the available SDM data. To this aim, we included in our analysis also MD simulation of the prototypical agonist 2MeADP.

The docking analysis suggested that the modulation of nucleotide ligand affinity is determined by (i) the loss of bidentate H-bond with N283^{6,58} caused by the steric limit imposed by the hydrophobic pocket at the interface of TM6 and TM7 hosting the *N*⁶-alkyl group; (ii) the steric limit of a contiguous hydrophobic pocket delimited by the N-Term and EL3 in hosting C2-alkyl groups forcing bulky substituents to be exposed to the EC aqueous environment; (iii) the mitigation of solvent exposure of hydrophobic C2 substituents with the introduction of a properly spaced functional group amenable to establish additional H-bonds with charged residues in EL2 and N-Term; and (iv) the loss of the *anti*-conformation imposed by the introduction of a C8-substituent.

The MD analysis of the MRS2500-hP2Y₁R complex identified the most relevant ligand protonation state, which agreed with the available SDM data, validated docking hypotheses ii–iv, and provided an alternative explanation for hypothesis i. In particular, MD simulation confirmed the preference for (i) the North conformation of the ribose ring of 2MeSADP, which explained the higher affinity of *N*-methanocarba compared to the corresponding (*S*)-methanocarba isomers and (ii) the *anti*-configuration of the glycosidic bond for all considered ligands, corroborating the detrimental effect of introducing a C8 substituent. The analysis of salt-bridges (Table 6) also confirmed the availability of the K196 (EL2) and K41 (N-term) side chains for additional H-bond interactions with the ligand, as those residues did not engage in salt-bridges with nearby negatively charged residues during 3.6 μ s of MD simulation. Concerning hypothesis i, we did not detect the presence of a stable bidentate H-bond between the adenine core of MRS2500 (**23**) and the side chain of N283^{6,58}. This observation was also supported by analyzing the electrostatic potential of residues surrounding the *N*⁶ substituent of the nucleotide-like antagonist as well as with this substituent's role in determining the functional affinity of the ligands. We therefore hypothesize that the affinity decrease as a function of increasing the size of the *N*⁶-alkyl substituent in nucleotide-like antagonists arises from the solvent exposure of the adenine

core imposed by the small size of the hydrophobic cavity delimited by A286^{6.61} and V302^{7.31}. This hypothesis is supported by the observation that during the MD simulation of all the nucleotide ligands considered, the adenine core establishes van der Waals interactions with the R287^{6.62} and L44 (N-Term) side chains that created a hydrophobic spot hosting the adenine ring. Our analysis is in agreement with available SDM data showing the loss of 2MeSADP binding as a result of an L44A mutation (Table 1).

In line with these results, the docking analysis failed to provide an explanation for the modulation of the functional activity as a function of the presence of a 6-NH₂ and the 5'-phosphate ester in 3',5'-bisphosphate nucleotides, as the predicted binding modes of structurally related agonists and antagonists were nearly identical and involved the same interaction pattern.

The MD analysis of the hP2Y₁R modeled in complex with MRS2268 (**17**, a close analogue of MRS2500 (**23**)) provided further insights suggesting that the formation of a bidentate H-bond with N283^{6.58}, which was not present in the MD simulation of the MRS2500-hP2Y₁R complex, might be involved in agonist recognition and, presumably, be implicated in receptor activation. Moreover, in the simulation of both the -3-charged species considered for MRS2268, an additional H-bond was established between the 6-NH₂ group and adenine N7 of the ligand and the side chain of N299^{7.28}, a residue whose role in ligand binding and receptor activation has not yet been assessed by SDM.

The docking analysis of non-nucleotide antagonists suggested that (i) the reciprocal placement of the three aromatic features plays a significant role in the binding at the P2Y₁R and that (ii) a bidentate H-bond with L102^{2.55} is not strictly required for compound affinity. The BPTU-hP2Y₁R complex MD analysis confirmed these hypotheses and identified additional residues that might play a role in stabilizing the ligand in the allosteric binding site, such as F109^{2.62} and M123^{3.24}.

The analysis of salt-bridge interactions established during the MD simulation provided a structural explanation for the role of D204 (EL2) and K280^{6.55} in affecting 2MeSADP affinity and efficacy, and in affecting nucleotide ligand binding, by suggesting a role for this ion pair in delimiting the lower portion of the orthosteric binding site hosting 3',5'-bisphosphate ligands. Our analysis of salt-bridges was also in agreement with previously published MD simulation performed by another research group,⁶⁸ identifying the D204 (EL2)-R310^{7.39} salt bridge as a specific feature of the allosteric modulation mediated by BPTU. Moreover, our analysis of saltbridges also identified residues whose conformational changes might: (i) reshape the orthosteric binding cavity in the presence or absence of an orthosteric ligand (e.g., R287^{6.62} and K41 (N-Term)); (ii) stabilize the receptor inactive state (D329); and (iii) help accommodate 3',5'-bisphosphate agonists.

The analysis of the water molecule distribution in the final state of the MD simulated ligand-receptor complexes highlighted that when the orthosteric site was unoccupied or occupied by a nucleotide agonist, a higher number of water molecules diffused into the TM bundle. The fewer water molecules diffusing into the cavity when MRS2500 was bound were ascribed to a salt-bridge interaction established between the 3'-phosphate and the R195

(EL2) side chain, presumably forming a steric lock. Interestingly, the distribution of water molecules within the TM domain in 2MeSADP-hP2Y₁R surrounded a cluster of hydrophobic residues lying below the orthosteric binding site, that have been shown to be implicated in modulating the agonist efficacy.^{31,37,38,41} We speculate that this cavity might represent an accessory binding site to host a second nucleobase.

Finally, the analysis of TM movements and superimposition among the different final structures extracted from the MD trajectories suggested that the simulated time was not enough to appreciate significant conformational changes and that the receptor persisted in an inactive-like state. Indeed, conformational changes associated with receptor activation occur on a longer time-scale and presumably involve energy barriers that are usually inaccessible within a few μ s of classical MD simulation. Moreover, as X-ray evidence has shown for the P2Y₁₂R,^{33,42} there is a chance that the receptor active states in the P2Y family might differ considerably from the inactive states. Hopefully, an X-ray structure of the P2Y₁R in complex with an agonist will help overcome these limitations by providing a structural template to unravel the structural factors governing receptor activation.

5. CONCLUSIONS

We provided a structural basis to explain and predict steric and conformational factors affecting the binding affinity and functional efficacy of both orthosteric nucleotide ligands and allosteric non-nucleotide antagonists at the hP2Y₁R. The validation of the binding modes of these two structural classes provides a predictive tool for new analogue design. Moreover, the analysis of sequence conservation among P2Y₁-like receptor subtypes, for which X-ray structures are not yet available, can guide the design of subtype selective binders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Contacts between the P2Y₁R X-ray structure and the cocrystallized ligand MRS2500 **23** (A) and BPTU **41** (B). H-bonds, π - π stacking interactions, and hydrophobic contacts are depicted as orange dashed lines, cyan dotted lines, and continuous green lines, respectively. (C) High-resolution X-ray crystallographic structures of the hP2Y₁R showing the binding sites for orthosteric antagonist MRS2500 (**23**) (purple atoms) and allosteric antagonist (NAM) BPTU (**41**) (gray atoms). The surfaces of the binding site region in contact with MRS2500 and BPTU are shown in purple and gray, respectively.

Figure 2.

Docking analysis of the effect of substituents on the adenine core in nucleoside-like antagonists binding at the MRS2500-bound hP2Y₁R X-ray structure (cyan ribbons). (A) *N*⁶-substitution: Superposition of the docking poses of 2'-deoxyadenosine 3',5'-bisphosphates **8** (yellow carbon sticks), **9** (pink carbon sticks), **10** (green carbon sticks), and **11** (orange carbon sticks). (B) C2-alkyl-substitution: Superposition of the docking poses of 2-methylthio-2'-deoxyadenosine 3',5'-bisphosphate **14** (green carbon sticks) and 2-hexyl-2'-deoxy-(*N*)-methanocarpa-adenosine 3',5'-bisphosphate **26** (orange carbon sticks) and the X-ray binding pose of MRS2500 (compound **23**, pink carbon sticks). (C) C2-alkynyl-substitution: Superposition of the docking poses of **27** (green carbon sticks), **28** (magenta carbon sticks), **29** (orange carbon sticks). (D) Superposition of the docking poses of 8-bromo-2'-deoxyadenosine 3',5'-bisphosphate **3** (magenta carbon sticks) and 8-methyl-2'-deoxyadenosine 3',5'-bisphosphates **6** (blue carbon sticks). Side chains of residues surrounding the ligands are shown in sticks (gray carbons), and polar interactions are indicated by dashed orange lines. Steric clashes are represented as solid red lines. Nonpolar hydrogen atoms are omitted. The surface of the binding site region in contact with the *N*⁶ and C2 substituent is shown in gray.

Figure 3.

Docking analysis of the effect of phosphate esters regioisomers in nucleoside-like antagonists binding at the MRS2500-bound hP2Y₁R X-ray structure (cyan ribbons). (A) Superposition of the docking poses of 3',5'-bisphosphate derivative **8** (orange carbon sticks) and 2',5'-bisphosphate derivative **12** (yellow carbon sticks) and the X-ray binding mode of MRS2500 (compound **23**, pink carbon sticks). (B) Docking pose of 2',3'-bisphosphate derivative **7** (yellow carbon sticks). Side chains of some residues important for ligand recognition are shown in sticks (gray carbons), and polar interactions are indicated by red dashed lines. Nonpolar hydrogen atoms are omitted.

Figure 4.

Docking analysis of the effect of ribose substitutes in nucleotide-like antagonists binding at the MRS2500-bound hP2Y₁R X-ray structure (cyan ribbons). (A) Methanocarba derivatives: Super-position of the docking poses of 2'-deoxy-(*N*)-methanocarbaadenosine 3',5'-bisphosphate **17** (yellow carbon sticks) and 2'-deoxy-(*S*)-methanocarba-adenosine 3',5'-bisphosphate **18** (orange carbon sticks). (B) Other ring systems: Superposition of the docking pose of 1,5-anhydrohexitol-containing bisphosphate analogue **31** (yellow carbon sticks) and the X-ray binding mode of MRS2500 (compound **23**, pink carbon sticks). (C) Acyclic systems: Super-position of the docking poses of acyclic adenine nucleotides **34** (orange carbon sticks), **40** (magenta carbon sticks), **35** (pink carbon sticks), and **36** (yellow carbon sticks). Side chains of some residues important for ligand recognition are shown in sticks (gray carbons), and polar interactions are indicated by red dashed lines. Nonpolar hydrogen atoms are omitted.

Figure 5.

Docking analysis of the binding of non-nucleoside antagonists binding at the BPTU-bound hP2Y₁R X-ray structure (gray ribbons). (A) Effect of ring A substitution: Docking pose of meta-substituted BPTU analogue **42** (orange carbon sticks). (B) Effect of ether linker replacement: Superposition of the docking poses of tetrahydroquinoline derivative **47** (green carbon sticks) and 7-hydroxyl indoline derivative **50** (purple carbon sticks). (D) Effect of urea moiety bioisosteres: Superposition of the docking poses of diarylamine derivatives **52** (blue carbon sticks) and **54 f** (yellow carbon sticks). Side chains of some residues important for ligand recognition are shown in sticks (gray carbons), and polar and π - π interactions are indicated by dashed orange and cyan lines, respectively. Nonpolar hydrogen atoms are omitted.

Figure 6.

(top) Final state of the simulation of the MRS2500-hP2Y₁R complex for the -3a species (selected trajectory: run s2): (A) Side view facing TM6, TM7, and TM1 and (B) top view. (bottom) Final state of the simulation of the (C) 2MeSADP-hP2Y₁R complex (selected trajectory: run s1) and the (D) MRS2268-hP2Y₁R complex for the -3b species (selected trajectory: run s3). Side chains of some residues important for ligand recognition are shown in sticks (cyan carbons) with polar interactions depicted as dashed orange lines. Nonpolar ligand hydrogen atoms and all protein hydrogen atoms are omitted. Residues surrounding the *N*⁶ group of MRS2500 in panels A and B are depicted as wired surface color-coded according to the residue type (blue positively charged; green polar or aromatic; white hydrophobic). Water molecules surrounding the ligand in panels C and D are represented as a transparent blue surface.

Figure 7.

Distribution of water molecules (cyan transparent surface) in the final state of the MD simulation of hP2Y₁R (cyan ribbons): (A) complex with MRS2500 (–3a species); (B) complex with BPTU; (C) apo form (run s3); (D) complex with 2MeSADP; (E) complex with MRS2268 (–3a species); and (F) complex with MRS2268 (–3b species). Distribution of water molecules (cyan beads) during the MD simulation of hP2Y₁R in complex with 2MeSADP: (G) initial state; (H) progression of water molecules after 200 ns. Residues surrounding regions filled with water molecules are highlighted as sticks.

Chart 1. Structure of Representative P2Y₁R Ligands Discussed in This Study

Chart 2.
Graphic Summary of Collected SAR Data for (A) Nucleotide and Non-nucleotide (B) Ligands

Table 1Summary of Available Mutagenesis Data for Orthosteric Ligands at the hP2Y₁R^a

P2Y ₁ R residue (BW number ³⁴)	interaction with MRS2500 in the X-ray complex	mutagenesis data available
Proximity to Adenine Core		
C42 (N-term)	surrounds C2-iodo group	C42A: reduces receptor surface expression and 2MeSADP efficacy ³⁸
L44 (N-term)	hydrophobic contact with adenine	L44A: abolishes 2MeSADP binding ⁹
N283 (6.58)	bidentate H-bond with N ⁶ and N ⁷	N283A: abolishes 2MeSADP binding ⁹
A286 (6.61)	proximity to N ⁶ methyl group	A286W: no effect on 2MeSADP binding, strongly reduces MRS2500 binding ⁹
R287 (6.62)	π -cation interaction with adenine	R287A/Q/E: reduces 2MeSADP efficacy. ³⁸ R287K: partial rescue of 2MeSADP efficacy. ³⁸
D300 (7.29)	ND ^b	D300A: slightly reduces 2MeSADP efficacy ³⁸
Y303 (7.32)	π - π interaction with adenine, H-bond with 3'-phosphate	Y303F: no effect on 2MeSADP binding, strongly reduces MRS2500 binding ⁹
Proximity to 3'-Phosphate		
K46 (N-term)	salt-bridge with 3'-phosphate	K46A: no effect on 2MeSADP binding, reduces MRS2500 binding ⁹
Y110 (2.63)	H-bond with 3'-phosphate	Y110F: abolishes 2MeSADP binding ⁹
R195 (EL2)	salt-bridge with 3'-phosphate	R195A: no effect on 2MeSADP binding, reduces MRS2500 binding. ⁹ R195A: no effect on 2MeSADP efficacy. ³⁸
Proximity to (N)-Methanocarba Ring		
Y203 (EL2)	hydrophobic contact with methanocarba ring	Y203A: abolishes 2MeSADP binding. ⁹ Y203A: reduces 2MeSADP efficacy and binding and slightly reduce MRS2179 ^c binding. ³¹ Y203F: partial rescue of 2MeSADP efficacy and binding, no effect on MRS2179 ^c binding. ³¹
Proximity to 5'-Phosphate		
D204 (EL2)	backbone H-bond with 5'-phosphate	D204A/N/E: reduces 2MeSADP efficacy ³⁸
T205 (EL2)	two H-bonds with 5'-phosphate	T205A: abolishes 2MeSADP binding ⁹
Y306 (7.35)	H-bond with 5'-phosphate	Y306F: strongly reduces binding of 2MeSADP and MRS2500. ⁹ Y306A/F: reduces 2MeSADP efficacy and binding, minor effect on MRS2179 ^c binding. ³¹
Q307 (7.36)	ND ^b	Q307A: reduces 2MeSADP efficacy; ³⁷ slightly reduces MRS2179 ^b binding, reduces 2MeSADP potency and MRS2179 ^c affinity ⁴¹
R310 (7.39)	salt-bridge with 5'-phosphate, water mediated interaction with 3'-phosphate	R310A: abolishes 2MeSADP efficacy. ³⁷ R310 K: partial rescue of 2MeSADP efficacy; ³⁷ reduces 2MeSADP potency, no effect on MRS2179 ^c affinity ⁴¹

^aThe residues are grouped according to their proximity to substitutions around the nucleoside scaffold as observed in the MRS2500-hP2Y₁R X-ray complex. Residues conserved at other P2Y₁R-like subtypes (according to the alignment reported in Figure S2) are highlighted in bold.

^bNot detected.

^cMRS2179: 2'-deoxy-N⁶-methyladenosine 3',5'-bisphosphate.

Table 2Summary of MD Simulation Performed^a

ligand/system	species	number of replicas × simulation time (ns)	total simulation time (μs)	
			per species	per ligand/system
MRS2500	−4	3 × 200	0.6	2.4
	−3a	3 × 200	0.6	
	−3b	3 × 200	0.6	
	−2	3 × 200	0.6	
MRS2268	−4	3 × 200	0.6	3.0
	−3a	3 × 400	1.2	
	−3b	3 × 200	0.6	
	−2	3 × 200	0.6	
2MeSADP	−3	3 × 200	0.6	0.6
BPTU	NA ^b	4 × 400	NA	1.6
apo	NA	3 × 200	NA	0.6

^aSpecies legend: −4, fully deprotonated 3′,5′-bisphosphate; −3a, deprotonated 5′-phosphate; −3b, deprotonated 3′-phosphate; −2 half deprotonated 3′,5′-bisphosphate.

^bNot applicable.

Table 3

Nucleotide-like Compounds Discussed in This Study^a

no.	adenine substitution				ribose substitution				IC ₅₀ or as noted ^b (EC ₅₀) [μ M]	ref
	R (n) C2	R ¹ N ⁶	R ² C8	R ³ C2'	R ⁴ C3'	R ⁵ C5'	C5'			
Ribose Derivatives										
1 ^c	H	NH ₂	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	5.76 $\pm$ 0.68 (6.26 $\pm$ 2.52)	15
2	Cl	NH ₂	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	2.01 $\pm$ 0.83	15
3	H	NH ₂	Br	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	36.7 $\pm$ 8.6	15
4	H	NH ₂	H	OPO ₃ H ₂	H	Cl	NE ^h			15
5	H	NH ₂	H	OCH ₃	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	12.4 $\pm$ 2.9 (12.9 $\pm$ 3.4)	15
6	H	NH ₂	CH ₃	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	75.1 $\pm$ 14.5	16
7	H	NH ₂	H	OPO ₃ H ₂	OPO ₃ H ₂	H	>100 ^d			17
8 ^e	H	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.330 $\pm$ 0.059	15
9	H	NHCH ₂ CH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	1.08 $\pm$ 0.38	15
10	H	NH(CH ₂) ₂ CH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	>100 ^d	15
11	H	N(CH ₃) ₂	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	46.7 $\pm$ 20.8	15
12	H	NHCH ₃	H	OPO ₃ H ₂	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.324 $\pm$ 0.054	15
13 ^c	Cl	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.206 $\pm$ 0.053	16
14	SCH ₃	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.362 $\pm$ 0.119	16
15 ^e	SCH ₂ CH ₃	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.98 $\pm$ 0.27	16

no.	adenine substitution				ribose substitution					IC ₅₀ or as noted ^b (EC ₅₀) [μ M]	ref
	R (n) C2	R ¹ N ⁶	R ² C8	R ³ C2'	R ⁴ C3'	R ⁵ C5'					
16	S(CH ₂) ₂ CH ₃	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.449 ± 0.190	16
17	H	NH ₂	Methanocarba Derivatives								
18 ^{c,g}	H	NH ₂	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	NE ^h (0.155 ± 0.021 ^f)	17
19	H	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	~40 (18.9 ± 5.7 ^c)	17
20	F	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.157 ± 0.060	13
21	Cl	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.356 ± 0.122	13
22 ^e	Br	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.0516 ± 0.0008	17
23 ^e	I	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.037 ± 0.006	13
24	I	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.0084 ± 0.0008	13
25	CH ₃	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	1.56 ± 0.54	19
26	(CH ₂) ₅ CH ₃	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.049 ± 0.001	13
27	C≡CH	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.452 ± 0.221	13
28	C≡C(CH ₂) ₂ -CO ₂ H	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.095 ± 0.039	19
29	C≡C(CH ₂) ₂ -CONH-(CH ₂) ₄ NH ₂	NHCH ₃	H	H	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	OPO ₃ H ₂	0.023 ± 0.003	20
30										0.132 ± 0.017	20
Other Ring Systems											
31											
32											
33-40											

adenine substitution				ribose substitution			IC ₅₀ or as noted ^b (EC ₅₀) [μ M]	ref
no.	R (n) C2	R ¹ N ⁶	R ² C8	R ³ C2'	R ⁴ C3'	R ⁵ C5'		
Acyclic Derivatives								
33	H (<i>n</i> = 2)	NH ₂			OPO ₃ H ₂	OPO ₃ H ₂	>50	22
34	Cl (<i>n</i> = 2)	NH ₂			OPO ₃ H ₂	OPO ₃ H ₂	7.75 $\pm$ 1.41	22
35	Cl (<i>n</i> = 0)	NHCH ₃			OPO ₃ H ₂	OPO ₃ H ₂	4.56 $\pm$ 1.38	23
36 ^e	Cl (<i>n</i> = 1)	NHCH ₃			OPO ₃ H ₂	OPO ₃ H ₂	0.48 $\pm$ 0.13	23
37	H (<i>n</i> = 2)	NHCH ₃			OPO ₃ H ₂	OPO ₃ H ₂	1.60 $\pm$ 0.47	22
38	Cl (<i>n</i> = 2)	NHCH ₃			OPO ₃ H ₂	OPO ₃ H ₂	0.840 $\pm$ 0.130	16, 22
39	H (<i>n</i> = 3)	NHCH			OPO ₃ H ₂	OPO ₃ H ₂	>30	22
40	H (<i>n</i> = 4)	NHCH			OPO ₃ H ₂	OPO ₃ H ₂	>50	22

^aCompounds were previously reported and tested at the P2Y₁R.

^bCompounds from refs 14, 15, 16, 23, and 24 were tested at the tP2Y₁R. Compounds from refs 13 and 19 were tested at the hP2Y₁R. If agonist effect is present, an EC₅₀ (μ M) is indicated.

^cCompound **1** has partial (12% of maximal) agonist activity. Compound **13** has partial (35% of maximal) agonist activity. Compound **18** has partial (50% of maximal) agonist activity.

^dSmall inhibition at 30–100 μ M.

^e**8**, MRS2179; **13**, MRS2216; **17**, MRS2268; **21**, MRS2279; **23**, MRS2500; **36**, MRS2298; **38**, MRS2286.

^fCompound **17** displays agonist activity (% of maximal increase = 92%) but not antagonist activity.

^gRibose-like moiety is a derivative of **18**, bicyclo[3.1.0]hexane; **30**, cyclopropane; **31**, cyclobutane; **32**, 1,5-anhydrohexitol.

^hNo antagonist effect.

Table 4

Non-nucleotide Compounds Discussed in This Study^d

No.	R	R ¹	X	K _i (nm) ^a	Ref.
41 ^c	4-OCF ₃ -Ph	2- <i>t</i> -Bu	O	6.0 ± 0.6	24
<i>Substitutions on ring A</i>					
42	4-Cl-Ph	3-CF ₃	O	69 ± 23	24
43	CH ₂ -Ph	3-CF ₃	O	>5000	24
44	2,4-di-F-Ph	4-Me	O	>5000	24
<i>Replacement of ether linker</i>					
45	4-Me-Ph	2- <i>t</i> -Bu	NMe	>5000	24
46				8.7 ± 1.7	27

46-49		52-55			
No.	R	R ¹	X	K _i (nm) ^a	Ref.
47				7.3 ± 0.6	27
41-45		50, 51			
48				830 ± 5	27

No.	R	R ¹	X	K _i (nm) ^a	Ref.
49				4.3 ± 2.6	27
50				<i>b</i>	28
51				<i>b</i>	28

Replacement of urea moiety

No.	R	R ¹	X	K _i (nM) ^a	Ref.
52				11	30
53				2500	30
54				45	30
55				>36,000	27

^aCompounds were tested at the hP2Y₁R.

^bBinding K_i values were not reported for compounds **50** and **51**, but their reported IC₅₀ values in calcium mobilization determined using a fluorescent imaging plate reader (FLIPR) are 0.14 ± 0.04 and 0.39 ± 0.28 nM, respectively.

^dCompounds were previously reported and tested in binding at the P2Y₁R.

^c**41**, BPTU.

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Table 5

Average RMSD Values Calculated during the MD Production Runs^a

ligand	species	run no.	RMSD [Å]					
			C α	ligand	3'p ^b	5'p ^c /aP	adenine	N-meth ^d
MRS2500	-4	s1	2.428	2.247	2.886	2.871	1.720	1.853
	-4	s2	2.428	1.866	2.047	2.199	1.499	1.870
	-4	s3	2.698	2.696	3.220	3.609	2.082	2.161
	-3a	s1	1.451	1.136	1.920	0.891	0.924	0.622
	-3a	s2	1.569	0.951	1.276	0.776	0.888	0.669
	-3a	s3	1.943	2.992	0.825	1.186	4.390	1.225
	-3b	s1	1.774	1.471	1.824	1.981	1.109	0.782
	-3b	s2	2.294	1.503	1.854	2.000	1.167	0.999
	-3b	s3	1.743	1.965	3.103	2.750	1.131	1.050
	-2	s1	1.566	1.950	2.172	2.111	1.788	1.640
	-2	s2	1.702	1.715	2.314	1.180	1.752	1.275
	-2	s3	1.867	1.887	2.670	1.496	1.974	0.954
MRS2268	-4	s1	1.556	1.239	1.486	1.871	0.800	0.652
	-4	s2	2.018	1.385	1.767	2.025	0.828	0.969
	-4	s3	1.479	1.406	2.073	1.857	0.719	0.990
	-3a	s1 ^e	1.656	1.586	1.824	2.024	1.257	1.216
	-3a	s2 ^e	2.252	1.979	2.873	2.168	1.166	1.848
	-3a	s3 ^e	2.444	1.532	2.38	0.947	1.334	1.209
	-3b	s1	1.909	1.589	1.580	1.930	1.351	1.448
	-3b	s2	1.946	1.135	0.683	2.116	0.794	0.660
	-3b	s3	1.845	1.831	2.205	2.064	1.464	1.553
	-2	s1	2.155	0.938	1.037	0.956	0.920	0.734
	-2	s2	2.063	2.072	2.320	2.880	1.506	1.603
	-2	s3	1.980	1.148	1.381	1.268	0.916	0.959
2MeSADP	-3	s1	1.593	1.247	2.019	1.242	0.889	1.108
	-3	s2	1.575	2.098	2.415	3.619	1.200	1.803

RMSD [Å]									
ligand	species	run no.	C α	ligand	ϕ , ψ , χ	5' p', α P	adenine	N-meth ^d	
BPTU	-3	s3	1.789	1.626	2.161	1.721	1.498	1.295	
	NA	s1 ^e	2.323	1.835					
	NA	s2 ^e	3.036	2.197					
	NA	s3 ^e	2.355	1.825					
	NA	s4 ^e	2.522	1.743					
apo	NA	s1	1.905						
	NA	s2	1.855						
	NA	s3	1.811						

^aThe trajectories were aligned on protein C α , and the first frame coordinates were taken as reference. Selected trajectories for video visualization and detailed visual inspection are highlighted in bold. The simulations were run for 200 ns unless otherwise specified.

^b3'-Phosphate group in MRS2500 and MRS2268 and β -phosphate group in 2MeSADP.

^c5'-Phosphate group in MRS2500 and MRS2268 and α -phosphate group in 2MeSADP.

^d(N)-Methanocarba ring in MRS2500 and MRS2268 and ribose ring in 2MeSADP.

^eSimulation length: 400 ns.

Table 6

Persistency of Salt-Bridge Interactions Expressed as Percent of the Total Simulation Time for Each Ligand/Species in Which the O–N Distance of the Residues Composing the Ion Pair Was Less than 4.0 Å^a

ion pair	MRS2500 (–3a) ^b	MRS2268 (–3a) ^c	MRS2268 (–3b) ^b	BPTU ^d	apo ^b	2MeSADP ^b
D289-R212	94.10	71.03	65.40	62.45	73.53	63.47
D208-K41	22.22	83.85	68.12	33.49	32.95	17.43
D204-K280	67.63	68.63	57.70	6.02	1.23	1.00
D204-R310	13.07	2.74	0.27	59.86	7.65	2.38
D204-R128	1.02	22.81	1.15	11.99	1.93	1.12
D204-R287	0.00	0.00	0.00	1.58	26.32	0.00
D208-R287	2.92	6.41	26.58	26.26	57.97	7.50
D329-R149	0.03	56.69	25.57	52.38	14.83	50.37
D329-K257	33.80	4.05	6.85	31.16	14.27	50.87
D329-R333	27.23	11.11	14.35	2.28	45.88	7.12
D300-K46	0.00	0.00	5.12	31.19	0.73	0.00
D300-R301	0.00	57.89	19.70	0.21	0.05	0.00
D289-R285	5.87	51.71	9.38	4.12	6.65	8.20
D121-K125	1.65	5.95	11.90	12.52	25.02	17.18
D250-R256	0.55	52.49	0.05	2.19	2.47	0.40

^aPersistency values >50% are highlighted in bold.

^bTotal simulation time: 0.6 μs.

^cTotal simulation time: 1.2 μs.

^dTotal simulation time: 1.6 μs.