Interaction of New, Very Potent Non-Nucleotide Antagonists with Arg256 of the Human Platelet P2Y\textsubscript{12} Receptor

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ABSTRACT
The P2Y\textsubscript{12} receptor plays a crucial role in platelet aggregation. In the present study, we analyzed the properties of non-nucleotide antagonists at the recombinant human P2Y\textsubscript{12} receptor and searched for amino acids involved in the molecular interaction. Receptor function was assessed by measuring the cAMP response element (CRE)-directed luciferase expression and searching for amino acids involved in the molecular interaction. ADP induces the aggregation of human platelets by the activation of G\textsubscript{q}-coupled P2Y\textsubscript{1} receptors and G\textsubscript{i}-coupled P2Y\textsubscript{12} receptors (Dorsam and Kunapuli, 2004; Cattaneo, 2007; Gachet, 2008; for an overview of P2Y receptors, see Abbracchio et al., 2006; von Kügelgen, 2006; Jacobson et al., 2007; Gachet, 2008; for an overview of P2Y receptors, see Abbracchio et al., 2006; von Kügelgen, 2006; Jacobson et al., 2007; Gachet, 2008).}

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ADP induces the aggregation of human platelets by the activation of G\textsubscript{q}-coupled P2Y\textsubscript{1} receptors and G\textsubscript{i}-coupled P2Y\textsubscript{12} receptors (Dorsam and Kunapuli, 2004; Cattaneo, 2007; Gachet, 2008; for an overview of P2Y receptors, see Abbracchio et al., 2006; von Kügelgen, 2006; Jacobson et al., 2007; Gachet, 2008). The irreversible blockade of the platelet P2Y\textsubscript{12} receptor by active metabolites of thienopyridine antithrombotic drugs such as clopidogrel (Savi et al., 2000; Algaier et al., 2008). In summary, the results demonstrate that PSB-0739 is the most potent competitive non-nucleotide antagonist at the human P2Y\textsubscript{12} receptor described so far. The results also indicate that the sulfonic acid residue at ring D is involved in the interaction of antagonists derived from reactive blue 2 with the residue Arg256 of the human P2Y\textsubscript{12} receptor.
some patients, the long duration of action of the thienopyridine compounds may cause prolonged bleeding incidents. For these patients, the use of potent, but competitive antagonists of the P2Y12 receptor for pharmacotherapy may be a beneficial and better controllable alternative. Cangrelor is a nucleotide antagonist that has been developed for this purpose (Ingall et al., 1999; Cattaneo, 2007). However, nucleotide-derived compounds have a poor bioavailability after peroral administration and a short plasma half-life time. The non-nucleotide analog ticagrelor (AZD6140) has recently been reported to act as an orally active, competitive antagonist at the platelet P2Y12 receptor (Springthorpe et al., 2007).

In a previous study, we showed that the residue Arg256 of the human P2Y12 receptor plays a prominent role in the recognition of both the agonists ADP and 2-methylthio-ADP and the non-nucleotide antagonist reactive blue 2 (RB-2) by the receptor protein (Hoffmann et al., 2008). The data suggest that the antagonist RB-2 interacts with the agonist at the orthosteric ligand binding site of the receptor compatible with competitive antagonism of RB-2 at the P2Y12 receptor. RB-2 has previously been used as a P2-receptor antagonist in a number of studies on native P2Y receptors (e.g., Burnstock and Warland, 1987; von Kügelgen et al., 1993; Boyer et al., 1994). The molecular structure of RB-2 contains three sulfonic acid residues. RB-2 is defined as a mixture of two constitutional isomers with a sulfonic acid residue in the meta- and para-position at ring F, respectively (CAS number 12236-82-7; see also Glänzel et al., 2003, 2005). The data of our present study indicate that the sulfonic acid residue at ring D of antagonists based on the structure of RB-2 (Fig. 1) is involved in the interaction with the basic residue Arg256 of the receptor protein. Moreover, we demonstrate that one of the analogs of RB-2 is the most potent competitive antagonist at the human P2Y12 receptor known so far. Some of the results have been presented in abstract form at a meeting (Hoffmann et al., 2009).

Materials and Methods

Molecular Biological Experiments and Cell Culture Techniques. The sequences encoding for the human P2Y12 receptor, mutant P2Y12-receptor constructs, and the human P2Y13 receptor were cloned into the expression vector pDNA5/FRT-V5-His-TOPO (Invitrogen, Karlsruhe, Germany) by use of standard molecular biology techniques. Chinese hamster ovary (CHO) Flp-In cells (Invitrogen) were transfected by use of the pcDNA5-FRT expression vector combined with the pOG44 vector (Invitrogen) and Lipofectamine 2000 (Invitrogen). Cells stably expressing the receptor constructs were selected 2 days after transfection by culturing in Ham's F-12 medium (Invitrogen) supplemented with Glutamax I, 10% fetal bovine serum, and 500 µg/ml hygromycin (Invitrogen). Cells stably expressing the receptor constructs were selected 2 days after transfection by culturing in Ham's F-12 medium (Invitrogen) supplemented with Glutamax I, 10% fetal bovine serum, and 500 µg/ml hygromycin (Invitrogen). A previous study showed that CHO Flp-In cells express wild-type P2Y12 receptors and the mutant constructs at almost identical densities (Hoffmann et al., 2008). Cells from passages 3 to 50 of the isogenic CHO Flp-In cells were used for further experiments.

Analysis of CRE-Dependent Luciferase Activity. In most experiments, the interaction of agonists and antagonists was studied by analyzing changes in the cAMP response element (CRE)-dependent expression of luciferase in CHO Flp-In cells expressing wild-type human P2Y12 receptors or mutant P2Y12-receptor constructs as
described previously (Hoffmann et al., 2008). In brief, the cells were transiently transfected with the pCRE-luc vector (Stratagene, Amsterdam, The Netherlands) by use of Lipofectamine 2000 (Invitrogen). After a period of 18 h, the cells were cultured on 24-well plates for additional 24 h. The culture medium was then removed and the cells were incubated with HBSS buffer (without HEPES, pH 7.3) for 3.5 h at 36.5°C. Cellular cAMP production was stimulated by addition of 1 μM forskolin. Solvent (control) or 2-methylthio-ADP was added together with forskolin, when used. The P2Y12-receptor antagonists were given 10 min before the agonist 2-methylthio-ADP. The reaction was stopped after 3.5 h by removal of the reaction buffer followed by the addition of 50 μl of HBSS and 50 μl of Bright-GLO luciferase assay solution (Promega, Mannheim, Germany). The activity of luciferase in the supernatant was analyzed by use of a single photon luminometer (Berthold Technologies, Bad Wildbad, Germany). The forskolin-induced (CRE-dependent) increases in luciferase activity in the presence of 2-methylthio-ADP were expressed as percentage of the responses to forskolin in the absence of 2-methylthio-ADP (percentage of respective control).

**Analysis of Cellular Cyclic AMP Accumulation.** In some experiments, changes in cellular cAMP levels were determined as described previously (Hoffmann et al., 2008). In brief, CHO Flp-In cells stably expressing the human P2Y12 receptor or the human P2Y13 receptor and 1321N1 astrocytoma cells stably expressing the human P2Y11 or P2Y14 receptors were cultured on 24-well plates for 24 h (culture media; see above and Hillmann et al., 2009). After removal of the culture medium, cells were incubated with HBSS buffer at 36.5°C for 2 h. Cellular cAMP production was then accelerated by addition of forskolin at the concentration of 10 μM (cells expressing P2Y12 or P2Y13 receptors) or 3 μM (cells expressing P2Y14 receptors) at 36.5°C for 10 min. Solvent (control) or 2-methylthio-ADP (P2Y12 and P2Y13 receptors; up to 1 mM) or UDP-glucose (P2Y14 receptors; 300 nM) was added together with forskolin. ATP (30 μM) was used to stimulate 1321N1 astrocytoma cells stably expressing the human P2Y11 receptor. The antagonist PSB-0739 was given 10 min before the agonist. The reaction was stopped after 10 min by removal of the reaction buffer followed by the addition of a hot lysis solution. cAMP levels in the supernatant were then quantified by incubation of an aliquot with cAMP-binding protein and cAMP (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and liquid scintillation counting after removal of the unbound cAMP by charcoal. cAMP levels per well were calculated by regression analysis from a standard curve determined for each experiment.

**Testing at Further P2-Receptor Subtypes.** In addition, effects of PSB-0739 on the agonist-induced increases in intracellular calcium concentration were tested in 1321N1 astrocytoma cells stably expressing the human P2Y1, P2Y2, P2Y4, P2Y6, and P2Y13 receptors or in rat PC12 cells expressing the native P2X2 receptor, and in rat C6 cells expressing the native P2X7 receptor after incubation of the cells with fluo-4 acetoxy methylester or Oregon green BAPTA-1/acetoxy methylester (Invitrogen) (mixture of reactive blue 2 meta and reactive blue 2 para; Ega Chemie, Mannheim, Germany; cibarcon blue 3GA, MG 38-1 (for structure see Glänzel et al., 2003, 2005), reactive blue 2 meta, and reactive blue 2 para (synthesized by Markus Glänzel, Department of Pharmacology and Toxicology, University of Freiburg; for structures see Glänzel et al., 2003, 2005); PSB-0739 (see Fig. 1), PSB-0826 (see Fig. 1); PSB compounds synthesized in the laboratory of Christa E. Müller, Pharmaceutical Sciences Bonn, University of Bonn, as described previously by Baqi and Müller, 2007; Weyler et al., 2008; Baqi et al., 2009a,b; Bay u 9421 (see Fig. 1) and suramin hexosamide salt (Bayer, Wuppertal, Germany). Stock solutions of drugs were prepared either with distilled water or with a mixture of ethanol and dimethyl sulfoxide (Sigma Chemie; used for forskolin only). The solvents added to the buffer were also used for control experiments.

**Results**

The interaction of antagonists with the prototypic agonist 2-methylthio-ADP was studied in CHO Flp-In cells stably expressing recombinant human wild-type P2Y12 receptors and mutant receptor constructs by use of a reporter gene assay (see Hoffmann et al., 2008). Stimulation by 1 μM forskolin for 3.5 h markedly increased the CRE-dependent luciferase expression by approximately 2.4-fold over basal activity in cells expressing wild-type receptors (Table 1). Similar increases were obtained in cells expressing the S101A and R256A mutant constructs (Table 1). In cells expressing the wild-type P2Y12 receptor, the agonist 2-methylthio-ADP inhibited the response to forskolin in a concentra-
tion-dependent manner with a half-maximal concentration (IC₅₀) of 1 nM and a maximal inhibition of approximately 70% (Table 1 and Fig. 2A). Very similar effects of 2-methylthio-ADP were observed in cells expressing the S101A mutant construct (Table 1), whereas 2-methylthio-ADP showed a lower potency and a reduced maximal effect in cells expressing the R256A mutant construct (Table 1 and Fig. 3).

Effects of RB-2, the Pure meta- and para-Isomers and the Regioisomer (ortho) Cibacron Blue 3GA. In cells expressing the wild-type P2Y₁₂ receptor, RB-2 (1 μM) shifted the concentration-response curve of the agonist 2-methylthio-ADP markedly to the right (Fig. 2A). The shift caused by RB-2 was concentration-dependent as shown by additional experiments using a lower (0.3 μM) and a higher concentration (3 μM) of RB-2 (Fig. 2B). Linear regression analysis of log (dose ratio – 1) against log (concentration of RB-2) revealed a slope not different from unity and (after refitting with a slope fixed to unity) a pA₂ value of 7.4 (Fig. 2B). Next, the effects of the pure constitutional isomers of RB-2 were tested. The pure meta-isomer of RB-2 (3 μM) shifted the concentration-response curves of 2-methylthio-ADP both in cells expressing wild-type receptors and in cells expressing R256A mutant constructs (Fig. 3, A and B). However, the degree of the shifts differed (Fig. 3, A and B). The apparent pKᵢ values amounted to 7.8 and 7.0, respectively (Table 2; p < 0.01). Similar results were obtained with the pure para-isomer of RB-2. In cells expressing wild-type receptors and R256A mutant constructs, there were clear, but again different shifts of the concentration-response curves of 2-methylthio-ADP to the right (Fig. 3, C and D) with apparent pKᵢ values of 7.3 and 6.7, respectively (Table 2; p < 0.01). An antagonistic action with a clear difference in potency at the wild-type receptor and the R256A mutant was also ob-

Fig. 2. 2-Methylthio-ADP (2-MeSADP)-mediated inhibition of the forskolin-induced CRE-dependent luciferase expression in CHO Flp-In cells stably expressing the human P2Y₁₂ receptor and interaction with RB-2. A, concentration-response curves. B, Schild plot analysis. Cells expressing the wild-type receptor were transiently transfected with the pCRE-luc vector and were stimulated by 1 μM forskolin for 3.5 h before measuring the luciferase activity. Values are expressed as percentage of relative light units (RLU) determined in experiments with forskolin, but without 2-methylthio-ADP (percentage of respective control; con). A, concentration-response curves of 2-MeSADP in the absence (continuous line) and presence of 1 μM RB-2 (interrupted line). Means ± S.E. of 3 to 15 experiments. The forskolin-induced increases in RLU amounted to 2,958,439 ± 230,435 in the absence and 2,744,407 ± 190,136 in the presence of RB-2, respectively. ++, p < 0.01, significant differences versus respective control; +++, p < 0.001, significant differences versus respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post test). B, Schild plot analysis of data from experiments using RB-2 at concentrations of 0.3, 1, and 3 μM. The slope did not differ from unity; therefore, the regression analysis was recalculated with a slope fixed to 1.

Methods

Effects of Novel Analogs of RB-2 with Two Sulfonate Residues. Next, we studied the effects of novel analogs of RB-2 with only two sulfonate residues in CHO cells expressing the wild-type human P2Y₁₂ receptor (A, C) or the R256A-mutant construct (B, D). The figures show concentration-response curves for 2-MeSADP in the absence (continuous lines) or presence of the antagonists (interrupted lines). Values are expressed as percentage of relative light units (RLU) determined in experiments with forskolin, but without 2-methylthio-ADP (percentage of respective control; con). Means ± S.E. of 5 to 20 experiments. The forskolin-induced increases in RLU amounted to 2,514,523 ± 222,190 in the absence and 2,324,229 ± 221,798 in the presence of RB-2 meta (A), 1,646,847 ± 144,611 in the absence and 1,466,221 ± 99,489 in the presence of RB-2 para (B), 2,435,338 ± 123,086 in the absence and 2,405,545 ± 121,352 in the presence of RB-2 para (C), and 1,561,389 ± 111,913 in the absence and 1,614,620 ± 105,327 in the presence of RB-2 para (D), respectively. +, p < 0.05; ++, p < 0.01, significant differences versus respective control. +++, p < 0.001, significant differences versus respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post test). For further details see legend to Fig. 2.
Apparent pK_B or pA_2 Values at Human P2Y_{12} Receptor Constructs

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† p < 0.05; ‡ p < 0.01 significant differences from respective values determined in cells expressing wild-type P2Y_{12} receptors (pF test).

‡ Data determined in CHO Flp-In cells transiently transfected with the pCRE-luc vector (taken from Hoffmann et al., 2008).

Fig. 4. Interaction of 2-methylthio-ADP (2-MeSADP) with purified cibacron blue 3GA (sulfonate in ring-F-ortho-position) in CHO cells expressing the wild-type human P2Y_{12} receptor (A) or the R256A-mutant construct (B). The figures show concentration-response curves for 2-MeSADP in the absence (continuous lines) or presence of cibacron blue 3GA (interrupted lines). Values are expressed as percentage of relative light units (RLU) determined in experiments with forskolin, but without 2-methylthio-ADP (percentage of respective control, con). Mean ± S.E. of 4 to 12 experiments. The forskolin-induced increases in RLU amounted to 1,289,273 ± 157,397 in the absence and 1,414,876 ± 165,524 in the presence of cibacron blue 3GA (A) and 842,685 ± 59,847 in the absence and 842,085 ± 130,760 in the presence of cibacron blue 3GA (B), respectively. * p < 0.05, † p < 0.01, significant differences versus respective control. ++, p < 0.01, significant differences versus respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post test). For further details see legend to Fig. 2.

Fig. 5. Interaction of 2-methylthio-ADP (2-MeSADP) with the analog MG 38-1 in CHO cells expressing the wild-type human P2Y_{12} receptor (A) or the R256A-mutant construct (B). The figures show concentration-response curves for 2-MeSADP in the absence (continuous lines) or presence of MG 38-1 (interrupted lines). Values are expressed as percentage of relative light units (RLU) determined in experiments with forskolin, but without 2-methylthio-ADP (percentage of respective control, con). Mean ± S.E. of 7 to 20 experiments. The forskolin-induced increases in RLU amounted to 1,325,325 ± 157,397 in the absence and 1,258,339 ± 127,028 in the presence of MG 38-1 (A) and 985,462 ± 101,225 in the absence and 971,238 ± 121,753 in the presence of MG 38-1 (B), respectively. * p < 0.05, ** p < 0.01, significant differences versus respective control. ++, p < 0.01, significant differences versus respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post test). For further details see legend to Fig. 2.

To confirm the very high antagonistic potency of PSB-0739 at the human P2Y_{12} receptor by the use of a distinct method, we analyzed receptor-mediated changes in cellular cAMP content in a concentration-dependent manner with a half-maximal concentration (IC_{50}) of 0.6 nM and a maximal inhibition of approximately 90% (n = 7–9; not shown). PSB-0739 (30 nM) shifted the concentration-response curve to the right with an apparent pK_B value of 10.1 (not shown; note that this pK_B value is close to the pA_2 value of 9.8 determined by the reporter gene assay).

The human P2Y_{12} Receptor and the human P2Y_{13} Receptor share pharmacological properties. To determine the selectivity of PSB-0739 at the P2Y_{12} receptor versus the P2Y_{13} receptor, the interaction of 2-methylthio-ADP and PSB-0739 was also tested in CHO Flp-In cells stably expressing recombinant human P2Y_{13} receptors. In these cells, 2-methylthio-ADP (10 nM to 1 μM) inhibited the forskolin-induced increases in cellular cAMP with a maximal inhibition of approximately 35% (n = 9–12); PSB-0739 (30 nM and 1 μM tested) did not block the effect of 2-methylthio-ADP (not shown). Moreover, PSB-0739 (1 μM) did not affect the responses to stimulation of the recombinant human P2Y_{2}, and P2Y_{14}-receptors stably expressed in 1321N1 astrocytoma cells, native P2X_{7}-receptors in rat PC12 cells and native P2X_{7} receptors in rat C6 cells, respectively (not shown). In

TABLE 2

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‡ Data determined in CHO Flp-In cells transiently transfected with the pCRE-luc vector (taken from Hoffmann et al., 2008).
Testing of Analogs of PSB-0739 with Only One Sulfonate Residue. To identify the sulfonate residue involved in the interaction of PSB-0739 with Arg256 of the P2Y12-receptor protein, two analogs of PSB-0739 with only one sulfonate residue were tested by use of again the reporter gene assay and CHO cells expressing the P2Y12-receptor or mutant constructs. PSB-0801 with a sulfonate residue at ring D (see Fig. 1) was markedly less potent than PSB-0739 (Fig. 7A and Table 2). PSB-0801 shifted the concentration-response curves of 2-methylthio-ADP by obviously different degrees in cells expressing wild-type receptors and R256A mutant constructs, respectively (Fig. 7, A and B). The corresponding apparent pK_B values amounted to 7.4 (wild-type receptor) and 6.7 (R256A mutant; Table 2), respectively. In contrast, PSB-0826 with a sulfonate residue at ring C had higher and almost identical potencies at wild-type and mutant receptors with apparent pK_B values of 8.4 at the wild-type receptor (Fig. 7C) and of 8.3 at the R256A mutant (Fig. 7D), respectively. PSB-0801 and PSB-0826 did not affect the responses to forskolin (see legend to Fig. 7).

Testing of Reactive Red 2, Suramin and Bay u 9421. Finally, the effects of three antagonists without an anthraquinone structure were studied in CHO cells expressing the P2Y12-receptor or mutant constructs. Reactive red 2 (two sulfonate residues), suramin (six sulfonate residues), and the suramin analog Bay u 9421 (four sulfonate residues; see Fig. 1) all acted as antagonists at the human P2Y12 receptor with apparent pK_B values of 6.5, 5.5, and 5.9, respectively (Table 2). For each antagonist, almost identical antagonistic potencies were determined in cells expressing wild-type receptors, R256A mutant constructs, or S101A mutant constructs (Table 2).

Discussion

In a previous study on CHO Flp-In cells stably expressing the recombinant human P2Y12-receptor or mutant constructs, we showed that the receptor-mediated changes in the forskolin-induced, CRE-dependent expression of luciferase...
reflect receptor-mediated changes in intracellular cAMP concentrations (Hoffmann et al., 2008). To avoid known problems of radioligand-binding assays using labeled nucleotides (e.g., high-affinity binding to a number of proteins including nucleotidases), we used the CRE-dependent reporter gene assay to characterize the pharmacological properties of RB-2 and analogs. In our previous study, we also showed that wild-type P2Y₁₂ receptors, S101A mutant constructs, and R256A mutant constructs were expressed in CHO Flp-In cells with almost identical densities (Hoffmann et al., 2008).

Our present data confirm the notion that RB-2 acts as a competitive antagonist at the human P2Y₁₂ receptor. The pA₂ value of 7.4 fits very well to pA₂ and pKᵢ₀ values of RB-2 at the P2Y₁₂ receptor determined previously (Boyer et al., 1994; Hoffmann et al., 2008). Our results show for the first time that the synthesized pure meta- and para-isomers of RB-2 (carrying a sulfonate residue in meta- or para-position at ring F) as well as the ortho-isomer cibacron blue 3GA (sulfonate in ortho-position at ring F) have similar antagonistic potencies at the human P2Y₁₂ receptor.

A smaller analog of RB-2 without an extra ring (ring F), namely PSB-0739, showed a markedly higher antagonistic potency with a pKᵢ₀ value in the subnanomolar range. Our data indicate that PSB-0739 acts as competitive antagonist. To our knowledge, PSB-0739 is the most potent competitive antagonist acting at the human P2Y₁₂ receptor described so far (compare potencies in Table 2; see Springthorpe et al., 2007, for ticagrelor and analogs). Testing the effects of PSB-0739 at P2Y₁₂, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₄, P2X₂, and P2X₃ receptors and ecto-5'-nucleotidase revealed selectivity for the P2Y₁₂ receptor by factors greater than 100 (Baqi et al., 2009a; present results). Hence, PSB-0739 will serve as a valuable experimental tool for the pharmacological characterization of P2 receptors.

The data of the study also indicate that the sulfonate residue at the ring D of PSB-0739 interacts with Arg256 of the receptor protein. Arg256 in the upper third of transmembrane region 6 of the human P2Y₁₂ receptor has previously been shown to contribute to both the recognition of agonists by the receptor (Cattaneo et al., 2003; Hoffmann et al., 2008) and the recognition of the antagonist reactive RB-2 (Hoffmann et al., 2008). In agreement with an important role of Arg256 in the function of the P2Y₁₂ receptor, a patient carrying a R256Q/R265W mutation in the P2Y₁₂ receptor showed a prolonged bleeding time (Cattaneo et al., 2003).

Corresponding basic residues (Lys or Arg) in transmembrane region 6 of the P2Y₁ receptor (Jiang et al., 1997; Hoffmann et al., 1999; Guo et al., 2002), the P2Y₂ receptor (Erb et al., 1995; Hillmann et al., 2009), and the P2Y₁₁ receptor (Qi et al., 2001; Zylberg et al., 2007) have also been demonstrated to contribute to agonist and antagonist recognition. The synthesized pure meta- and para-isomers of RB-2 and the ortho-isomer cibacron blue 3GA also showed reduced potencies at the R256A mutant construct of the P2Y₁₂ receptor in comparison with those determined at the wild-type receptor. This argues against a major contribution of a sulfonate residue at ring F in the interaction with the residue Arg256 of the receptor. In agreement with this notion, reduced potencies at the R256A mutant construct were also observed for the smaller compounds PSB-0739, PSB-0801, and MG 38-1 without a ring F. Moreover, PSB-0826 (the analog of PSB-0739 lacking the sulfonate residue at ring D) showed very similar potencies at the wild-type receptor and the R256A mutant construct indicating that the sulfonate residue of PSB-0739 at ring D (and, most likely, the respective sulfonate residue of other analogs of RB-2) interacts with Arg256 of the P2Y₁₂ receptor. RB-2 acts as an antagonist at the human P2Y₁₂ receptor with a lower potency. RB-2 has recently been shown to interact with the residues Tyr114, Arg180, and Tyr198 of the human P2Y₁₂ receptor (Hillmann et al., 2009) indicating that the interaction sites of P2Y-receptor antagonists differ markedly at different receptor subtypes.

For chemically unrelated antagonists such as suramin, an interaction with Arg256 of the P2Y₁₂ receptor is unlikely as shown by almost identical potencies of suramin, its analog Bay u 9421, and reactive red 2 at wild-type and mutant receptors. None of the compounds tested in the present study interacted with Ser101 of the human P2Y₁₂ receptor. It had previously been shown that the nucleotide antagonist canagrelor acts in a noncompetitive manner in cells expressing S101A-mutant constructs of the P2Y₁₂ receptor (Hoffmann et al., 2008).

In summary, our data demonstrate the potent antagonistic action of PSB-0739 at the human P2Y₁₂ receptor. The data also indicate that the sulfonate residue of PSB-0739 at ring D interacts with Arg256 of the P2Y₁₂ receptor.

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