Interation of New, Very Potent Non-Nucleotide Antagonists with Arg256 of the Human Platelet P2Y₁₂ Receptor

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ABSTRACT

The P2Y₁₂ receptor plays a crucial role in platelet aggregation. In the present study, we analyzed the properties of non-nucleotide antagonists at the recombinant human P2Y₁₂ 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 in Chinese hamster ovary cells. The cellular cAMP production was accelerated by forskolin; 2-methylthio-ADP was used to activate the wild-type P2Y₁₂ receptor or mutant constructs. 2-Methylthio-ADP inhibited the CRE-dependent luciferase expression with an IC₅₀ value of approximately 1 nM. The anthraquinone derivative reactive blue 2 used at increasing concentrations shifted the concentration-response curve of 2-methylthio-ADP to the right in a manner compatible with competitive antagonism (pA₂ value, 7.4). Its analog, 1-amino-4-[4-phenylamino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (PSB-0739), showed a markedly higher antagonistic potency with a pA₂ value of 9.8. In cells expressing the R256A-mutant receptor, the potencies of both reactive blue 2 (apparent pKᵦ, 5.9) and PSB-0739 (apparent pKᵦ, 9.1) were decreased. The same was true for the pure reactive blue 2 meta- and para-isomers and for the ortho-isomer cibacron blue 3GA. In contrast, the analog, 1-amino-4-[4-anilino-phenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate, lacking a sulfonic acid residue at ring D (PSB-0826), showed similar pKᵦ values at wild-type (8.4) and R256A-mutant receptors (8.3). In summary, the results demonstrate that PSB-0739 is the most potent competitive non-nucleotide antagonist at the human P2Y₁₂ 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 P2Y₁₂ receptor.

ADP induces the aggregation of human platelets by the activation of Gₛ-coupled P2Y₁ receptors and Gₛ-coupled P2Y₁₂ 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). Recently, it was shown that the active metabolites of clopidogrel (Savi et al., 2000) and prasugrel (Sugidachi et al., 2001) has been proven to be useful in pharmacotherapy of thrombotic diseases including heart attack and stroke (Savi and Herbert, 2005; Jakubowski et al., 2007). It was shown that the active metabolites of clopidogrel and prasugrel interact in an irreversible manner with residues Cys97 and Cys175 of the human P2Y₁₂ receptor that are likely to form a disulfide bridge in the intact receptor protein (Savi et al., 2006; Algaier et al., 2008). In

ABBREVIATIONS: AZD6140, (15S,2S,3R,SS)-3-[7-[(1R,2S)-2-(3,4-difluorophenyl)cyclopropylamino]-5-(propylthio)-3R-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy)cyclopentane-1,2-diol; ANOVA, analysis of variance; CHO cells, Chinese hamster ovary cells; CRE, cAMP response element; HBSS, Hank’s balanced salt solution; 2-MeSADP, 2-methylthio-ADP, 2-methylthioadenosine 5'-diphosphate; BAPTA, 1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid; RB-2, reactive blue 2, amino-4-[4-[4-chloro-6-[[3 (or 4)-sulfophenyl]amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenylamino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid; PSB-0739, 1-amino-4-[4-phenylamino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate; PSB-0826, 1-amino-4-[4-anilino-phenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate, lacking a sulfonic acid residue at ring D; PSB-0801, 1-amino-2-methyl-4-[4-phenylamino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene monosodium salt; Bay u 9421, 2,2'-(6-chloro-1,3,5-triazine-2,4-diyl)-bis(azanediyl)-bis-[(N- (6-hydroxy-3,6-disulfonylphenyl-1-yl)benzamide tetrasodium salt; MG 38-1, (1-amino-4-[4-amino-3-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt.
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 pcDNA5/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). 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 P2Y13-receptor antagonist was 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., 2008). 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 P2Y11 or P2Y14 receptors) or 3 μM (cells expressing P2Y13 receptors) at 36.5°C for 10 min. Solvent (control) or 2-methylthio-ADP (P2Y13 and P2Y14 receptors; up to 1 μM) 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 P2Y12 receptor, 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 methyl ester or Oregon green BAPTA-1 acetoxy methyl ester (Invitrogen) as described previously (Kulick and von Kügelgen, 2002; Weyer et al., 2008; Hillmann et al., 2009). For this purpose, cells were stimulated by addition of 3 μM ADP (P2Y1 receptor), 100 μM ATP (P2X1 receptor), or 300 μM 3′-O-(4-benzoyl)benzoyl-ATP (P2X7 receptor) in the absence and presence of 1 μM PSB-0739, respectively.

**Data Analysis and Statistics.** Results are presented as means ± S.E. from n observations (analyzed wells). Differences between means were tested for significance by an analysis of variance (ANOVA) followed by the Bonferroni or Dunnett post test (Prism 4.03, Graph Pad, San Diego CA). Concentration-response data were fitted by nonlinear regression to estimate IC50 values (concentrations causing half-maximal inhibition) for 2-methylthio-ADP. Apparent pK1 values were calculated according to: pK1 = log (dose ratio − 1) − log[E]. pK2 values were determined by linear regression analysis (according to Arunlakshana and Schild, 1959). Differences between the estimated IC50 values or pK1 values were tested for significance by the F test (Prism 4.03). p < 0.05 or lower was the significance criterion.

**Chemicals.** The following drugs were used: adenosine 5′-diphosphate sodium salt (ADP), adenosine 5′-triphosphate sodium salt (ATP), forskolin, 2-methylthioadenosine 5′-diphosphate trisodium salt (2-methylthio-ADP, 2-MeSADP), reactive red 2 and uridine 5′-diphosphate-glucose (UDP-glucose; Sigma Chemie, Deisenhofen, Germany); 3′-O-(4-benzoyl)benzoyl-ATP sodium (Invitrogen); RB-2 (mixture of reactive blue 2 meta and reactive blue 2 para; Ega Chemie, Mannheim, Germany); ciferon 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), and PSB compounds synthesized in the laboratory of Christa E. Müller, Pharmaceutical Sciences Bonn, University of Bonn, as described previously by Baqi and Müller, 2007; Weyer et al., 2008; Baqi et al., 2009a,b; Bay u 9421 (see Fig. 1) and suramin hexosidum 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-

**TABLE 1**

<table>
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<th>Forskolin-induced increases in the CRE-dependent luciferase-expression (relative light units) and inhibition by 2-methylthio-ADP (2-MeSADP) in CHO Flp-In cells expressing human wild-type and mutant P2Y12-receptor constructs</th>
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<tr>
<td>Cells stably expressing the receptor constructs were transiently transfected with the pCRE-luc vector and stimulated by 1 μM forskolin in the absence or presence of 2-MeSADP for 3.5 h. Means ± S.E. from (n) observations.</td>
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<tr>
<td>CHO Flp-In Cells Expressing the Human P2Y12 Receptor</td>
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<tr>
<td>Basal RLU</td>
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<td>Forskolin-induced RLU</td>
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<td>Inhibition by 1 μM 2-MeSADP, %</td>
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<td>Inhibition by 2-MeSADP, log IC50</td>
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RLU, relative light units. *, p < 0.05; **, p < 0.01 significant differences from values determined at wild-type receptors (ANOVA followed by the Bonferroni post test or F test).
tion-dependent manner with a half-maximal concentration (IC$_{50}$) 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) Cibacon Blue 3GA.** In cells expressing the wild-type P2Y$_{12}$ 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 pA$_2$ 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 pK$_{B}$ 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 pK$_{B}$ 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 observed for the ortho-isomer cibacon blue 3GA (Fig. 4). The apparent pK$_{B}$ values amounted to 7.2 (wild-type receptor) and 5.7 (R256A mutant; Table 2; p < 0.05). None of the antagonists changed the forskolin-induced increases in luciferase expression in cells expressing wild-type receptors or R256A mutant constructs (see legends to Figs. 3 and 4).

**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 P2Y$_{12}$ 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 respective 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 para (A), 1,646,847 ± 144,611 in the absence and 1,466,221 ± 99,489 in the presence of RB-2 meta (B), 2,435,338 ± 123,936 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 < 0.05; ++, p < 0.01, significant differences versus respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post test).**

![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 P2Y$_{12}$ 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 respective light units (RLU) determined in experiments with forskolin, but without 2-methylthio-ADP (percentage of respective control, con). Mean ± 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 < 0.01, 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.**

![Fig. 3. Interaction of 2-methylthio-ADP (2-MeSADP) with the purified meta- and para-isomers of the non-nucleotide antagonist reactive blue 2 in CHO cells expressing the wild-type human P2Y$_{12}$ 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 respective 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 meta (B), 2,435,338 ± 123,936 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 < 0.05; ++, 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.
constructs stably expressed in CHO Flp-In cells
human wild-type P2Y12 receptors, 2-methylthio-ADP (0.1 nM levels. In CHO Flp-In cells stably expressing recombinant
we analyzed receptor-mediated changes in cellular cAMP
K
0739 (30 nM) shifted the concentration-response curve to the
half-maximal concentration (IC50) of 0.6 nM and a maximal
inhibiting the forskolin-induced increases in cellular cAMP by the use of a distinct method, [44x160]Figs. 5 and 6).
PSB-0739 affected the responses to forskolin (see legends to
compared with the shift determined in cells with wild-type
concentration-response curves of the agonist 2-methylthio-ADP to the
right (Fig. 6, C and D). The shift observed in cells
absence of the antagonist (ANOVA followed by the Bonferroni post test). For further details see legend to Fig. 2.
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: Apparent pK_B or pA_2 Values at Human P2Y_{12}-Receptor Constructs

<table>
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<th>Wild Type</th>
<th>S101A</th>
<th>R256A</th>
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<tr>
<td>Reactive blue 2 (mixture of meta and para)</td>
<td>7.4*</td>
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<tr>
<td>Reactive blue 2 meta</td>
<td>7.8</td>
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<tr>
<td>Reactive blue 2 para</td>
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<td>Cibacron blue 3GA (ortho-isomer)</td>
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</tr>
<tr>
<td>PSB-0801</td>
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<td></td>
<td>6.7**</td>
</tr>
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<td></td>
<td>8.3*</td>
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<tr>
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<td>Suramin</td>
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<td>5.2</td>
</tr>
<tr>
<td>Bay u 9421</td>
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<td>6.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Cangrelor</td>
<td>8.6*</td>
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<td>8.5†</td>
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* p < 0.05; † p < 0.01 significant differences from respective values determined in cells expressing wild-type P2Y_{12} receptors (P = test). pA_2 values calculated from the data shown in Figs. 2B (reactive blue 2) and 6B (PSB-0739).
† 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). Means ± S.E. of 4 to 12 experiments. The forskolin-induced increases in RLU amounted to 1,289,273 ± 135,254 in the absence and 1,414,876 ± 165,524 in the presence of cibacron 3 GA (A) and 822,640 ± 59,847 in the absence and 842,085 ± 130,760 in the presence of cibacron 3 GA (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). Means ± 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 ± 121,735 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.
1321N1 astrocytoma cells stably expressing recombinant human P2Y_{11} receptors, PSB-0739 (1 μM) had only a minor effect (<20% inhibition of ATP (30 μM)-induced increase in cAMP levels; not shown).

**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 P2Y_{12}-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 wild-type human P2Y_{12} receptor or mutant constructs. PSB-0801 with a sulfonate residue at ring C had only a minor effect (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. 7A, 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 P2Y_{12}-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 P2Y_{12} 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 P2Y_{12}-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 adenine 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 P2Y_{12} 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 P2Y_{12} receptor. The pA_{2} value of 7.4 fits very well to pA_{2} and pK_{B} values of RB-2 at the P2Y_{12} 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 P2Y_{12} receptor.

A smaller analog of RB-2 without an extra ring (ring F), namely PSB-0739, showed a markedly higher antagonistic potency with a pK_{B} 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 P2Y_{12} 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 P2Y_{1}, P2Y_{2}, P2Y_{4}, P2Y_{6}, P2Y_{11}, P2Y_{13}, P2Y_{14}, P2X_{2}, and P2X_{7} receptors and ecto-5'-nucleotidase revealed selectivity for the P2Y_{12} 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 P2Y_{12} 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 P2Y_{12} receptor, a patient carrying a R256Q/R265W mutation in the P2Y_{12} receptor showed a prolonged bleeding time (Cattaneo et al., 2003).

Corresponding basic residues (Lys or Arg) in transmembrane region 6 of the P2Y_{1} receptor (Jiang et al., 1997; Hoffmann et al., 1999; Guo et al., 2002), the P2Y_{2} receptor (Erb et al., 1995; Hillmann et al., 2009), and the P2Y_{11} receptor (Qi et al., 2001; Zyliberg 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 P2Y_{12} 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 P2Y_{12} receptor. RB-2 acts as an antagonist at the human P2Y_{2} receptor with a lower potency. RB-2 has recently been shown to interact with the residues Tyr114, Arg180, and Tyr198 of the human P2Y_{12} 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 P2Y_{12} 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 P2Y_{12} receptor. It had previously been shown that the nucleotide antagonist canagrel acts in a noncompetitive manner in cells expressing S101A-mutant constructs of the P2Y_{12} receptor (Hoffmann et al., 2008).

In summary, our data demonstrate the potent antagonistic action of PSB-0739 at the human P2Y_{12} receptor. The data also indicate that the sulfonate residue of PSB-0739 at ring D interacts with Arg256 of the P2Y_{12} receptor.

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References


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