British Journal of Pharmacology (2002) 135, 2004–2010
www.nature.com/bjp

2-Chloro N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′, 5′-bisphosphate is a selective high affinity $P2Y_1$ receptor antagonist

[1]José L. Boyer, [1]Mary Adams, [2]R. Gnana Ravi, [2]Kenneth A. Jacobson & *,[1]T. Kendall Harden

[1]Department of Pharmacology, University of North Carolina School of Medicine, CB#7365, Chapel Hill, North Carolina, NC 27599, U.S.A. and [2]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, MD 20892, U.S.A.

1 We reported previously that bisphosphate derivatives of adenosine are antagonists of the $P2Y_1$ receptor and that modification of the ribose in these analogues is tolerated in the $P2Y_1$ receptor binding pharmacophore.

2 Here we delineate the pharmacological activity of one such non-nucleotide molecule, 2-chloro N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2279), in which the ribose is replaced by a cyclopentane ring constrained in the (N)-conformation by a cyclopropane moiety.

3 MRS2279 antagonized 2MeSADP-stimulated inositol phosphate formation in turkey erythrocyte membranes with competitive kinetics ($pK_B = 7.75$). High affinity competitive antagonism by MRS2279 was also observed at the human $P2Y_1$ receptor ($pK_B = 8.10$) stably expressed in 1321N1 human astrocytoma cells. Antagonism was specific for the $P2Y_1$ receptor since MRS2279 had no effect on activation of the human $P2Y_2$, $P2Y_4$, $P2Y_6$, or $P2Y_{11}$ receptors by their cognate agonists.

4 MRS2279 also did not block the capacity of ADP to act through the Gi/adenylyl cyclase linked P2Y receptor of platelets to inhibit cyclic AMP accumulation.

5 In contrast, the $P2Y_1$ receptor is known to be obligatory in the process of ADP-induced platelet aggregation, and MRS2279 competitively inhibited ADP-promoted platelet aggregation with an apparent affinity ($pK_B = 8.05$) similar to that observed at the human $P2Y_1$ receptor heterologously expressed in 1321N1 cells.

6 Taken together these results illustrate selective high affinity antagonism of the $P2Y_1$ receptor by a non-nucleotide molecule that should prove useful for pharmacological delineation of this receptor in various tissues.

British Journal of Pharmacology (2002) **135**, 2004–2010

Keywords: P2Y receptors; $P2Y_1$ receptor antagonist; 2-chloro N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate; phospholipase C; adenylyl cyclase; platelet aggregation

Abbreviations: 2MeSADP, 2-Methylthioadenosine 5′-diphosphate; DMEM, Dulbecco's Modified Eagle's Medium; EGTA, Ethylene glycol-bis(beta-aminoethyl ether)- N,N,N′,N′-tetraacetic acid; GTPγS, Guanosine 5′-O-(3-thiotriphosphate); HEPES, 4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulphonic acid; MRS2179, 2′-deoxy-N^6-methyladenosine-3′,5′-bisphosphate; MRS2216, 2-chloro-2′-deoxy-N^6-methyladenosine-3′,5′-bisphosphate; MRS2279, 2-chloro-N^6-methyl-(N)-methanocarba-2′-deoxyadenosione-3′,5′-bisphosphate; MRS2286, 2-[2-(2-chloro-6-methylamino-purin-9-yl)-ethyl]-propane-1,3-bisoxy (diaminophosphate); MRS2298, Phosphoric acid mono-[2-(2-chloro-6-methylamino-purin-9-ylmethyl)-3-phosphonooxy-propyl] ester; PLC-βt, Turkey phospholipase C-beta

Introduction

Appreciation of the importance of adenine and uridine nucleotides as extracellular signalling molecules has been heightened during the past decade by identification of up to 15 different mammalian receptors for nucleotides (Fredholm *et al*., 1997; Ralevic & Burnstock, 1998). These receptors are readily subdivided into the ligand-gated ion channel P2X receptors and the G protein-coupled P2Y receptors. The metabotropic P2Y receptors are essentially ubiquitously expressed and regulate myriad physiological effects ranging from muscle contraction and epithelial cell Cl^- secretion to immunological/inflammatory responses of circulating lymphocytes and platelet aggregation (Dubyak & El-Moatassim, 1993; Ralevic & Burnstock, 1998).

Whereas a central role for extracellular nucleotides in many physiological effects is well-accepted, few nucleotide-regulated responses have been aligned unambiguously with a given P2 receptor subtype. This follows from several problems associated with the study of nucleotide-regulated responses. First, a complex array of ectoenzymes hydrolyze and interconvert extracellular nucleotides (Zimmermann, 1996; Harden *et al*., 1997). Given that the P2Y receptor subtypes are differentially activated by adenine and uridine tri- and diphosphates and selective stable agonists for these receptors are not available, determination of P2Y receptor identity in tissues using agonists alone has proven difficult. Second, few selective antagonists for the P2Y receptors have been available, and many molecules utilized as antagonists to date not only block certain of the P2Y receptors but also interact with P2X receptors and many other proteins (Harden *et al*., 1995, 1998).

*Author for correspondence; E-mail: tkh@med.unc.edu

Our laboratory has focused on the $P2Y_1$ receptor as a potentially important therapeutic target. This receptor is relatively broadly distributed in the central nervous system and in peripheral tissues (Ralevic & Burnstock, 1998; Webb & Barnard, 1999), and its role in the platelet aggregation response to ADP has been a topic of increased interest (Leon *et al*., 1997, 1999; Jin *et al*., 1998; Hechler *et al*., 1998; Fabre *et al*., 1999). Our initial observation that adenosine bisphosphate molecules are competitive antagonists of the $P2Y_1$ receptor (Boyer *et al*., 1996a) has been followed by directed chemical syntheses of new molecules exhibiting potential as selective competitive antagonists of the $P2Y_1$ receptor (Camaioni *et al*., 1998; Kim *et al*., 2000). We recently reported the synthesis of a series of ribose-modified-2′-deoxyadenosine bisphosphate analogs (Nandanan *et al*., 2000). Fusion of a cyclopropane bridge into a cyclopentane ring indicated that a constrained carbocyclic ring was tolerated in place of the ribose moiety in molecules that retained relatively high affinity for the $P2Y_1$ receptor of turkey erythrocytes. We now report the pharmacological properties of the most promising of these molecules, 2-chloro-N^6- methyl-(N)-methanocarba -2′-deoxyadenosine-3′,5′-bisphosphate (MRS2279). Our results reveal that a non-nucleotide bisphosphate molecule exhibits very high affinity and selectivity among the P2Y receptors for the $P2Y_1$ receptor. This molecule does not bind to the Gi/adenylyl cyclase-linked P2Y receptor of platelets, and therefore, can be utilized as a high affinity probe for differentiating the role of the $P2Y_1$ receptor versus a similar receptor for ADP found in platelets and other tissues.

Methods

Synthesis of N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate

The IUPAC name of N^6-mehyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2279) is (*1R,2S,4S,5S*)-1-[(phosphato)methyl]-4-(2-chloro-6-methylaminopurin-9-yl) bicyclo [3.1.0]-hexane-2-phosphate. MRS2279 was synthesized as previously described in detail (Nandanan *et al*., 2000).

Assay of $P2Y_1$ receptor-promoted inositol lipid hydrolysis in turkey erythrocyte membranes

$P2Y_1$-receptor-promoted activation of phospholipase C was studied in turkey erythrocyte membranes as we have described (Boyer *et al*., 1989, 1996a). Briefly, erythrocytes were incubated in inositol-free medium (DMEM; Gibco, Gaithersburg, MD, U.S.A.) with 0.5 mCi of 2-[^{3}H]-myo-inositol (20 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO, U.S.A.) for 18–24 h in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Membranes were prepared and phospholipase C activity was measured in 25 μl of ^{3}H-inositol-labelled membranes (approximately 175 μg of protein, 200–500,000 c.p.m. per assay) in a medium containing (mM): $CaCl_2$ 0.424, $MgSO_4$ 0.91, EGTA 2, KCl 115, KH_2PO_4 5, and HEPES 10, pH 7.0. Assays (200 μl final volume) contained 1 μM GTPγS and the indicated concentrations of nucleotide analogues. Membranes were incubated at 30°C for 5 min, and total [^{3}H]-inositol phosphates were quantified by anion-exchange chromatography. Typical values for inositol phosphate accumulation were approximately 200–300 c.p.m. (basal), 2000–3000 c.p.m. (1 μM GTPγS alone), and 15,000–20,000 c.p.m. (30 nM 2MeSADP + 1 μM GTPγS). The range of triplicate values was within 10% of the mean.

Assay of inositol phosphate accumulation in P2Y receptor-expressing 1321N1 cells

The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, or $P2Y_{11}$ receptors were stably expressed in 1321N1 human astrocytoma cells using retroviral vectors as previously described (Schachter *et al*., 1996). Cells were labelled overnight with [^{3}H]-inositol and agonist-promoted [^{3}H]-inositol phosphate accumulation was quantitated after a 10 min incubation in the presence of 10 mM LiCl and the cognate agonist for each receptor. The agonists used were 2MeSADP or ADP for $P2Y_1$ receptor expressing cells, UTP for $P2Y_2$ receptor-expressing cells, UTP for $P2Y_4$ receptor-expressing cells, UDP for $P2Y_6$ receptor-expressing cells, and ATP for $P2Y_{11}$ receptor-expressing cells. Typical values for inositol phosphate accumulation were 500–700 c.p.m. for basal and 4000–10,000 c.p.m. for accumulation in the presence of a maximally effective concentration of cognate agonist for each receptor. The range of triplicate values was within 10% of the mean.

Preparation of washed platelets

Venous blood was obtained from healthy volunteers and mixed with 20% of the final volume of acid/citrate/dextrose. The blood was centrifuged $180 \times g$ for 20 min, and the platelet-rich plasma was removed and incubated for 1 h in the presence of 1 mM aspirin. The platelets were centrifuged at $1000 \times g$ and resuspended to a density of 2×10^8 platelets ml^{-1} in HEPES-buffered Tyrode solution containing 0.2% BSA and 0.05 U ml^{-1} apyrase.

Assay of cyclic AMP accumulation in human platelets

Cyclic AMP accumulation was measured as described previously (Meeker & Harden, 1982). Briefly, platelets isolated from 50 ml of blood were labelled with 1 μCi ml^{-1} [^{3}H]-adenine for 1 h at 37°C. The platelets were then washed and resuspended in (mM): NaCl 137 , KCl 2.7, $MgCl_2$ 1, NaH_2PO_4 3, glucose 5 and HEPES 10, pH 7.4. Incubations were for 10 min in the presence of 200 μM 3-isobutyl-1-methyl xanthine, and the reaction was stopped with 10% trichloroacetic acid. [^{3}H]-Cyclic AMP was quantitated after chromatography over Dowex and alumina columns.

Platelet aggregation

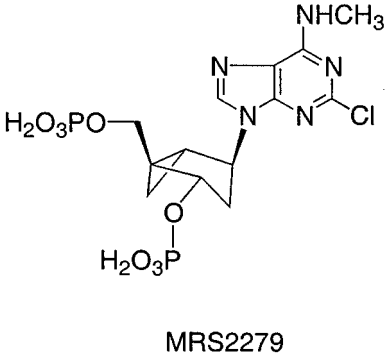
Platelet aggregation was measured using a four-channel Chrono-Log aggregometer (Haverton, PA, U.S.A.). Briefly, a 500 μl aliquot of washed platelets, supplemented with 2 mM $CaCl_2$ and 1 mg ml^{-1} fibrinogen, was stirred at 37°C and the indicated concentrations of ADP were added and aggregation monitored during an 8 min incubation. Antagonist effects of MRS2279 were studied by preincubating platelets for 2 min with the $P2Y_1$ antagonist prior to addition of ADP. The baseline for the aggregation response was set using 500 μl of HEPES-buffered Tyrode solution.

Results

We recently reported the synthesis of a series of methanocarbocyclic 2′-deoxyadenosine bisphosphate analogues (Nandanan *et al*., 2000). The fused cyclopropane ring, depending on its position on the cyclopentane ring, fixes the carbocyclic nucleoside into a rigid Northern (N) or Southern (S) envelope conformation. Our preliminary data indicated that the N conformation is favoured in recognition at the $P2Y_1$ receptor (Nandanan *et al*., 2000), and we have chosen one such molecule, 2-chloro-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2279; Figure 1) to study as a potentially useful high affinity non-nucleotide antagonist of the $P2Y_1$ receptor.

Turkey erythrocyte membranes have been developed as a cell-free model for the study of a P2Y receptor and its associated signalling cohorts (Harden *et al*., 1987; Boyer *et al*., 1989). The involved receptor ($P2Y_1$ receptor), G protein ($G\alpha11$), and phospholipase C (PLC-βt) all were identified, purified, and cloned (Boyer *et al*., 1996b). This model system has been highly useful for identification of $P2Y_1$ receptor agonists (Boyer *et al*., 1995, 1996c) and antagonists (Nandanan *et al*., 2000; Camaioni *et al*., 1998; Kim *et al*., 2000). Our preliminary studies suggested high affinity of MRS2279 for the $P2Y_1$ receptor of turkey erythrocytes (Nandanan *et al*., 2000). Therefore, the activity of MRS2279 was examined in detail by generating a series of concentration effect curves for the agonist 2MeSADP in the presence of increasing concentrations of MRS2279 (Figure 2). MRS2279 alone had no effect on inositol phosphate accumulation in the turkey erythrocyte membrane preparation. However, coaddition of MRS2279 with 2MeSADP resulted in a shift to the right of the 2MeSADP activation curve that was dependent on the concentration of MRS2279. Schild analysis revealed that the antagonism observed with MRS2279 was competitive (slope = 0.97), and the calculated pK_B ($pK_B = 7.75 \pm 0.10$; $n = 3$ experiments) indicated a very high binding affinity of MRS2279 for the avian receptor.

The $P2Y_1$ receptor of turkey erythrocyte membranes has proven to be an excellent predictor of activity of agonist and antagonist molecules at the human $P2Y_1$ receptor (Boyer *et al*., 1996a, b; 1998). As illustrated in Figure 3, this trend also holds for MRS2279. Inositol phosphate accumulation was measured in 1321N1 cells stably expressing the human $P2Y_1$ receptor. MRS2279 alone had no effect on inositol phosphate accumulation. However, this bisphosphate analogue shifted to the right the concentration effect curve of 2MeSADP for stimulation of inositol phosphate accumulation. Schild analysis revealed that antagonism was competitive, and the pK_B (8.10 ± 0.19; $n = 4$ experiments) was similar to that observed at the avian receptor. The affinity observed with MRS2279 indicates that this molecule is the most potent competitive antagonist of the human $P2Y_1$ receptor reported to date.



MRS2279

Figure 1 Structure of 2-chloro N^6-methyl-(N)-methanocarbaxyadenosine-3′, 5′-bisphosphate (MRS2279).

To establish the P2Y receptor selectivity of MRS2279, we studied the inositol phosphate response to cognate agonists of the human $P2Y_2$ receptor (UTP), the human $P2Y_4$ receptor (UTP), the human $P2Y_6$ receptor (UDP), and the human $P2Y_{11}$ receptor (ATP) all stably expressed in 1321N1 cells. As illustrated in Figure 4, 30 μM MRS2279 completely antagonized the stimulatory effects of 2MeSADP at the $P2Y_1$ receptor but had no effect on the capacity of the cognate agonists of the $P2Y_2$, $P2Y_4$, $P2Y_6$, or $P2Y_{11}$ receptors to promote inositol phosphate accumulation. Therefore, the high affinity antagonism of the human $P2Y_1$ receptor exhibited by MRS2279 also is accompanied by high selectivity for this P2Y receptor.

The physiological effects of ADP on platelet secretion and aggregation apparently occur due to the action of two different G protein-coupled receptors: the Gq/phospholipase C-linked $P2Y_1$ receptor and the recently cloned (Hollopeter *et al*., 2001) $P2Y_{12}$ receptor, which couples through Gi to inhibit adenylyl cyclase (Jin *et al*., 1998; Hechler *et al*., 1998; Leon *et al*., 1999; Fabre *et al*., 1999). The presence of these two receptors exhibiting similar agonist selectivity has complicated both the study of the physiological effects of ADP in regulation of platelet aggregation and resolution of the relative role of the adenylyl cyclase-linked $P2Y_{12}$ receptor versus the $P2Y_1$ receptor in the action of ADP. As such, the synthesis of MRS2279 may provide an additional high affinity tool to study the role of ADP in platelet physiology. As illustrated in Figure 5, ADP inhibited the capacity of PGE_1 + forskolin to stimulate cyclic AMP accumulation in intact human platelets. In contrast to its potent activity at the ADP-activated $P2Y_1$ receptor, MRS2279 exhibited no effect on ADP-promoted inhibition of cyclic AMP accumulation through the Gi/adenylyl cyclase-linked P2Y receptor of platelets (Figure 5).

Given the activity of MRS2279 in blocking $P2Y_1$ receptors and its lack of activity in blocking the platelet adenylyl cyclase-linked $P2Y_{12}$ receptor, we examined the capacity of MRS2279 to block ADP-induced platelet aggregation. As illustrated in Figure 6A, MRS2279 antagonized the action of ADP in inducing aggregation. Schild analyses of these data indicated that antagonism was competitive (Figure 6B), and the pK_B of this effect ($pK_B = 8.05 \pm 0.10$; $n = 3$ experiments) was similar to that observed for MRS2279 at either the avian or recombinant human $P2Y_1$ receptor. ADP-induced shape change of platelets was inhibited by MRS2279 with the same apparent affinity as that illustrated for inhibition of aggregation (data not shown). Thus, as has been previously illustrated pharmacologically (Jin *et al*., 1998; Hechler *et al*., 1998; Savi *et al*., 1998) and by $P2Y_1$ receptor gene disruption (Leon *et al*., 1999; Fabre *et al*., 1999), these data with MRS2279 illustrate that the $P2Y_1$ receptor plays an obligatory role in ADP-promoted platelet aggregation.

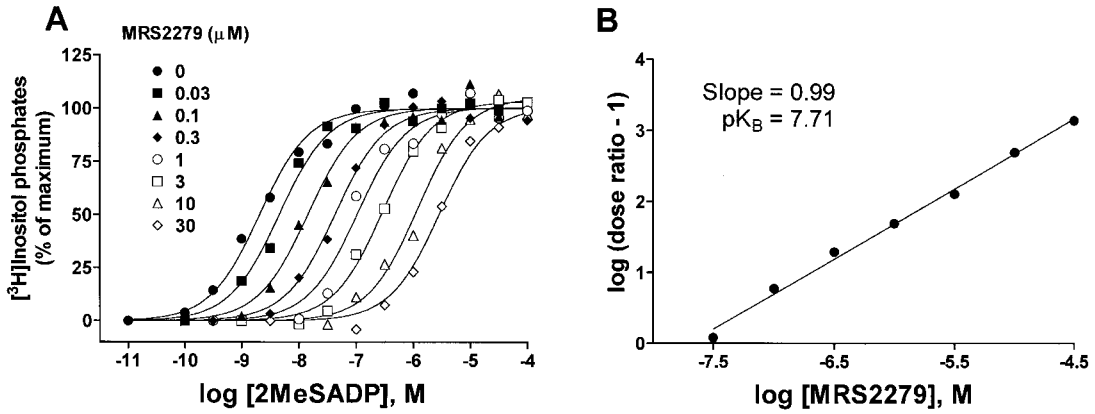


Figure 2 Competitive antagonism of 2MeSADP stimulated phospholipase C activity in turkey erythrocyte membranes. (A), phospholipase C activity was measured in turkey erythrocyte membranes as described in Methods in the presence of the indicated concentrations of 2MeSADP in the absence or presence of the indicated concentrations of MRS2279. The data are presented from duplicate determinations from an experiment that is representative of results obtained in four separate experiments. (B), a Schild plot is presented of the data presented in (A).

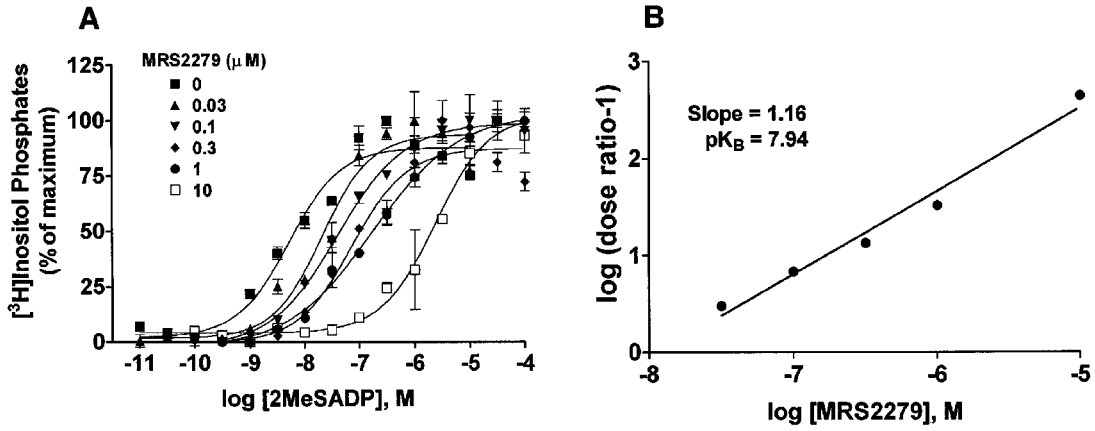


Figure 3 Competitive antagonism of 2MeSADP-stimulated phospholipase C activity in 1321N1 cells stably expressing the human $P2Y_1$ receptor. (A), $P2Y_1$ receptor-expressing 1321N1 cells were labelled with ^{3}H-inositol and ^{3}H-inositol phosphate accumulation was measured as described in Methods. Assays were in the presence of the indicated concentrations of 2 MeSADP alone or 2MeSADP in the presence of the indicated concentrations of MRS2279. The data are presented as the average of triplicate determinations from an experiment that is representative of three separate experiments.

Discussion

The results described here illustrate very high affinity antagonism of the $P2Y_1$ receptor by 2-chloro N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate. Moreover, this antagonism is specific for the $P2Y_1$ receptor among the metabotropic P2Y receptors. No effect was observed on the four other cloned human Gq/phospholipase C linked P2Y receptors, and MRS2279 also did not block the $P2Y_{12}$ receptor for ADP on platelets that couples in an inhibitory fashion to adenylyl cyclase. Thus, we have developed a non-nucleotide high affinity $P2Y_1$ receptor antagonist that exhibits notable selectivity.

The work described here follows from our original observation that bisphosphate-substitution of adenosine results in molecules that act as competitive antagonists at the $P2Y_1$ receptor (Boyer *et al*., 1996a). A broad series of bisphosphate analogues subsequently was synthesized (Camaioni *et al*., 1998; Boyer *et al*., 1998) including the widely utilized $P2Y_1$ receptor antagonist, 2′-deoxy-N^6-methyladenosine-3′,5′-*bis*phosphate (MRS2179), and its 2-chloro analogue (MRS2216). Both of these antagonists are adenine-9-riboside 3′,5′-bisphosphate derivatives that exhibit K_i values for the $P2Y_1$ receptor in the 100–300 nM range. We have moved away from riboside nucleotides in recent chemical synthesis (Nandanan *et al*., 2000; Kim *et al*., 2000, 2001), and the carbocyclic nucleotide analogue MRS2279 studied here is the most potent $P2Y_1$ receptor antagonist reported to date. Similarly, phosphoric acid mono-[2-(2-chloro-6-methylamino-purin-9-ylmethyl)-3-phosphonooxy-propyl] ester (MRS2298) was almost as potent as the riboside MRS2179 for inhibition of $P2Y_1$ receptor-mediated activation of phospholipase C (Kim *et al*., 2001). Although the widely used riboside bisphosphate MRS2179 exhibits no agonist activity at $P2Y_1$ receptors, several other riboside bisphosphates are partial agonists. $P2Y_1$ receptor agonist activity was not observed

with MRS2279 or any other acyclic bisphosphate molecule we have studied.

The adenosine bisphosphate backbone of MRS2279 contains four additional modifications that in composite enhance $P2Y_1$ receptor selectivity and affinity by 3–4 orders of magnitude (Camaioni *et al*., 1998; Nandanan *et al*., 2000). These include a methyl group addition at the N^6-position of the adenine base, a 2-chloro substitution of the adenine base, removal of the 2′-hydroxyl of the ribose, and a cyclopropane (methanocarba) bridge in a cyclopentane ring replacing the ribose. The methanocarba substitution of MRS2279 constrains the pseudo ribose in an envelope (N)-(2′-exo) conformation. We previously reported that a (S)-(2′-endo) conformation was many fold less favourable for $P2Y_1$ receptor binding (Nandanan *et al*., 2000). Molecular modelling of the $P2Y_1$ receptor pharmacophore based on the 2.8 A three dimensional structure of rhodopsin and our previous mutational analyses of the $P2Y_1$ receptor indicated that the (N)-conformation maximizes electrostatic interactions between the negatively charged bisphosphates and positively charged amino acids of the binding pocket (Kim *et al*., 2000).

This and other work on acyclic analogues (Kim *et al*., 2000) indicate that the ribose *per se* is not necessary for ligand binding, and a non-ribose spacer of similar length between the N position of the adenine base and the two phosphates will suffice for full receptor recognition. This observation is important because retention of $P2Y_1$ receptor binding in molecules lacking a ribose essentially assures that binding will not occur to the thousands of other nucleotide binding proteins known to exist in the human genome. Therefore, receptor selectivity is highly likely and development of an MRS2279 radioligand is underway to take advantage of this evolution of a high affinity non-nucleotide molecule. We have not tested the activity of MRS2279 at any of the ATP-activated ionotropic P2X receptors, although a

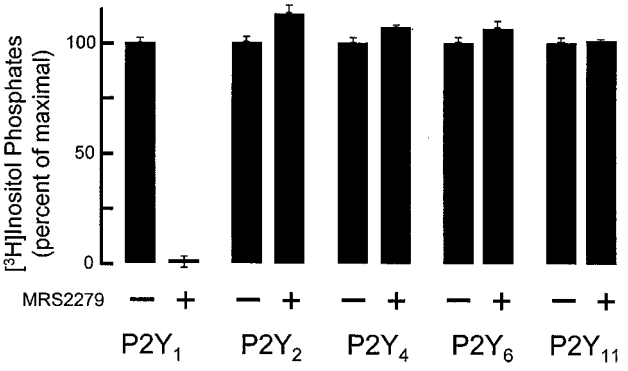


Figure 4 Selectivity of antagonism of the human $P2Y_1$ receptor by MRS2279. 1321N1 human astrocytoma cells stably expressing the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptor were incubated, respectively, with 10 μM 2MeSADP, 10 μM UTP, 10 μM UTP, 10 μM UDP, or 10 μM ATP without (−) or with (+) 30 μM MRS2279. The data are presented as mean ± s.e.mean of three separate experiments with stimulation with agonist alone given a value of 100% of maximum in each experiment.

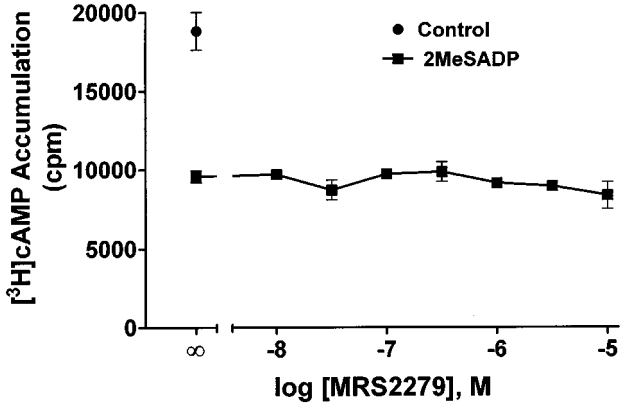


Figure 5 Effect of MRS2279 on 2MeSADP-promoted inhibition of adenylyl cyclase activity in human platelets. Cyclic AMP accumulation was measured as described in Methods. Assays were in the presence of 10 μM PGE_1 and 50 μM forskolin (solid circle) or 10 μM PGE_1, 50 μM forskolin, and 10 nM 2MeSADP in the presence of the indicated concentrations of MRS2279 (solid square). The data are presented as the mean ± s.e.mean of three separate experiments.

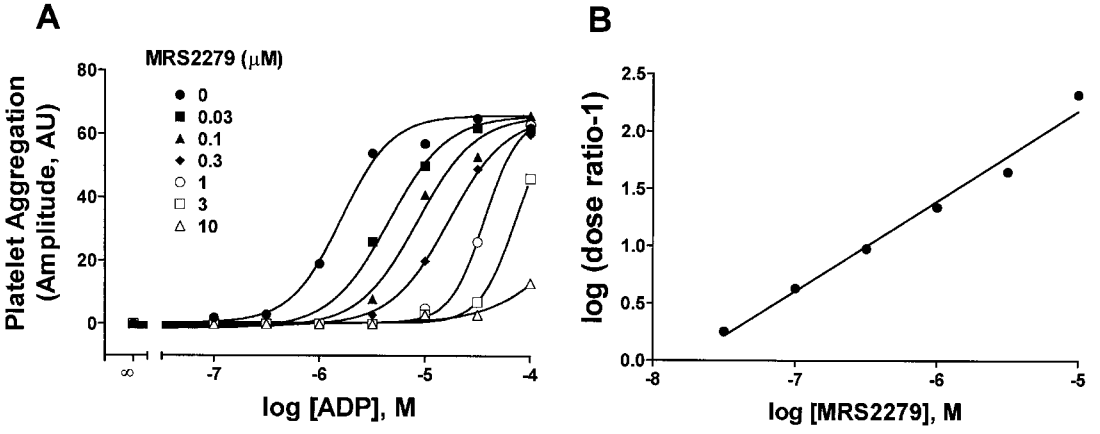


Figure 6 Effect of MRS2279 on ADP-promoted platelet aggregation. (A), platelet aggregation was measured as described in Methods. Assays were in the presence of the indicated concentrations of ADP alone or the indicated concentrations of ADP with the indicated concentrations of MRS2279. The cyclic AMP response to PGE_1 + forskolin in the absence of ADP also is indicated (solid circle). The data are the mean of duplicate determinations and are representative of results from three experiments. (B), a Schild plot of the data in (A) is presented.

closely related acyclic derivative, 2-[2-(2-chloro-6-methylamino-purin-9-yl)-ethyl]-propane-1,3-bisoxy (diammonium-phosphate) (MRS2286), was inactive at the rat $P2X_1$ receptor (Brown *et al*., 2000). The earlier bisphosphate nucleotide analogue MRS2179 developed by our laboratory as a high affinity antagonist for the $P2Y_1$ receptor (Boyer *et al*., 1996c), interacted with the $P2X_1$ receptor but with 20–40 fold lower affinity than at the $P2Y_1$ receptor (Brown *et al*., 2000).

The work of several groups (Hechler *et al*., 1998; Jin *et al*., 1998; Kunapuli, 1998; Savi *et al*., 1998; Fagura *et al*., 1998) has amplified the idea that both the Gq-coupled $P2Y_1$ receptor and the recently cloned (Hollopeter *et al*., 2001) adenylyl cyclase-linked $P2Y_{12}$ receptor are involved in ADP action in platelets. This concept was initially based in part on utilization (Hechler *et al*., 1998; Jin *et al*., 1998; Savi *et al*., 1998) of the adenosine bisphosphate molecules that were identified as the lead molecules in the eventual development of MRS2279 (Boyer *et al*., 1996a). As we illustrate here MRS2279 now provides a very high affinity antagonist for the platelet $P2Y_1$ receptor that does not bind to the second ADP-activated receptor on the platelet, the $P2Y_{12}$ receptor. MRS2279 blocked ADP-promoted aggregation of human platelets with a K_i identical to its K_i for antagonism of the recombinant human $P2Y_1$ receptor. Thus, as previously concluded by others the $P2Y_1$ receptor is indispensable in the platelet aggregation response to ADP.

The high affinity and absolute specificity of MRS2279 for the $P2Y_1$ receptor over the other metabotropic P2Y receptors provides a molecule that should be widely useful in delineation of the physiological role of the $P2Y_1$ receptor in the central nervous system and in peripheral tissues. Binding affinity in the low nanomolar range also provides an attractive non-nucleotide antagonist molecule that can be radiolabelled as a probe for $P2Y_1$ receptor quantification and characterization.

This research was supported by United States Public Service Grants HL54889 and GM38213. The authors are indebted to Savitri Maddileti for technical support and Meredith Jones and David Rinker for assistance in preparing the manuscript.

References

BOYER, J.L., DOWNES, C.P. & HARDEN, T.K. (1989). Kinetics of activation of phospholipase C by P_2 purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.,* **264,** 884–890.

BOYER, J.L., MOHANRAM, A., CAMAIONI, E., JACOBSON, K.A. & HARDEN, T.K. (1998). Competitive and selective antagonism of $P2Y_1$ receptors by N^6-methyl 2′-deoxyadenosine 3′,5′-*bis*phosphate. *Br. J. Pharmacol.,* **124,** 1–3.

BOYER, J.L., O'TUEL, J.W., FISCHER, B., JACOBSON, K.A. & HARDEN, T.K. (1995). Potent agonist action of 2-thioether derivatives of adenine nucleotides at adenylyl cyclase-linked P2Y-purinoceptors. *Br. J. Pharmacol.,* **116,** 2611–2616.

BOYER, J.L., ROMERO, T., SCHACHTER, J.B. & HARDEN, T.K. (1996a). Identification of competitive antagonists of the $P2Y_1$ receptor. *Mol. Pharmacol.,* **50,** 1323–1329.

BOYER, J.L., SCHACHTER, J.B., SROMEK, S.M., PALMER, R.K., JACOBSON, K.A., NICHOLAS, R.A. & HARDEN, T.K. (1996b). Avian and human homologues of the $P2Y_1$ receptor: pharmacological, signaling, and molecular properties. *Drug Dev. Res.,* **39,** 253–261.

BOYER, J.L., SIDDIQI, S.M., FISCHER, B., JACOBSON, K.A. & HARDEN, T.K. (1996c). Identification of potent P_{2Y}-purinoceptor agonists that are derivatives of adenosine 5′-monophosphate. *Br. J. Pharmacol.,* **118,** 1959–1964.

BROWN, S.G., KING, B.F., KIM, Y.-C., BURNSTOCK, G. & JACOBSON, K.A. (2000). Activity of novel adenine nucleotide derivatives as agonists and antagonists at recombinant rat P2X receptors. *Drug Dev. Res.,* **49,** 253–259.

CAMAIONI, E., BOYER, J.L., MOHANRAM, A., HARDEN, T.K. & JACOBSON, K.A. (1998). Deoxyadenosine-bisphosphate derivatives as potent antagonists at $P2Y_1$ receptors. *J. Med. Chem.,* **41,** 183–190.

DUBYAK, G.R. & EL-MOATASSIM, C. (1993). Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.,* **265,** C577–C606.

FABRE, J.E., NGUYEN, M., LATOUR, A., KEIFER, J.A., AUDOLY, L.P., COFFMAN, T.M. & KOLLER, B.H. (1999). Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat. Med.,* **5,** 1199–1202.

FAGURA, M.S., DAINTY, I.A., McKAY, G.D., KIRK, I.P., HUMPHRIES, R.G., ROBERTSON, M.J., DOUGALL, I.G. & LEFF, P. (1998). $P2Y_1$-receptors in human platelets which are pharmacologically distinct from $P2Y_{AOP}$-receptors. *Br. J. Pharmacol.,* **124,** 157–164.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DUBYAK, G.R., HARDEN, T.K., JACOBSON, K.A., SCHWABE, U. & WILLIAMS, M. (1997). Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol. Sci.,* **18,** 79–82.

HARDEN, T.K., BOYER, J.L. & NICHOLAS, R.A. (1995). P_2-purinergic receptors: subtype-associated signaling responses and structure. *Annu. Rev. Pharmacol. Toxicol.,* **35,** 541–579.

HARDEN, T.K., BARNARD, E.A., BOEYNAEMS, J.M., BURNSTOCK, G., DUBYAK, G.R., HOURANI, S.M.O. & INSEL, P.A. (1998). P2Y receptors. In *The IUPHAR compendium of receptor characterization and classification* ed Girdlestone D pp. 209–217, London: IUPHAR Media.

HARDEN, T.K., LAZAROWSKI, E.R. & BOUCHER, R.C. (1997). Release, metabolism and interconversion of adenine and uridine nucleotides: implications for G protein-coupled P2 receptor agonist selectivity. *Trends Pharmacol. Sci.,* **18,** 43–46.

HARDEN, T.K., STEPHENS, L., HAWKINS, P.T. & DOWNES, C.P. (1987). Turkey erythrocyte membranes as a model for regulation of phospholipase C by guanine nucleotides. *J. Biol. Chem.,* **262,** 9057–9061.

HECHLER, B., LEON, C., VIAL, C., VIGNE, P., FRELIN, C., CAZENAVE, J.P. & GACHET, C. (1998). The P2Y1 receptor is necessary for adenosine 5′-diphosphate-induced platelet aggregation. *Blood,* **92,** 152–159.

HOLLOPETER, G., JANTZEN, H.M., VINCENT, D., LI, G., ENGLAND, L., RAMAKRISHNAN, V., YANG, R.B., NURDEN, P., NURDEN, A., JULIUS, D. & CONLEY, P. (2001). Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature,* **409,** 202–207.

JIN, J., DANIEL, J.L. & KUNAPULI, S.P. (1998). Molecular basis for ADP-induced platelet activation. II. The $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J. Biol. Chem.,* **273,** 2030–2034.

KIM, Y.-C., GALLO-RODRIGUEZ, C., JANG, S.-Y., NANDANAN, E., ADAMS, M., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (2000). Acyclic analogues of deoxyadenosine 3′,5′-bisphosphates as $P2Y_1$ receptor antagonists. *J. Med. Chem.,* **42,** 746–755.

KIM, H.S., BARAK, D., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (2001). Acyclic and cyclopropyl analogues of adenosine bisphosphate antagonists of the $P2Y_1$ receptor: Structure activity relationships and receptor docking. *J. Med. Chem.,* **44,** 3092–3108.

KUNAPULI, S.P. (1998). Multiple P2 receptor subtypes on platelets: a new interpretation of their function. *Trends Pharmacol. Sci.,* **19,** 391 – 394.

LEON, C., HECHLER, B., FREUND, M., ECKLY, A., VIAL, C., OHLMANN, P., DIERICH, A., LEMEUR, M., CAZENAVE, J.P. & GACHET, C. (1999). Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J. Clin. Invest.,* **104,** 1731 – 1737.

LEON, C., HECHLER, B., VIAL, C., LERAY, C., CAZENAVE, J.-P. & GACHET, C. (1997). The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS. Lett.,* **403,** 26 – 30.

MEEKER, R.B. & HARDEN, T.K. (1982). Muscarinic receptor-mediated activation of phosphodiesterase. *Mol. Pharmacol.,* **22,** 310 – 319.

NANDANAN, E., JANG, S.Y., MORO, S., KIM, H.O., SIDDIQUI, M.A., RUSS, P., MARQUEZ, V.E., BUSSON, R., HERDEWIJN, P., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (2000). Synthesis, biological activity, and molecular modeling of ribose-modified deoxyadenosine bisphosphate analogues as P2Y1 receptor ligands. *J. Med. Chem.,* **43,** 829 – 842.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413 – 492.

SAVI, P., BEAUVERGER, P., LABOURET, C., DELFAUD, M., SALEL, V., KAGHAD, M. & HERBERT, J.M. (1998). Role of P2Y1 purinoceptor in ADP-induced platelet activation. *FEBS, Lett.,* **422,** 291 – 295.

SCHACHTER, J.B., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1996). Second messenger cascade specificity and pharmacological selectivity of the human P_{2Y1} receptor. *Br. J. Pharmacol.,* **118,** 167 – 173.

WEBB, T.E. & BARNARD, E.A. (1999). Molecular biology of P2Y receptors expressed in the nervous system. *Prog. Brain Res.,* **120,** 23 – 31.

ZIMMERMANN, H. (1996). Extracellular purine metabolism. *Drug Dev. Res.,* **39,** 337 – 352.

(Received October 4, 2001
Revised November 19, 2001
Accepted February 13, 2002)