Diisothiocyanate derivatives as potent, insurmountable antagonists of $P2Y_6$ nucleotide receptors

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Abstract

The physiological role of the $P2Y_6$ nucleotide receptor may involve cardiovascular, immune and digestive functions based on the receptor tissue distribution, and selective antagonists for this receptor are lacking. We have synthesized a series of symmetric aryl diisothiocyanate derivatives and examined their ability to inhibit phospholipase C (PLC) activity induced by activation of five subtypes of recombinant P2Y receptors. Several derivatives were more potent at inhibiting action of UDP at both human and rat $P2Y_6$ receptors expressed in 1321N1 human astrocytes than activation of human $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors. The inhibition by diisothiocyanate derivatives of 1,2-diphenylethane (MRS2567) and 1,4-di-(phenylthioureido) butane (MRS2578) was concentration-dependent and insurmountable, with IC_{50} values of 126 ± 15 nM and 37 ± 16 nM (human) and 101 ± 27 nM (rat), respectively. A derivative of 1,4-phenylendiisothiocyanate (MRS2575) inhibited only human but not rat $P2Y_6$ receptor activity. MRS2567 and MRS2578 at 10 μM did not affect the UTP (100 nM)-induced responses of cells expressing $P2Y_2$ and $P2Y_4$ receptors, nor did they affect the 2-methylthio-ADP (30 nM)-induced responses at the $P2Y_1$ receptor or the ATP (10 μM)-induced responses at the $P2Y_{11}$ receptor. Other antagonists displayed mixed selectivities. The selective antagonists MRS2567, MRS2575 and MRS2578 (1 μM) completely blocked the protection by UDP of cells undergoing TNFα-induced apoptosis. Thus, we have identified potent, insurmountable antagonists of $P2Y_6$ receptors that are selective within the family of PLC-coupled P2Y receptors.

Keywords

$P2Y_6$ nucleotide receptor; GPCR; Pyrimidines; Purines; Isothiocyanate; Apoptosis

1. Introduction

In addition to their intracellular functions in signaling and genetic coding, nucleotides have an important role as extracellular signaling molecules. The P2 receptors consist of two superfamilies: 8 G protein-coupled 7TM receptors (P2Y) and 7 ligand-gated ion channels (P2X) [1–3]. The $P2Y_{1,2,4,6,11}$ receptors are coupled preferentially to the activation of phospholipase C (PLC), via $G_{q/11}$ proteins. The $P2Y_{12,13,14}$ receptors are coupled preferentially to the inhibition of adenylate cyclase. The agonist preference of these eight

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receptors differs greatly and includes both adenine and uracil nucleotides [4]. The pyrimidine-selective receptors include: $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ receptors, which are activated by UTP, UDP and UDP-glucose, respectively. $P2Y_2$ receptors are activated by both ATP and UTP. The remaining subtypes are activated by adenine nucleotides.

The physiological role of the $P2Y_6$ receptor may involve cardiovascular, immune, and digestive functions, based on the receptor tissue distribution [4–8]. $P2Y_6$ receptors are associated with vasoconstriction [9]. Recently, we have reported that activation by UDP of $P2Y_6$ receptors inhibited apoptosis induced by TNFα through a mechanism dependent on PKC and Erk [10]. Also, it was reported that extracellular nucleotides stimulated contractions of human cerebral arteries primarily by activation of the $P2Y_6$ receptor [11], suggesting antagonists of the $P2Y_6$ receptor may be useful in the treatment of vasospasm.

Selective antagonists for the $P2Y_6$ receptor have not previously been reported [4]. The lack of selective receptor probes for the $P2Y_6$ and other subtypes of nucleotide receptors has impeded the ability to examine their pharmacology. DIDS (**1**) and its dihydro derivative, H_2DIDS (**2**) (Fig. 1) have been shown to inhibit the PLC activity induced by UDP at rat $P2Y_6$ receptors [12]. It appears that both isothiocyanate groups are needed for this activity, since SITS (**3**) was inactive. In this study we have explored the structure–activity relationship (SAR) of aryl diisothiocyanates at P2Y receptors. We tested these compounds as antagonists of human $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors stably expressed in 1321N1 astrocytoma cells and the human $P2Y_{11}$ receptor stably expressed in CHO cells.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. General procedure for the synthesis of 4 and 5—A mixture of the diamine (**12** or **13**, 1.0 mmol), sodium bicarbonate (4.0 mmol), water (10 ml) and chloroform (30 ml) was stirred at room temperature. After 10 min thiophosgene (4 mmol, Aldrich Chemical Co.) was added and the mixture stirred for 2 h at room temperature. The phases were separated, the organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by silica gel chromatography eluting with ethyl acetate-petroleum ether (5:95) to furnish the product as a solid (yield 65–70%). *trans*-1,2-Di-(4-isothiocyanatophenyl)ethylene (**4**). ^{1}H NMR ($CDCl_3$): δ 7.14 (s, 2H), 7.34 (t, J = 12 Hz, 4H), 7.58 (d, J = 9 Hz, 4H). FAB-MS 295.1 (M + 1), 1,2-di-(4-isothiocyanatophenyl)ethane (**5**). ^{1}H NMR ($CDCl_3$): δ 2.87 (s, 4H), 6.92–7.22 (m, 8H). FAB-MS 297.1 (M + 1).

2.1.2. General procedure for the synthesis of 6–11—Either 1,3- or 1,4-phenylenediisothiocyanate (**14** or **15**, 5 mmol) [13] was dissolved in dry acetonitrile (20 ml). To the above solution was added alkyl diamine (1 mmol) in acetonitrile (10 ml), and the resulting reaction mixture was stirred at room temperature for 1 h. Solvent was removed by evaporation, and the residue was purified by silica gel chromatography eluting with methanol–chloroform (5:95) to furnish as a solid (yield 55–60%). 1,2-Di-[(4-isothiocyanatophenyl)-thioureido] ethane (**6**). ^{1}H NMR (DMSO-d_6): δ 3.61–3.78 (m, 4H), 7.51 (d, J = 8.7 Hz, 4H), 7.56 (d, J = 7.2 Hz, 4H), 8.00 (brs, 2H), 9.76 (brs, 2H). FAB-MS 445.1 (M + 1). 1,3-Di-[(4-isothiocyanatophenyl)-thioureido] propane (**7**). ^{1}H NMR (DMSO-d_6): δ 1.62–1.83 (m, 2H), 3.38–3.60 (m, 4H), 7.42 (d, J = 12 Hz, 4H), 7.52 (d, J = 12 Hz, 4H), 7.95 (brs, 2H), 9.65 (brs, 2H). FAB-MS 459.1 (M + 1). 1,4-Di-[(4-isothiocyanato phenyl)-thioureido] butane (**8**). ^{1}H NMR (DMSO-d_6): δ 1.51 (brs, 4H), 3.42 (brs, 4H), 7.20–7.58 (m, 8H), 7.85 (brs, 2H), 9.62 (brs, 2H). FAB-MS 473.1 (M + 1).

1,2-Di-[(3-isothiocyanato phenyl)-thioureido]ethane (**9**). ^{1}H NMR (DMSO-d_6): δ 3.71 (brs, 4H), 7.08–7.20 (m, 2H), 7.28–7.42 (brs, 4H), 7.61 (brs, 2H), 8.03 (brs, 2H), 9.72 (brs, 2H).

FAB-MS 445.1 (M + 1). 1,3-Di-[(3-isothiocyanato-phenyl)-thioureido] propane (**10**). ^{1}H NMR (DMSO-d_6): δ 1.76–2.01 (m, 2H), 3.32 (brs, 4H), 6.95–7.45 (m, 6H), 7.55 (brs, 2H), 8.00 (brs, 2H), 9.65 (brs, 2H). FAB-MS 459.1 (M + 1). 1,4-Di-[(3-isothiocyanato phenyl)-thioureido] butane (**11**). ^{1}H NMR (DMSO-d_6): δ 1.58 (m, 4H), 3.45 (brs, 4H), 7.04–7.20 (m, 2H), 7.25–7.40 (m, 4H), 7.65 (m, 2H), 7.95 (brs, 1H), 9.62 (brs, 2H). FAB-MS 473.1 (M + 1).

2.2. Cell culture and membrane preparation

Human 1321N1 astrocytoma cells stably transfected with the hP2Y$_{1-6}$ receptors and CHO cells stably transfected with the human P2Y$_{11}$ receptors [14,15] were grown at 37 °C in a humidified incubator with 5% CO_2/95% air in Dulbecco's modified Eagle's medium (JRH Biosciences, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 Units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. The cells were grown to ~60% confluence for the experiments.

For membrane preparation, human astrocytoma cells expressing human P2Y$_1$ receptors were grown to approximately 80% confluence and then harvested. The cells were homogenized and suspended and then centrifuged at $100 \times g$ for 5 min at room temperature. The pellet was resuspended in 50 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (pH 7.4). The suspension was homogenized with a polytron homogenizer (Brinkmann) for 10 s and was then recentrifuged at $20,000 \times g$ for 20 min at 4 °C. The resultant pellets were resuspended in Tris buffer (pH 7.4), and the suspension was stored at −80 °C until the binding experiments. The protein concentration was measured with the Bradford assay [16].

2.3. Determination of inositol phosphates

The quantity of inositol phosphates was measured by a modification of the method of Kim et al. [17] and Gao et al. [18]. Agonists were dissolved as stock solutions in Tris buffer (pH 7.4), and antagonists were dissolved in DMSO (5 mM) and stored at −20 °C. The antagonists were not stable to storage in aqueous medium. The P2Y$_{1-6}$-1321N1 and P2Y$_{11}$-CHO cells were grown to confluence in 6-well plates in the presence of myo-[3H]inositol (2 µCi/ml) for 24 h. Cells were then treated for 30 min at 37 °C with antagonists or buffer in the presence of 20 mM LiCl, followed by another 30 min incubation at 37 °C with the appropriate agonist. Agonists used were: P2Y$_1$, 2-MeSADP; hP2Y$_2$, UTP; hP2Y$_4$, UTP; hP2Y$_6$, UDP; hP2Y$_{11}$, ATP. The reaction was terminated upon aspiration of the medium and addition of cold formic acid (20 mM). After 30 min, supernatants were neutralized with NH_4OH, and applied to Bio-Rad Dowex AG1-X8 anion exchange columns. All of the columns were then washed with water followed by a 60 mM sodium formate solution containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with 1 M ammonium formate containing 0.1 M formic acid, and radioactivity were measured using a liquid scintillation counter.

2.4. Radioligand binding assays

P2Y$_1$ receptor binding experiments were performed as previously described [19]. Briefly, membranes (40 µg protein) from astrocytoma cells stably expressing human P2Y$_1$ receptors were incubated with [3H]MRS2279 (8 nM) for 30 min at 4 °C in a total assay volume of 200 µl. For adenosine A$_1$ receptor binding, an agonist radioligand [3H]R-PIA (2.0 nM) was incubated with membranes (40 µg protein/tube) from CHO cells stably expressing human adenosine A$_1$ receptors for 60 min at 25 °C [20]. Radiolabeled ligand concentrations used in all assays approximated the K_d values of the receptor. Binding reactions were terminated by filtration through Whatman GF/B glass-fiber filters under reduced pressure with a MT-24 cell harvester (Brandel), and radioactivity was determined with a 1414 liquid scintillation counter (Wallac, Win Spectral, Perkin Elmer Life Sciences).

2.5. Induction of apoptosis

TNFα was used to induce apoptosis. Medium containing 5 ng/ml cycloheximide was added to the cells grown to ~60% confluence. Cycloheximide was included in all experiments concerning TNFα-induced apoptosis. The cells were treated with both UDP and TNFα for 4 h. Antagonists (1 μM) were added to the incubation medium 20 min prior to addition of UDP (100 nM) and TNFα (20 ng/ml). After 4 h the media was changed and was left for 16 h. The media contained 5 ng/ml cycloheximide during the entire incubation. Cell death was observed 16 h later.

2.6. Degree of cell death

After treatment, cells were washed once with PBS and were stained with propidium iodide (PI) solution (final concentration; 2 μg/ml). The numbers of unstained (live) and stained (dead) cells were measured with a Cytoflour 4000 (Perseptive Biosystems) fluorescence plate reader, with excitation at 485 nm and emission at 645 nm.

2.7. Reagents

myo-[^{3}H]Inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals. Dowex AG 1-X8 resin was purchased from Bio-Rad. DMEM and FBS were from Life Technologies. [^{3}H]MRS2279 was from Perkin-Elmer, and [^{3}H]R-PIA was from Amersham. All other reagents were purchased from Sigma Chemical Co.

2.8. Statistical analysis

Pharmacological parameters were analyzed by Graph-PAD Prism software. Data were expressed as mean ± S.E.

3. Results

3.1. Chemistry

Symmetric aryl diisothiocyanate derivatives (**4**–**11**, Table 1) were synthesized as shown in Fig. 1. Diaryl-4,4′-diisothiocyanates (**4** and **5**) were prepared in good yields from their corresponding amines **12** and **13**, respectively, by a standard procedure using thiophosgene and sodium bicarbonate [13]. The symmetric diisothiocyanates (**6**–**11**) were synthesized in good yield by a different route, i.e. from the interaction of alkyl diamines and large excess of either 1,3- or 1,4-phenylenediisothiocyanates (Fig. 2).

3.2. Inhibition of agonist-induced production of inositol phosphates by aryl diisothiocyanate derivatives

The ability of the diisothiocyanate derivatives to inhibit agonist-induced accumulation of inositol phosphates in 1321N1 human astrocytes expressing P2Y$_6$ receptors and other P2Y subtypes was studied. Fig. 3 indicates that the concentration–response curve for UDP acting at the P2Y$_6$ receptor (EC$_{50}$ 230 ± 83 nM) was inhibited by the compounds **5** and **11**, but in an insurmountable manner. Table 1 indicates that similar effects were obtained for other compounds in the series and at other subtypes of P2Y receptors. Typically, the effect was inhibited with the diisothiocyanate in a micromolar concentration range. Full concentration–response curves for the inhibition of the effects of UDP at P2Y$_6$ receptors were measured for compounds **5** (MRS2567) and **11** (MRS2578) (Fig. 4), derivatives of 1,4- and 1,3-phenylendiisothiocyanate, respectively. Results indicated that the production of inositol phosphates in human P2Y$_6$-transfected 1321N1 astrocytoma cells in response to 300 nM UDP was inhibited in a concentration-dependent manner with IC$_{50}$ values of 126 ± 15 nM and 37 ± 16 nM, respectively. Also, these two selective inhibitors of the hP2Y$_6$ receptor activation had approximately identical effects on rat P2Y$_6$ receptors. MRS2567 and

MRS2578 inhibited the response to 300 nM UDP at the rat $P2Y_6$ receptor expressed in 1321N1 cells in a concentration-dependent manner, with IC_{50} values of 101 ± 27 nM and 98 ± 11 nM, respectively. MRS2567 and MRS2578 at 10 μM did not affect the UTP (100 nM)-induced responses of cells expressing human $P2Y_2$ or $P2Y_4$ receptors, nor did they affect the 2-MeSADP (30 nM)-induced responses of cells expressing the $P2Y_1$ receptor. In addition, they did not affect the ATP (10 μM)-induced responses of cells expressing the $P2Y_{11}$ receptor. Thus, MRS2567 and MRS2578 selectively blocked $P2Y_6$ receptor activity versus activity at $P2Y_1$, $P2Y_2$, $P2Y_4$ or $P2Y_{11}$ receptors. However, MRS2577 blocked activity at both $P2Y_4$ and $P2Y_6$ receptors, without affecting other subtypes.

It was interesting that another derivative of 1,4-phenylendiisothiocyanate (MRS2575) inhibited only human $P2Y_6$ receptor activity. This compound at 10 μM concentration had no effect on rat $P2Y_6$ or human $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors (Table 1). This antagonist inhibited in an insurmountable manner UDP activity at the $P2Y_6$ receptor. Inhibition of human $P2Y_6$ receptor activity by MRS2575 occurred in a concentration-dependent manner with an IC_{50} value of 155 ± 49 nM (Fig. 5A and B).

3.3. Aryl diisothiocyanate derivatives in receptor binding experiments

Binding experiments at the human A_1 AR expressed in CHO cell membranes indicated that compounds MRS2567 (**5**), MRS2575 (**7**) and MRS2578 (**11**) at 10 μM concentration displayed no significant inhibition of radioligand binding (data not shown).

The diisothiocyanate derivatives MRS2576 (**8**) and MRS2570 (**9**) inhibited agonist effects at $hP2Y_1$ receptors as well as at $P2Y_6$ receptors, and consequently we examined the ability to interfere with radioligand binding at the former subtype. The high affinity antagonist [^{3}H]MRS2279 was used as a $P2Y_1$ receptor radioligand in binding to membranes from astrocytoma cells stably expressing human $P2Y_1$ receptors. Specific binding of [^{3}H]MRS2279 was defined in these experiments using the antagonist MRS2179 (10 μM). Essentially no inhibition of binding of [^{3}H]MRS2279 was detected in the presence of 10 μM MRS2576 and MRS2570 (data not shown), both of which antagonized the $P2Y_1$ receptor-mediated effects of 2-MeSADP.

3.4. Inhibition of the anti-apoptotic effects of a $P2Y_6$ receptor agonist

The study of P2Y receptors has been a challenge, largely due to the paucity of pharmacological tools for identification of receptor subtypes. Since truly selective antagonists for most of the subtypes have not yet been developed, the characterization of P2Y receptors has mainly consisted of monitoring agonist responses, such as activation of PLC. One functional effect of $P2Y_6$ nucleotide receptor activation is its cellular protective role. Activation of the rat $P2Y_6$ receptor expressed in 1321N1 astrocytoma cells by UDP has been shown to protect the cells from undergoing apoptosis induced by TNFα [10,21]. The $P2Y_6$ receptor is a GPCR that is coupled to PLC, and the subsequent activation of protein kinase C (PKC) by the receptor has been shown to relate to this protective effect. We studied the present set of $P2Y_6$ receptor antagonists in a model of the anti-apoptotic effects of P2 receptor agonists.

We have extended previous results with the rat $P2Y_6$ receptor [10,21] to the human $P2Y_6$ receptor. The activation of the human $P2Y_6$ receptor by UDP (100 nM) in the presence of TNFα (20 ng/ml) and cycloheximide (5 ng/ml), which sensitizes the TNFα response, significantly reduced cell death (Fig. 6). Four antagonists of $P2Y_6$ receptors were used in the experiment, including the selective aryl diisothiocyanate derivatives MRS2567 (**5**), MRS2575 (**7**) and MRS2578 (**11**) and the non-selective derivative MRS2564 (**6**) (1 μM). Measurements of intracellular PI staining, a measure of cell death, indicated that MRS2567,

MRS2575 and MRS2578 completely blocked the protection by UDP (100 nM). Therefore, it was demonstrated using antagonists that $P2Y_6$ receptor activation by UDP played a crucial role in preventing 1321N1 astrocytoma cells from undergoing TNFα-induced apoptosis.

4. Discussion

The present study demonstrated that MRS2567 and MRS2578 block agonist effects at both human and rat $P2Y_6$ receptors, and MRS2575 selectively blocks effects at human $P2Y_6$ but not rat $P2Y_6$ receptors. This is the first report of selective antagonists for $P2Y_6$ receptors, albeit insurmountable. Previous studies have shown that DIDS and H_2DIDS block $P2Y_6$ receptors at concentrations of 10–100 μM and are clearly less potent than MRS2567, MRS2578 and MRS2575 [12]. Compounds MRS2567 and MRS2578 can block UDP-stimulated activity at concentrations less than 1 μM (for human $P2Y_6$ at IC_{50} values 126 ± 15 nM and 37 ± 16 nM, for rat $P2Y_6$ at IC_{50} values 101 ± 27 nM and 98 ± 11 nM, respectively). Interestingly, MRS2575 is a selective antagonist for human $P2Y_6$ receptors with an IC_{50} value of 155 ± 49 nM, while it has no effect on rat $P2Y_6$ receptors. Since other compounds in this series inhibited other P2Y receptor subtypes, a panel of such diisothiocyanates may be useful in characterizing a given pharmacological response to extracellular nucleotides. For example, the following mixed selectivities were observed: MRS 2564 ($P2Y_6$, $P2Y_{11}$), MRS 2576 ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$), and MRS 2577 ($P2Y_4$, $P2Y_6$).

It is likely that the isothiocyanate groups are involved in the antagonistic effects at $P2Y_6$ receptors. All of the derivatives **4**–**11** evaluated as P2Y receptor antagonists included two isothiocyanate groups. Isothiocyanate groups are potent electrophiles that are sufficiently stable in aqueous medium for biochemical experiments, yet may be chemically reactive towards certain nucleophilic groups on biopolymers [22], such as Lys and Cys side chains. The possible covalent reaction of these antagonists with the $P2Y_6$ receptor protein has not been demonstrated. Future studies will be needed to establish the location(s) of cross-linking, if any, with the receptor. The degree of inhibition and specificity of the antagonists depended on the chain length and position of ring substitution. Thus, these inhibitory effects follow a specific SAR pattern and are not the result of general reactivity of the electrophilic group. The selectivity of these derivatives was further indicated by their inability to block binding at the human A_1AR, which is know to be sensitive to other electrophilic reagents [13].

The inability of these aryl diisothiocyanate derivatives to produce a parallel rightward shift of the concentration–response curves suggests that they are insurmountable P2YR antagonists [23]. Moreover, the failure of selected derivatives to displace radioligand binding at the human $P2Y_1$R suggests that they are non-competitive antagonists at the $P2Y_1$ subtype. Since there is no radioligand for $P2Y_6$ receptors, it was not possible to carry out similar binding experiments at this subtype. An additional functional experiment using the antagonists has supported the finding [10,21] that activation of the human $P2Y_6$ receptor by the agonist UDP prevented TNFα-induced apoptosis, since selective antagonists MRS2575, MRS2567, and MRS2578 at a concentration of 1 μM completely blocked agonist-induced activation of the $P2Y_6$ receptor.

In summary, we have identified potent, insurmountable antagonists of $P2Y_6$ receptors that are selective within the family of PLC-coupled P2Y receptors. These selective antagonists could be used as pharmacological tools for defining the role of $P2Y_6$ and other P2Y receptors.

Abbreviations

CHO	Chinese hamster ovary
DIDS	4,4′-diisothiocya-natostilbene-2,2′-disulfonic acid disodium salt
MRS2279	2-chloro-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate
PIA	N^6-phenylisopropyladenosine
PLC	phospholipase C
TNFα	tumor necrosis factor α
Tris	tris(hydroxymethyl)aminomethane

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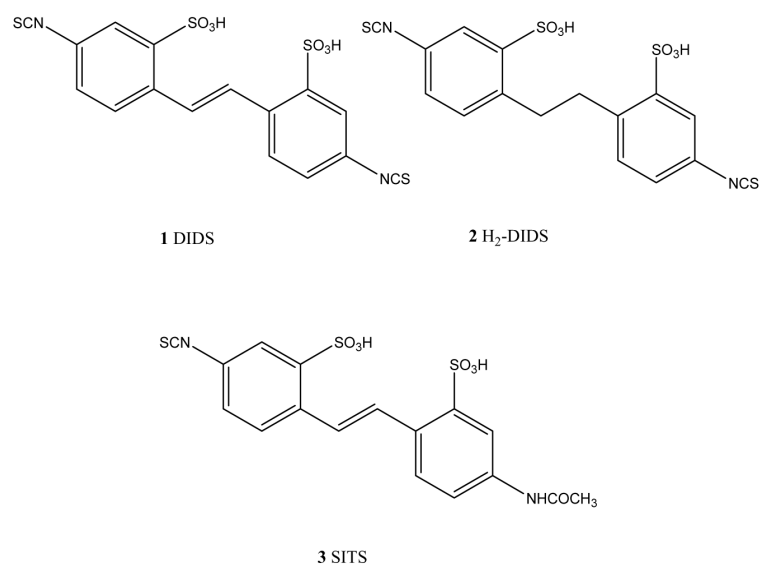
Fig. 1.
Structures of diisothiocyanates tested previously as inhibitors of P2Y receptors.
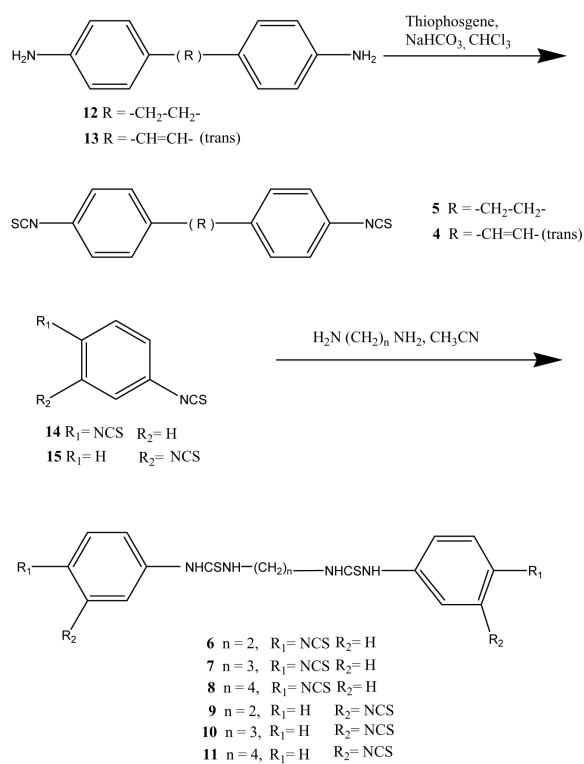
Fig. 2.
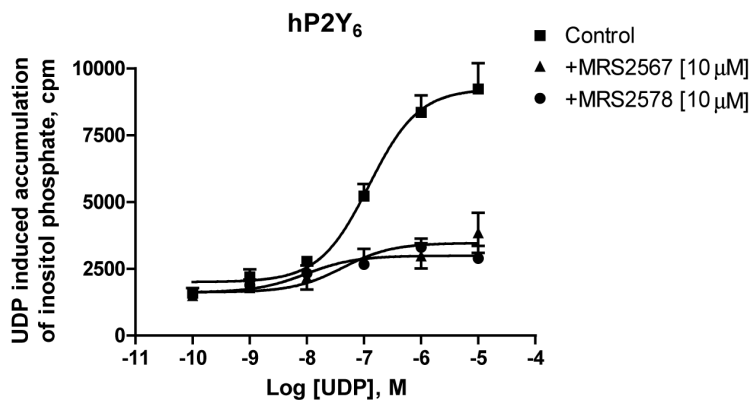Synthetic routes for the preparation of diisothiocyanate derivatives.

Fig. 3.
Inositol phosphate production in human P2Y$_6$-transfected 1321N1 human astrocytes. After labeling with *myo*-[^{3}H]inositol (1 μCi/10^6 cells) for 24 h, the cells were treated for 30 min at 37 °C with antagonists in the presence of LiCl, followed by addition of the agonist, UDP, for another 30 min. The quantity of inositol phosphates was analyzed after extraction though Dowex AG 1-X8 columns (see Section 2). Data shown are from the combined results of three independent experiments in triplicate. The EC$_{50}$ value is listed in Section 3.
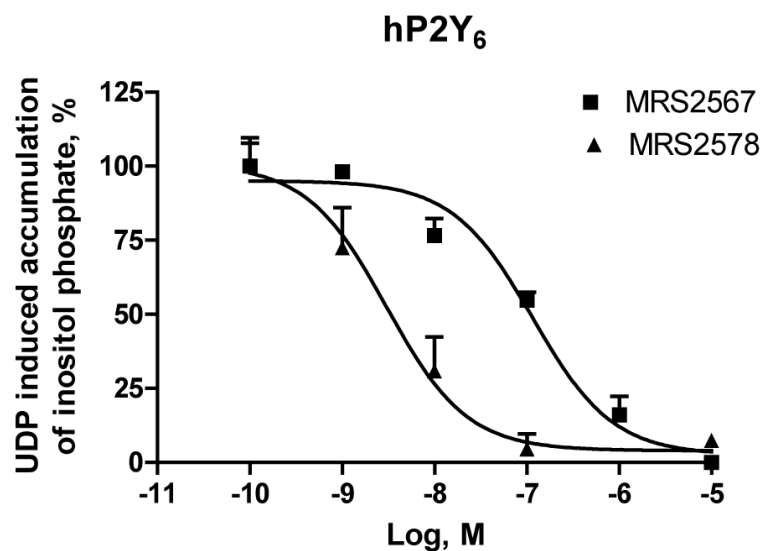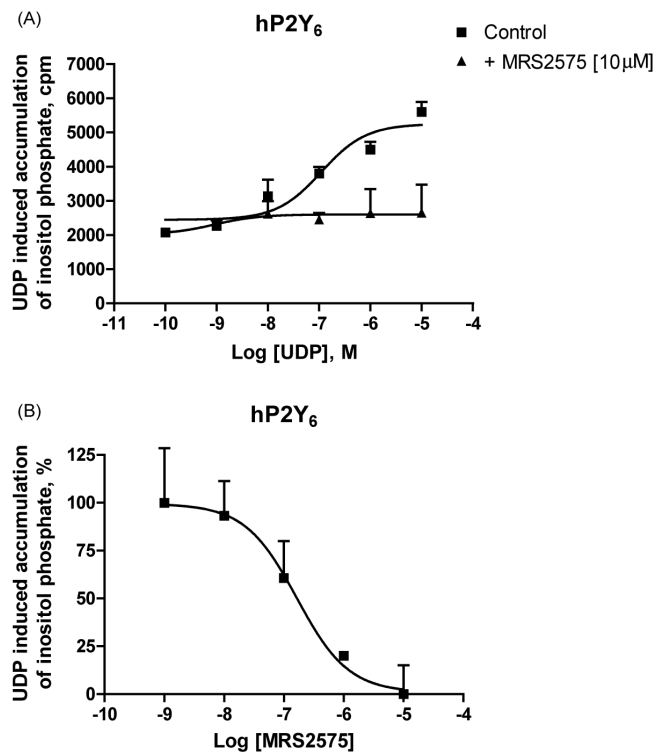
Fig. 4.
Concentration dependence of inhibition by compounds **5** and **11** of inositol phosphate production induced by UDP (300 nM) in human $P2Y_6$-receptor transfected 1321N1 human astrocytes. After labeling with *myo*-[^{3}H]inositol (1 μCi/10^6 cells) for 24 h, the cells were treated for 30 min at 37 °C with the antagonist or buffer in the presence of LiCl, followed by addition of agonist, UDP, for another 30 min. The quantity of inositol phosphates was analyzed after extraction though Dowex AG 1-X8 columns (see Section 2). Data shown represent the mean ± S.E.M. of three independent experiments in duplicate. IC_{50} values are listed in Section 3.

Fig. 5.
(A) Inositol phosphate production in human P2Y$_6$-transfected 1321N1 human astrocytes.
(B) Concentration dependence of inhibition by compound MRS2575 (7) of inositol phosphate production induced by UDP (300 nM) in human P2Y$_6$-receptor transfected 1321N1 human astrocytes. After labeling with *myo*-[^{3}H]inositol (1 μCi/10^6 cells) for 24 h, the cells were treated for 30 min at 37 °C with the antagonist or buffer in the presence of LiCl, followed by addition of agonist, UDP, for another 30 min. The quantity of inositol phosphates was analyzed after extraction though Dowex AG 1-X8 columns (see Section 2). Data shown represent the mean ± S.E.M. of three independent experiments in triplicate.
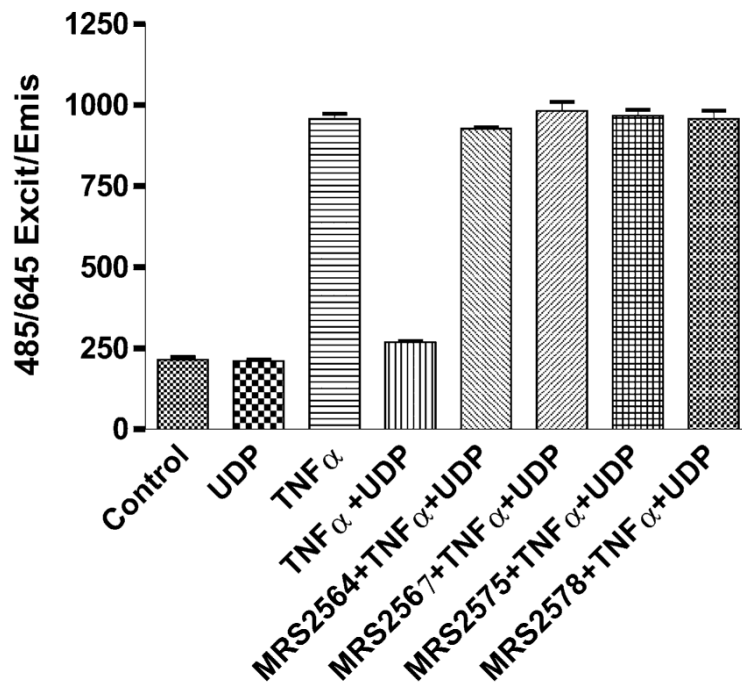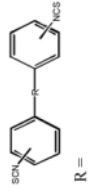
Fig. 6.
Effects of P2Y$_6$ receptor antagonists on cell death induced by the treatment of hP2Y$_6$ receptor-expressing 1321N1 astrocytoma cells with TNFα. Cells were treated with both UDP and TNFα for 4 h. Antagonists (1 μM) were added to the incubation medium 20 min prior to addition of UDP (100 nM) and TNFα (20 ng/ml). The media contained 5 ng/ml cycloheximide during the entire incubation. After 16 h of continuous treatment (as described in Section 2) the cell culture medium was replaced by medium containing PI (2 μg/ml). Since PI transverses only leaky or lysed cell membranes, DNA of dead cells can be stained and fluorescence emission measured, while living cells will not be stained. Fluorescence was measured using the Cytofluor 4000 microplate reader (excitation 485 nm/emission 645 nm), giving a direct number of dead cells. Results represent the mean value of two experiments, each performed in triplicate.

Table 1

Inhibition by aryl diisothiocyanate derivatives of the activation of PLC induced by P2Y receptor agonists

Compound (MRS number)	R =	Position of NCS	Percent inhibition[a]					
			$hP2Y_1$	$hP2Y_2$	$hP2Y_4$	$hP2Y_6$	$hP2Y_{11}$
4 (2568)	*trans*-CH=CH–	4	<5	<5	<5	<5	<5
5 (2567)	–CH₂–CH₂–	4	<5	<5	<5	95 ± 7	<5
6 (2564)	–NHCSNH(CH₂)₂NHCSNH–	4	<5	<5	<5	77 ± 4	62 ± 4
7 (2575)	–NHCSNH(CH₂)₃NHCSNH–	4	<5	<5	<5	96 ± 6	<5
8 (2576)	–NHCSNH(CH₂)₄NHCSNH–	4	90 ± 5	92 ± 4	77 ± 4	78 ± 2	<5
9 (2570)	–NHCSNH(CH₂)₂NHCSNH–	3	80 ± 4	40	<5	71 ± 10	<5
10 (2577)	–NHCSNH(CH₂)₃NHCSNH–	3	<5	<5	98 ± 2	86 ± 6	<5
11 (2578)	–NHCSNH(CH₂)₄NHCSNH–	3	20 ± 3	<5	<5	100 ± 6	<5

[a] Concentration of antagonists was 10 μM. Specific subtypes of human P2Y receptors were expressed in 1321N astrocytoma cells ($hP2Y_1$, $hP2Y_2$, $hP2Y_4$, and $hP2Y_6$) or in CHO cells ($hP2Y_{11}$) [13]. Agonists used were: $P2Y_1$, 2-MeSADP (30 nM); $hP2Y_2$, UTP (100 nM); $hP2Y_4$, UTP (100 nM); $hP2Y_6$, UDP (300 nM); $hP2Y_{11}$, ATP (10 μM). The concentrations of agonists used were roughly equivalent to their EC₅₀ values. The potency (IC₅₀ values) of MRS2567, MRS2575 and MRS2578 at both human and rat P2Y6 receptors were listed in the text.