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Synthesis and preliminary evaluation of [^{3}H]PSB-0413, a selective antagonist radioligand for platelet $P2Y_{12}$ receptors

Ali El-Tayeb, Kerstin J. Griessmeier and Christa E. Müller*

Pharmaceutical Institute, Pharmaceutical Sciences Bonn (PSB), University of Bonn, Kreuzbergweg 26, D-53115 Bonn, Germany

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Abstract—The selective antagonist radioligand [^{3}H]2-propylthioadenosine-5′-adenylic acid (1,1-dichloro-1-phosphonomethyl-1-phosphonyl) anhydride ([^{3}H]PSB-0413) was prepared by catalytic hydrogenation of its propargyl precursor with a high specific radioactivity of 74 Ci/mmol. In preliminary saturation binding studies, [^{3}H]PSB-0413 showed high affinity for platelet $P2Y_{12}$ receptors with a K_D value of 4.57 nM. Human platelets had a high density of $P2Y_{12}$ receptors exhibiting a B_{max} value of 7.66 pmol/mg of protein.

The $P2Y_{12}$ receptor belongs to the family of membrane receptors that are activated by nucleotides.[1] Two large subfamilies of nucleotide receptors exist, the P2X family, which are ligand-gated ion channels activated by ATP,[2] and the P2Y family, which are G protein-coupled receptors and may be activated by purine or pyrimidine nucleoside di- or triphosphates or even by dinucleotides or nucleotide sugars, depending on the receptor subtype.[3,4] The $P2Y_{12}$ receptor is an ADP receptor expressed on blood platelets (previous nomenclature: $P2Y_T$, T for thrombocytes) and in a lower density in the brain.[5] ATP acts as an antagonist at the $P2Y_{12}$ receptor.

In platelets, two more P2 receptor subtypes are expressed, the G_q-coupled $P2Y_1$ receptor that is also activated by ADP, and the ATP-activated $P2X_1$ receptor subtype.[6]

ADP is one of the major regulators of hemostasis and thrombosis. $P2Y_{12}$ receptors have been cloned and identified as the targets of antithrombotic thienopyridine drugs, such as clopidogrel and ticlopidine.[7]

The platelet $P2Y_{12}$ receptor has been extensively characterized in functional assays. However, characterization on the protein level has been hampered by the lacking of a selective radioligand. The non-selective radioligands [^{3}H]2-methylthio-ADP,[8] [β-^{32}P]2-methylthio-ADP[9], and [^{33}P]2-methylthio-ADP[10] have previously been used to label $P2Y_{12}$ receptors, but they showed high affinity for $P2Y_1$ receptors as well, which are also expressed on platelets.[8–10] In addition, phosphoric acid esters, including nucleotides, are metabolically unstable and may be cleaved by a number of enzymes, such as alkaline phosphatase and ectonucleotidases.[11]

Our goal was to develop a stable, high-affinity, subtype-selective antagonist radioligand for $P2Y_{12}$ receptors.

Ingall et al. had synthesized a series of 2-substituted ATP analogs stabilized by a $P_\beta P_\gamma$-dichloromethylene bridge.[12] One of the compounds, 2-propylthioadenosine-5′-adenylic acid (1,1-dichloro-1-phosphonomethyl-1-phosphonyl) anhydride (AR-C67085MX), was a potent and selective $P2Y_{12}$ antagonist exhibiting an IC_{50} value of 2.5 nM against ADP-induced aggregation of human platelets.[12] The compound was later shown to be an agonist at $P2Y_{11}$ receptors (EC_{50} = 1.5–8.9 μM),[13] and an antagonist at $P2Y_{13}$ receptors (IC_{50} = 213–630 nM),[14] but only at much higher concentrations than its affinity for $P2Y_{12}$ receptors.

Thus, we developed a strategy for the preparation of a tritiated derivative of the potent and selective $P2Y_{12}$ receptor antagonist AR-C67085MX. As a precursor for tritiation we selected the corresponding 2-propargyl derivative PSB-0412 (see Scheme 1).

The nucleosides **1a** and **1b** were synthesized according to previously published procedures.[15–17] Reaction of the nucleosides **1** with phosphorus oxychloride in trimethyl

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* Corresponding author. Fax: +49 228 73 2567; e-mail: christa.mueller@uni-bonn.de

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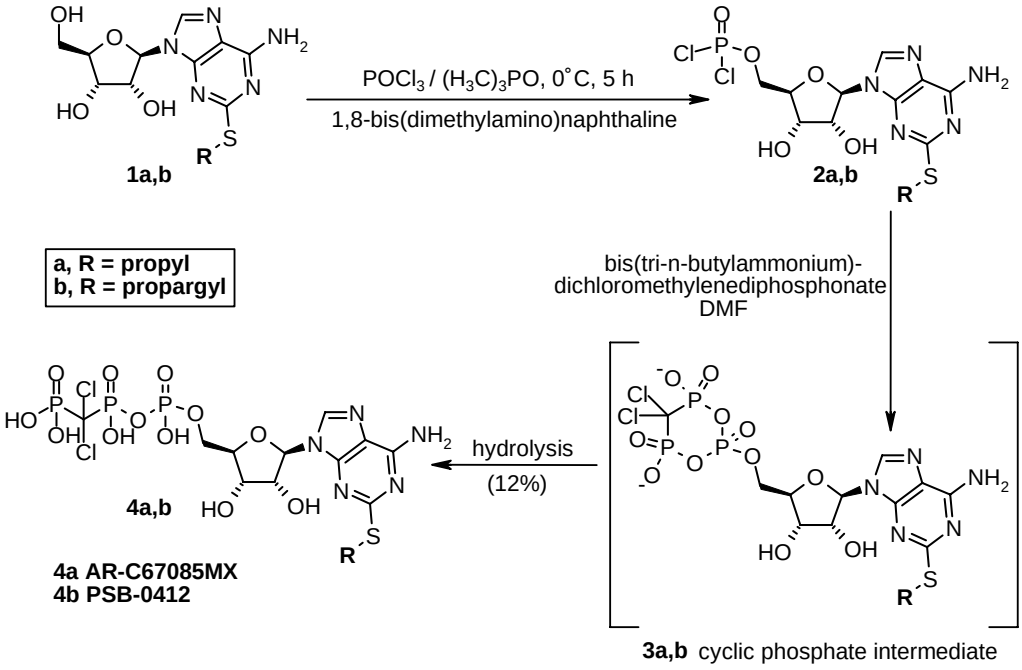
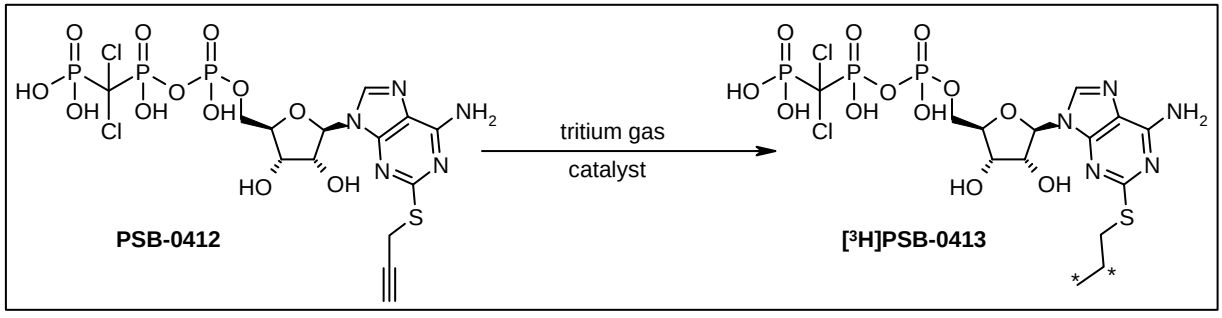


Scheme 1. Synthesis of nucleotide analogs.



Scheme 2. Preparation of [^{3}H]PSB-0413 from the propargyl precursor PSB-0412 by catalytic hydrogenation.

phosphate[18] followed by reaction with dichloromethylenediphosphonic acid in DMF afforded the corresponding triphosphate analogs, the propargyl-substituted radioligand precursor PSB-0412 (**4b**), and its propyl analog **4a** (AR-C67085MX), which was needed as a control (Scheme 1).

The synthesized nucleotides were purified by anion exchange chromatography using FPLC (ÄKTA FPLC, from Amersham Biosciences with Sephadex DEAE A-25 gel, XK 26 mm/20 cm length column) to remove nucleotides obtained as side-products, such as the corresponding AMP, ADP, and ATP derivatives.

In a second step, the products were further purified by reversed-phase HPLC[19] to remove inorganic impurities such as inorganic phosphates and buffer components.

The structures of the synthesized nucleotides were confirmed by ^{1}H, ^{13}C, and ^{31}P NMR data, in addition to LC/ESI-MS in both positive and negative modes.[20]

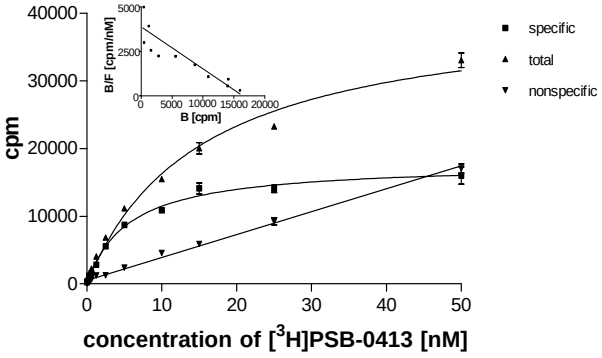


Figure 1. Representative saturation curve for [^{3}H]PSB-0413 binding to membranes of human platelets and corresponding Scatchard plot. The following binding parameters were calculated: K_D = 4.57 ± 0.51 nM, B_{max} = 7.66 pmol/mg protein.

The propargyl precursor PSB-0412 was subsequently subjected to catalytic hydrogenation using tritium gas (Scheme 2).[21] After HPLC purification,[22] [^{3}H]PSB-

0413 was obtained with a specific radioactivity of 2.74 TBq/mmol (74 Ci/mmol).

Saturation experiments[23] at $P2Y_{12}$ receptors natively expressed in human platelets using 12 different concentrations of [^{3}H]PSB-0413 ranging from 0.047 to 50 nM showed that the radioligand bound to a single class of binding sites with limited capacity exhibiting a K_D value of 4.57 ± 0.51 nM (Fig. 1). The membrane preparation showed a high expression level of $P2Y_{12}$ receptors (B_{max} = 7.66 ± 0.69 pmol/mg of protein). Non-specific binding was low and amounted to only 20% of total binding at a concentration of 5 nM (close to K_D).

Preliminary competition assays showed an expected rank order of potency typical for $P2Y_{12}$ receptors (data not shown): PSB-0412 > 2-methylthio-ADP ≫ ADPβS > ATP ⩾ ADP.

In conclusion, we have developed a selective, high-affinity radioligand for the $P2Y_{12}$ receptor expressed on blood platelets which should be useful for the characterization of the $P2Y_{12}$ receptors on the protein level in different cells and tissues. It will allow experiments to directly study interactions between the receptor protein and its ligands. The new radioligand will enable us to set up a screening assay in order to search for novel $P2Y_{12}$ receptor agonists and competitive antagonists.

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19. The nucleotides were dissolved in 5 mL of deionized water and injected into a RP-HPLC column (Knauer 20 mm ID, Eurospher-100 C18). The column was eluted with a solvent gradient of 0–25% of acetonitrile in 50 mM aq NH_4HCO_3 buffer for 25 min at a flow rate of 5 ml/min. UV absorption was detected at 254 nm. Fractions were collected and appropriate fractions were pooled, diluted with water, and lyophilized several times to remove the NH_4HCO_3 buffer yielding the nucleotides as white powders. Retention times were 16.5 and 13.6 min for AR-C67085MX and PSB-0412, respectively.
20. The nucleotide sample was dissolved at 1 mg/ml in H_2O/MeOH = 1:1, containing 2 mM NH_4CH_3COO. Then, 10 µl of the sample was injected into the HPLC column. Elution was performed with a gradient of water/methanol (containing 2 mM NH_4CH_3COO) from 90:10 to 0:100 for 30 min at a flow rate of 250 µl/min, starting the gradient after 10 min. UV absorption was detected from 190 to 400 nm using a DAD. The following mass peaks were obtained: 646 ($[M-H]^-$), 648 ($[M+H]^+$) for AR-C67085MX and 642 ($[M-H]^-$), 644 ($[M+H]^+$) for PSB-0412 in negative and positive modes, respectively.
21. Custom-labeling was performed by Amersham Biosciences, The Maynard Centre, Whitchurch, Cardiff, U.K.
22. [^{3}H]PSB-0413 was purified by HPLC (by Amersham Biosciences) with a Synergi Polar-RP 5 µm, 250 × 4.6 mm column. The product was eluted with 50 mM NH_4HCO_3 in water (solvent A) and acetonitrile (solvent B) applying a gradient from 0% B to 25% B over 25 min at a flow rate of 1 ml/min. UV absorption was detected at 254 nm.
23. Saturation binding experiments with [^{3}H]PSB-0413 (0.047 to 50 nM) to membrane preparations of outdated human blood platelets provided by the blood bank were performed by incubating membranes (100 µg/ml) for 1 h at rt in a buffer containing 50 mM Tris–HCl, 1 mM EDTA, 5 mM $MgCl_2$, and 100 mM NaCl (pH 7.4). Bound and free radioactivity was separated by rapid filtration through Whatman GF/B glass-fiber filters using a Brandel cell harvester. Filter-bound radioactivity was counted using a liquid scintillation counter. Non-specific binding was defined in the presence of 1 mM ADP. Assays were performed in duplicate in three independent experiments.