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ORIGINAL ARTICLE

The platelet $P2Y_{12}$ receptor under normal and pathological conditions. Assessment with the radiolabeled selective antagonist [^{3}H]PSB-0413

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Abstract Various radioligands have been used to characterize and quantify the platelet $P2Y_{12}$ receptor, which share several weaknesses: (a) they are metabolically unstable and substrates for ectoenzymes, (b) they are agonists, and (c) they do not discriminate between $P2Y_1$ and $P2Y_{12}$. We used the [^{3}H]PSB-0413 selective $P2Y_{12}$ receptor antagonist radioligand to reevaluate the number of $P2Y_{12}$ receptors in intact platelets and in membrane preparations. Studies in humans showed that: (1) [^{3}H]PSB-0413 bound to 425±50 sites/platelet (K_D=3.3±0.6 nM), (2) 0.5±0.2 pmol [^{3}H] PSB-0413 bound to 1 mg protein of platelet membranes (K_D=6.5±3.6 nM), and (3) competition studies confirmed the known features of $P2Y_{12}$, with the expected rank order of potency: AR-C69931MX>2MeSADP≫ADPβS>ADP, while the $P2Y_1$ ligand MRS2179 and the $P2X_1$ ligand α,β-Met-ATP did not displace [^{3}H]PSB-0413 binding. Patients with severe $P2Y_{12}$ deficiency displayed virtually no binding of [^{3}H]PSB-0413 to intact platelets, while a patient with a dysfunctional $P2Y_{12}$ receptor had normal binding. Studies in mice showed that: (1) [^{3}H]PSB-0413 bound to 634±87 sites/platelet (K_D=14±4.5 nM) and (2) 0.7 pmol±0.3 [^{3}H]PSB-0413 bound to 1 mg protein of platelet membranes (K_D=9.1±5.3 nM). Clopidogrel and other thiol reagents like pCMBS or DTT abolished the binding both to intact platelets and membrane preparations. Therefore, [^{3}H]PSB-0413 is an accurate and selective tool for radioligand binding studies aimed at quantifying $P2Y_{12}$ receptors, to identify patients with $P2Y_{12}$ deficiencies or quantify the effect of $P2Y_{12}$ targeting drugs.

Keywords $P2Y_{12}$ receptor · Competitive antagonist · Radioligand · [^{3}H]PBS-0413 · Platelets · Nucleotide analog · AR-C67085MX

Abbreviations

AR-C67085MX	2-Propylthioadenosine-5′-adenylic acid (1,1-chloro-1-phosphono-methyl-1-phosphonyl)anhydride
AR-C69931MX (cangrelor)	N^6-(2-Methylthioethyl)-2-(3,3,3-trifluoropropylthio)adenosine-5′-adenylic acid (1,1-chloro-1-phospho-nomethyl-1-phosphonyl)anhydride
[^{3}H]PSB-0413	[^{3}H]2-Propylthioadenosine-5′-adenylic acid (1,1-chloro-1-phospho-nomethyl-1-phosphonyl)anhydride
PSB-0412 (precursor of [^{3}H]PSB-0413)	2-Propargylthioadenosine-5′-adenylic acid (1,1-chloro-1-phospho-nomethyl-1-phosphonyl)anhydride
MRS2179	N^6-Methyl-2′-deoxyadenosine-3′,5′-bisphosphate

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MRS2500	2-Iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate)
*p*CMBS	*para*-Chloromercuribenzene sulfonic acid

Introduction

Platelet activation by ADP plays a crucial role in hemostasis and thrombosis, and their so-called P2 receptors are potential targets for antithrombotic drugs. Two G protein-coupled ADP receptors, $P2Y_1$ and $P2Y_{12}$, selectively contribute to platelet aggregation. The $P2Y_1$ receptor is responsible for ADP-induced shape change, weak and transient aggregation, while the $P2Y_{12}$ receptor is responsible for the completion and amplification of the response to ADP and to all platelet agonists including thromboxane A_2, thrombin, and collagen [1]. Due to its central role in the formation and stabilization of a thrombus, the $P2Y_{12}$ receptor is a well-established target of drugs like the thienopyridines (ticlopidine, clopidogrel, prasugrel) and ticagrelor which proved to have potent antithrombotic efficacy both in clinical trials in humans and in experimental models of thrombosis [2,3]. One important point is to be able to quantify the number of receptors expressed on platelets in order to assess the inter-individual variability in the general population, to characterize patients with inherited deficiencies, and to monitor and study patients treated with $P2Y_{12}$ targeting drugs [4].

Various radioligands have been used to characterize and quantify the platelet P2Y receptors such as, [^{14}C]ADP [5,6], [^{3}H]ADP [6,7], [^{3}H]2-methylthio-ADP [8], [β-^{32}P]2-methylthio-ADP [9,10], and [β-^{33}P]2-methylthio-ADP [11], but they all share several weaknesses: (a) They are metabolically unstable and may be cleaved by a number of enzymes such as alkaline phosphatase and ectonucleotidases; (b) being agonists, they may complicate the quantification when intact, living cells are used and receptors are internalized upon activation; (c) they do not discriminate between $P2Y_1$ and $P2Y_{12}$ receptors. In the last decade, the only one possibility to selectively quantify $P2Y_{12}$ receptors was to use the non-selective radiolabeled ligand 2-methylthio-ADP in the presence of a $P2Y_1$ antagonist such as N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2179) [12] or 2-iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2500) [13]. Concerning $P2Y_1$, recent studies used the very selective antagonist MRS2500 as a radioligand [14,15]. From these studies, it is thought that platelets express approximately 150 $P2Y_1$ receptor copies/cell. Concerning $P2Y_{12}$, based on earlier radioligand binding studies, the current idea is that individual platelets express around 450–1,000 copies. In the present study, we wished to more precisely quantify and characterize the $P2Y_{12}$ receptors in intact platelets as well as in membrane fractions. To do that, we used the recently described [^{3}H]PSB-0413 [16,17], which is a tritiated derivative of a selective antagonist of the $P2Y_{12}$ receptor, the 2-propylthioadenosine-5′-adenylic acid (1,1-chloro-1-phosphonomethyl-1-phosphonyl)anhydride (AR-C67085MX) compound. We also measured the binding to platelets from known patients, two with severe $P2Y_{12}$ deficiency [6,18] and one with a dysfunctional $P2Y_{12}$ receptor [19] and from clopidogrel treated mice.

Materials and methods

Subjects

For experiments on human platelets, blood was taken from ten healthy subjects and three patients with congenital defects of the platelet P2Y12 receptors. Two patients (A and B) displayed severe deficiency of the receptors, which was associated with a homozygous frameshift mutation resulting in premature truncation of the protein in patient A [6,20] or with heterozygous frameshift mutation resulting in premature truncation of the protein and haploinsufficiency in patient B [18,20]. The platelets from the third patient displayed abnormal $P2Y_{12}$-mediated responses to ADP, but normal number of dysfunctional $P2Y_{12}$ receptors, associated with Arg256 to Gln transition in one allele and a Arg265 to Trp transition in the other allele [19].

Chemicals

[^{3}H]PSB-0413 was prepared by catalytic hydrogenation with tritium gas (GE, Healthcare, Buckinghamshire, UK) of the propargyl precursor PSB-0412 as described [16]. Apyrase was purified from potatoes as previously described [21]. AR-C69931MX (Cangrelor) was provided by the Medicines Company (Parsippany, NJ, USA). MRS2179 was purchased from TOCRIS (Bristol, UK). ADP was from Sigma-Aldrich Corp. (St. Louis, MO, USA). Clopidogrel was from Sanofi-Aventis (Sanofi-Aventis, France). All other drugs were from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Cell culture

Human astrocytoma 1321N1 cells (reference no. 80630402, European Collection of Cell Cultures, UK) stably expressing the $P2Y_1$ or the $P2Y_{12}$ receptor and subcloned were used for binding experiments and cultured as described previously [22]. For binding experiments, cells were removed by trypsinization and resuspended in PBS at a concentration of 2.10^7 cells/ml.

Preparation of washed platelet suspension

Human blood was collected from a forearm vein and mouse blood from the aorta under ether anesthesia acid–citrate–dextrose anticoagulant. Washed platelets from human or mouse blood were prepared as previously described [21]. Platelets were suspended in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, 5 mM Hepes, pH 7.3) containing 0.35 % human serum albumin and apyrase (0.02 U/ml). The platelet count was adjusted at a concentration of 600,000 platelets/μl and the suspension maintained at 37 °C until use.

Preparation of platelet membranes

Human platelet membranes were prepared essentially as described [23]. Briefly, washed platelets were resuspended in Tyrode's buffer containing no Ca^{2+}, in the presence of 2 mM EDTA and apyrase (0.02 U/ml) at room temperature. These platelets were loaded with glycerol by centrifugation through a 0–30 % (*v*/*v*) glycerol gradient and lysed in a hypotonic Tris buffer containing 2 mM EDTA and a cocktail of protease inhibitors. After lysis, the broken platelets were layered onto a 30 % (*w*/*v*) sucrose cushion and centrifuged for 4 h at 60,000×*g*. The floating membranes were removed carefully with a plastic pipette, washed and pelleted by centrifugation for 1 h at 100,000×*g*. Mouse platelet membranes were prepared by nitrogen cavitation as described [24]. The broken platelets (crude membranes) were centrifuged at 30,000×*g* for 1 h. Human plasma membrane pellets and mouse crude membrane pellets were resuspended in 50 mM Tris HCl, pH 7.5 containing 3 % (*v*/*v*) glycerol. Protein concentrations from human and mouse platelet membranes were determined using the BCA assay and adjusted at 1 mg/ml.

Platelet aggregation

Aggregation was measured at 37 °C by a turbidimetric method in a Carat TX4 aggregometer (Entec GmbH, Ilmenau, Germany). Platelets were activated by addition of 5 μM ADP in the absence or presence of 100 nM of [^{3}H]PSB-0413 and human fibrinogen (0.8 mg/ml). The extent of aggregation was estimated quantitatively by measuring the maximum curve height above the baseline level.

Radioligand binding assay with human and mouse platelets

Binding experiments were performed with 200 μl of human or mouse washed platelets or with 20 μg of membrane proteins using [^{3}H]PSB-0413 ranging from 0.030 to 50 nM (hot saturation). Non-specific binding was defined in the presence of 1 mM ADP. Intact platelets were incubated at 37 °C for 5 min while platelet membranes were incubated at 25 °C for 60 min. The reaction was stopped by washing the filters with 3×5 ml of ice-cold washing buffer (Tris HCl 50 mM pH 7.5, EDTA 1 mM, $MgCl_2$ 5 mM, NaCl 100 mM). Bound and free radioactivity was separated by filtration through Whatman GF/B glass fiber filters for intact platelets or through nitrocellulose filters for platelet membranes using a Brandel cell harvester. Filter-bound radioactivity was counted using a liquid scintillation counter. Assays were performed in triplicate in three independent experiments.

Data analysis

Data binding was analyzed with the program EBDA-LIGAND [25]. The dissociation constant (K_D) of the radioligand and the inhibition constant for the drug (K_I) were calculated using the GraphPad software package (GraphPad, San Diego, CA, USA). The results are presented as the mean±SEM averaged from three or more independent experiments.

Results

[^{3}H]PSB-0413 is a selective $P2Y_{12}$ antagonist radioligand

Platelet aggregation induced by 5 μM ADP is completely inhibited by 100 nM of the selective antagonist radioligand [^{3}H]PSB-0413 (Fig. 1a) while the $P2Y_1$-dependent shape change, reflected by the initial decrease in light transmission upon addition of ADP, is preserved (Fig. 1b). The selectivity of [^{3}H]PSB-0413 was further confirmed using $P2Y_1$ and $P2Y_{12}$ transfected 1321N1 cell lines. Binding only occurred in $P2Y_{12}$ transfected cells, while no binding was observed in control or $P2Y_1$ transfected cells (Fig. 1c). Furthermore, the binding of [^{3}H]PSB-0413 was completely abolished in the presence of 10 μM of the $P2Y_{12}$ selective antagonist AR-C69931MX (Fig. 1c).

Binding properties of [^{3}H]PSB-0413 to intact human platelets

In a first series of experiments, we wanted to establish the general binding properties of [^{3}H]PSB-0413 toward intact platelets. The kinetic of association was very fast ($t_{1/2}$ of 26.7±3.9 s), and the steady-state was reached within 5 min (Fig. 2a). After 15 min incubation, the dissociation was initiated by the addition of 1 mM ADP and the residual binding measured over time during 30 min (Fig. 2b). The kinetic of dissociation was also fast ($t_{1/2}$=42.9±10.8 s), and dissociation was completed after 30 min incubation.

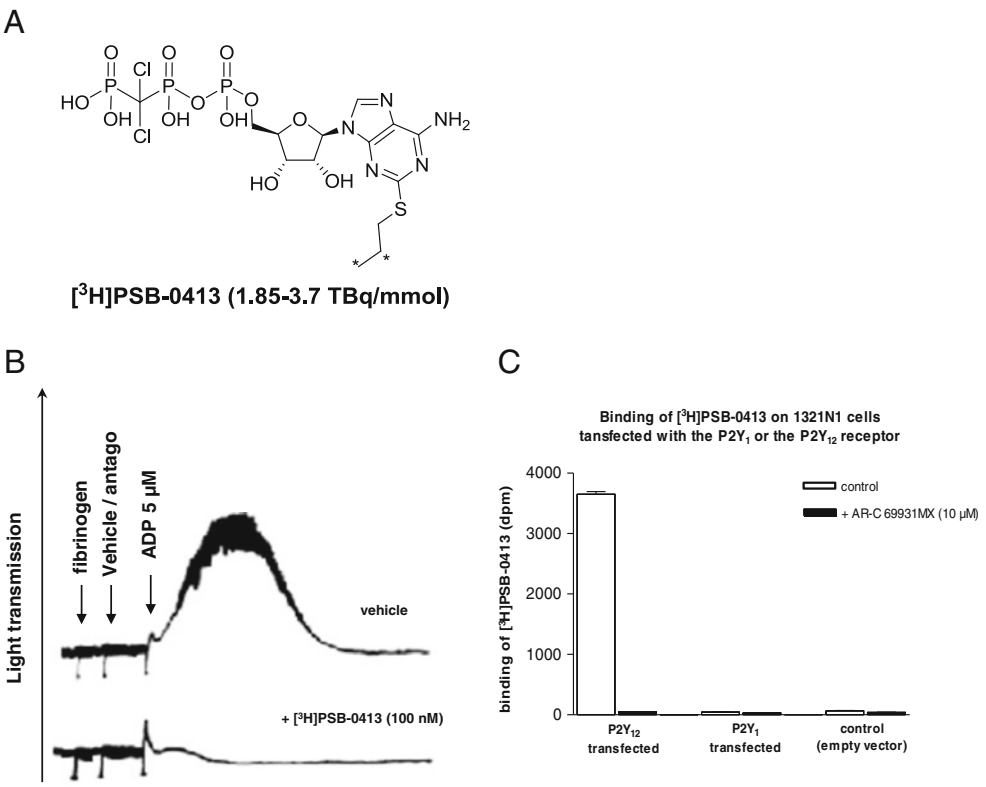

Fig. 1 Pharmacological characterization. **a** [^{3}H]PSB-0413 was obtained by catalytic hydrogenation using tritium gas with a specific radioactivity of 1.85 to 3.7 TBq/mmol where ^{3}H was fixed on the propyl moieties (*black stars*). **b** Washed platelets were activated with 5 μM ADP in the presence of human fibrinogen and in the absence or presence of 100 nM of [^{3}H]PSB-0413. **c** 1321N1 cell lines transfected with $P2Y_{12}$, $P2Y_1$ receptors or control (*empty vector*) were incubated with 1 nM of [^{3}H] PSB-0413 at 37 °C for 5 min. Binding was performed in the presence (*closed square*) or absence (*open square*) of 10 μM of the $P2Y_{12}$ antagonist AR-C69931MX. Assays were performed in triplicate in three independent experiments

Saturation on intact human platelets is reached with concentration range of 30 nM [^{3}H]PSB-0413, and under these conditions, non-specific binding in the presence of 1 mM of ADP was 7.5 % of total binding (Fig. 2c). Saturation curves generated on intact platelets from ten healthy volunteers revealed a single type of binding site with a high affinity (K_D) of 3.3±0.6 nM and number of binding sites (B_{max}) of 425±50 sites per platelet (Fig. 2c). Displacement experiments confirmed the known features of the $P2Y_{12}$ receptor with the expected rank order of potency of various ligands: K_I=1.5±0.26 nM (AR-C69931MX), 32.1±3.03 nM (2MeSADP), 7.3±1.76 μM (ADPβS), and 75±29.3 μM (ADP), while the $P2Y_1$ ligand MRS2179 and the $P2X_1$ ligand α,β-Me-ATP at 100 μM did not displace the binding of [^{3}H]PSB-0413 at concentrations below the millimolar range (Fig. 2d). Saturation experiments on intact platelets from two patients (A and B) with known severe $P2Y_{12}$ deficiency [6,18] displayed virtually no binding, while one patient with dysfunctional $P2Y_{12}$ (C) [19] displayed normal binding (Table 1).

Effects of thiol reagents on [^{3}H]PSB-0413 binding

Clopidogrel, one of the most prescribed $P2Y_{12}$ targeting drugs, is a prodrug of which the active liver metabolite is a thiol reagent which covalently binds to cysteine moieties of the $P2Y_{12}$ receptor. Other thiol reagents were previously reported to target the $P2Y_{12}$ receptor [4,9,26–28]. We thus wanted to check whether these compounds have an effect on the binding of [^{3}H]PSB-0413. The reducing agents *para*-chloromercuribenzene sulfonic acid (*p*CMBS) or dithiothreitol (DTT) inhibited the binding of [^{3}H]PSB-0413 on intact cells in a dose-dependent manner with K_I of 17.3±2.8 μM for *p*-CMBS (Fig. 3a) and a K_I of 62.8±15.2 μM for DTT (Fig. 3b). The evaluation of the effect of clopidogrel on [^{3}H]PSB-0413 binding to intact platelets was performed in mice treated by clopidogrel 50 mg/kg, a dose that inhibits 100 % platelet aggregation by ADP [29]. Control mouse platelets display 643±87 [^{3}H]PSB-0413 binding sites/platelet with a K_D=14±4.5 nM (Fig. 3c). The binding of [^{3}H]PSB-0413 to platelets from clopidogrel treated mice was completely abolished.

Binding of [^{3}H]PSB-0413 to platelet membranes

Saturation experiments showed that the radioligand bound to a single class of binding sites with a K_D=6.5±3.6 nM and a B_{max}=0.5±0.2 pmol/mg in human platelet membranes (Fig. 4a) and K_D=9.1±5.3 nM and B_{max}=0.7±0.3 pmol/mg in mouse platelet membranes (Fig. 4b). The binding of [^{3}H]PSB-0413 to mouse platelet membrane preparations was completely abolished after clopidogrel treatment. Similarly the direct competitive $P2Y_{12}$ antagonist AR-C69931MX inhibited the binding, while the $P2Y_1$ ligand MRS2179 had only minimal effect at concentrations above 100 μM (Fig. 4c).

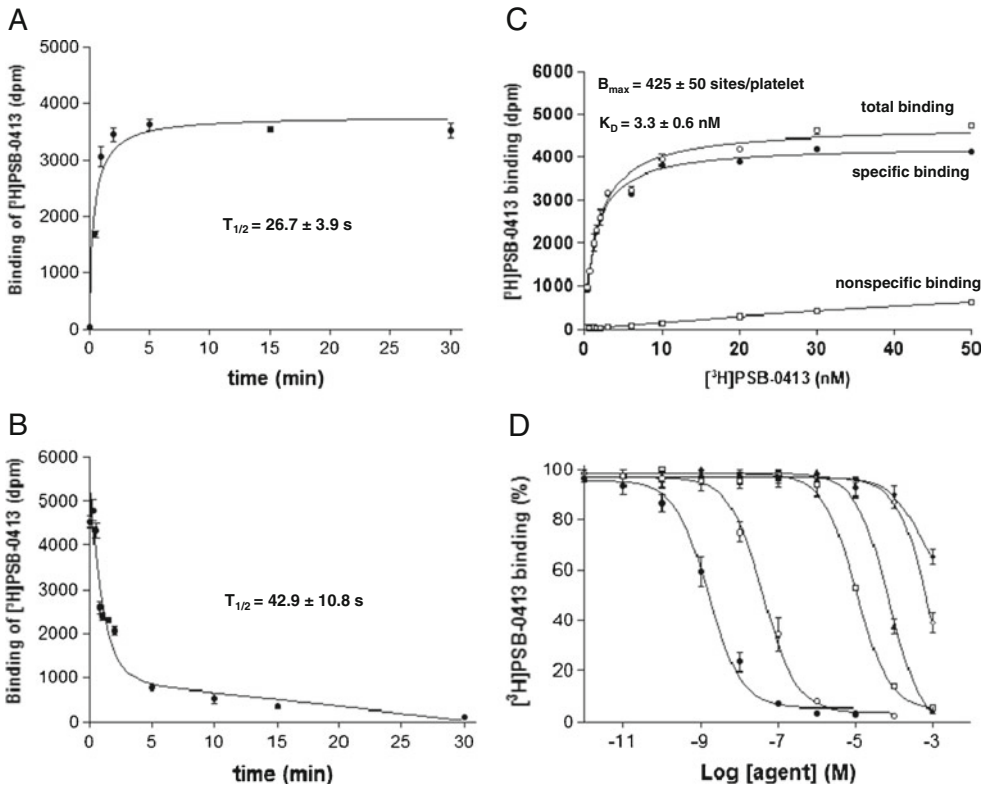

Fig. 2 Binding and pharmacological properties of [^{3}H]PSB-0413 on human intact platelets. **a** Association kinetic of [^{3}H]PSB-0413 binding on human intact platelets, measured at 37 °C over 30 min. **b** Dissociation kinetic was measured after [^{3}H]PSB-0413 binding had reached a steady state (15 min) and was initiated by addition of 1 mM ADP. Association and dissociation curves are representative from three independent experiments. **c** Saturation experiment on human intact platelets from ten healthy subjects was achieved using different concentrations of [^{3}H]PSB-0413 ranging from 0.05 to 50 nM (*closed circle*). Non-specific binding assessed in the presence of excess ADP (1 mM) and isotherm binding curves were calculated using the software EBDA-LIGAND in the hot-saturation mode. The saturation curve is representative of ten independent experiments. **d** Competition experiments were performed at 37 °C after a 5-min incubation with 5 nM of [^{3}H]PSB-0413 in the presence of increasing concentration of: AR-C69931MX (*closed circle*), 2MeSADP (*open circle*), ADPβS (*open square*), ADP (*closed triangle*), α,β-Me-ATP (*open diamond*), and MRS2179 (*closed diamond*). Competition curves are the mean of three independent experiments

Discussion

Reliable methodology for the quantification of $P2Y_{12}$ receptor binding sites as well as their characterization is crucial for advancing our understanding of a variety of conditions. For example, there is a debate on whether the platelet $P2Y_{12}$ receptor rapidly desensitizes and undergoes trafficking upon agonist activation [30–36]. Similarly, polymorphisms of the $P2Y_{12}$ receptor have been proposed to be associated with a gain of function in terms of platelet activation and an increased risk of cardiovascular disease [37–41], but the relative densities and binding properties of the platelet $P2Y_{12}$ receptor associated with these polymorphisms are not known. Finally, patients with inherited $P2Y_{12}$ defects may present with total absence of receptor expression [6,18,42] while other express abnormal receptors with modified binding properties or normal binding properties and, likely, defective signal transduction [19,42], which require fine characterization [4,20,42]. The [^{3}H]PSB-0413 selective $P2Y_{12}$ radioligand was reported recently with only preliminary evaluation as a selective tool using membrane preparations [16] but not intact platelets from healthy control, patients with inherited $P2Y_{12}$ defects, $P2Y_{12}$ targeting drugs treated patients or animals. The data reported here establish [^{3}H]PSB-0413 as a valuable new radioligand to investigate mechanistic aspects of the $P2Y_{12}$ receptor in these and other conditions.

Table 1 Binding studies of [^{3}H]PSB-0413 on intact platelets from patients with selective inherited $P2Y_{12}$ deficiencies

	Control	Patient A	Patient B	Patient C
B_{max} (sites/platelet)	425±50	0	0	420
K_D (nM)	3.3±0.6	nc	nc	3.6

Saturation experiments were performed on human intact platelets from two patients (A,B) with severe $P2Y_{12}$ deficiency [6,18] and one patient with dysfunctional $P2Y_{12}$ (C) [19] and on ten healthy donors as control. The number of binding sites (B_{max}) and the affinity constant (K_D) were calculated using EBDA-LIGAND software

nc not calculated

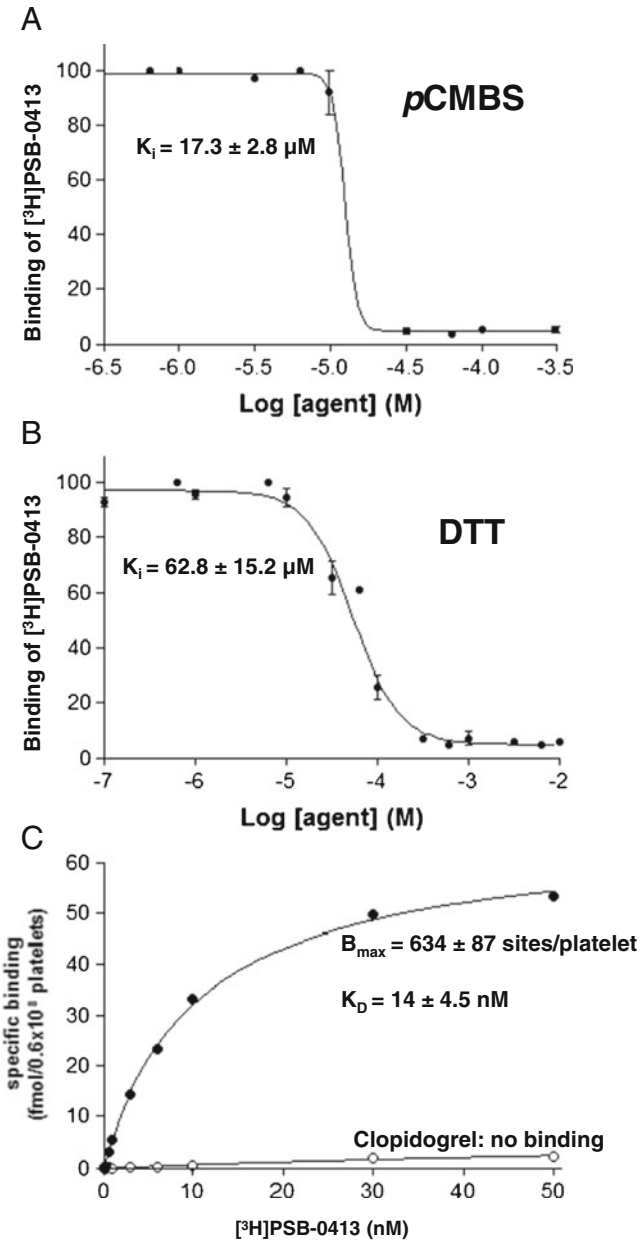

Fig. 3 Effects of thiol reagents on [^{3}H]PSB-0413 binding to intact human and mouse platelets. **a**, **b** Inhibition experiments were performed by incubating 5 nM of [^{3}H]PSB-0413 with intact platelets pretreated for 10 min with increasing concentrations of thiol reagents: **a** *p*CMBS and **b** DTT; inhibition curves are the mean of three independent experiments. **c** Saturation experiment was achieved on intact platelets from control mice (*closed circle*) or from mice that had been treated with 50 mg/kg clopidogrel (*open circle*) using concentrations of [^{3}H]PSB-0413 ranging from 0.05 to 50 nM. The saturation curve is representative from three independent experiments

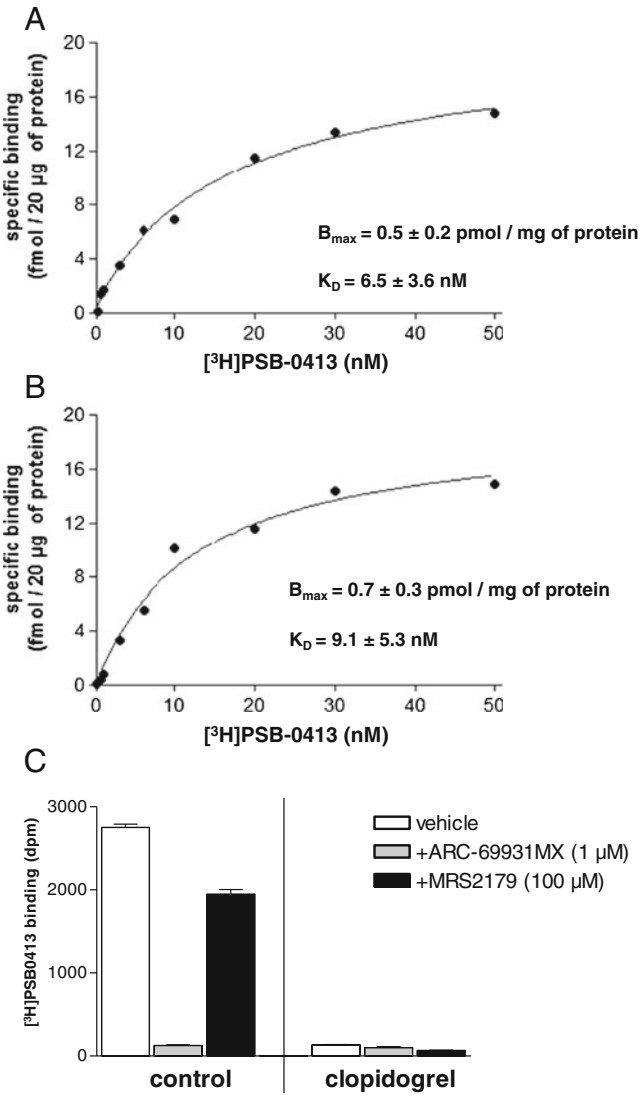

Fig. 4 Binding and pharmacological properties of [^{3}H]PSB-0413 on platelet membranes. Saturation experiments on **a** human or **b** mice platelet membrane preparations were achieved by incubating 20 µg of membrane proteins at 25 °C for 60 min with different concentrations of [^{3}H]PSB-0413 ranging from 0.030 to 50 nM. **c** Binding on platelet membranes from control or clopidogrel (50 mg/kg) treated mice was achieved using 5 nM of [^{3}H]PSB-0413 in the absence (*white square*) or presence of 1 µM AR-C69931MX (*gray square*) or 100 µM MRS2179 (*black square*). Assays were performed in triplicate in three independent experiments

Not surprisingly, we found that clopidogrel completely inhibited the binding of [^{3}H]PSB-0413 in intact mouse platelets, and it is not hazardous to speculate that it does the same in human platelets. However, we were happy to be able to measure the effect of clopidogrel in membrane preparations (Fig. 4c) as we were not able to do so in a previous study using [^{33}P]2MeSADP and membrane preparations from clopidogrel treated rats [11]. In fact, reasoning that clopidogrel acts through an active metabolite that covalently binds the cysteine residues of the $P2Y_{12}$ receptor, we thought to evaluate the impact of DTT itself on the binding of [^{3}H]PSB-0413. As expected, DTT strongly inhibited the binding as did the chemical *p*CMBS which is a well-known inhibitor of ADP-induced platelet aggregation [9,26–28,43]. Since DTT was present in our previous membrane preparations to preserve them from oxidation, it is not surprising

that we could not observe any difference between clopidogrel treated and control rats. We now have a clear explanation of the previous failure to measure the effect of clopidogrel on platelet membrane preparations.

Overall, the results presented here do not dramatically differ from previous studies using non-selective radioligands. However, new tools now exist to clearly distinguish and properly quantify the $P2Y_{12}$ receptor in the one hand and the $P2Y_1$ receptor on the other hand using [^{3}H]PSB-0413 and the [^{32}P]MRS2500 [14] or [^{125}I]MRS2500 [15] radioligands, respectively. Such studies will be very helpful not only in the platelet field but also in any other cell type and tissues where these receptors display regulated expression [44] .

Conclusion

[^{3}H]PSB-0413 is an accurate and selective tool for radioligand binding studies aimed at quantifying $P2Y_{12}$ receptors, to identify patients or quantify the effect of $P2Y_{12}$ targeting drugs.

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