Vascular Pharmacology 113 (2019) 47–56

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Adenosine receptor agonists deepen the inhibition of platelet aggregation by $P2Y_{12}$ antagonists

Magdalena Boncler*,[1], Joanna Wzorek[1], Nina Wolska, Dawid Polak, Cezary Watala, Marcin Rozalski

Department of Haemostasis and Haemostatic Disorders, Medical University of Lodz, Lodz, Poland

ARTICLE INFO

Keywords:
Platelet
Adenosine receptor
$P2Y_{12}$ antagonist
Dual anti-platelet therapy
Aggregation

ABSTRACT

Several adenosine receptor (AR) agonists have been shown in the past to possess anti-platelet potential; however, the adjunctive role of AR agonists in anti-platelet therapy with the use of $P2Y_{12}$ receptor inhibitors has not been elucidated so far.

This *in vitro* aggregation-based study investigates whether the inhibition of platelet function mediated by cangrelor or prasugrel metabolite can be potentiated by AR agonists. It evaluates the effect of non-selective (2-chloroadenosine), A_{2A}-selective (UK 432097, MRE 0094, PSB 0777) and A_{2B}-selective AR agonists (BAY 60-6583) on platelet function in relation to their toxicity, specificity towards adenosine receptor subtypes, structure and solubility.

UK 432097, 2-chloroadenosine, MRE 0094 and PSB 0777 were found to be more or less potent inhibitors of ADP-induced platelet aggregation when acting alone, and that they remained non-cytotoxic to the cells. These AR agonists were also effective in the potentiation of the effects exerted by $P2Y_{12}$ antagonists. Considering the estimated IC_{50} value, UK 432097, showing a relatively high binding affinity to the A_{2A} adenosine receptor, has been identified as the most potent anti-aggregatory agent. This compound diminished platelet aggregation at nanomolar concentrations and further augmented platelet inhibition by $P2Y_{12}$ antagonists by approx. 60% ($P < .01$).

Our results indicate the importance of adenosine receptors as therapeutic targets and point out challenges and potential benefits of therapeutic use of a combined therapy of $P2Y_{12}$ antagonist and AR agonist in cardioprotection. Our comparative analysis of the effects of AR agonists on platelet response in plasma and whole blood may indirectly suggest that other blood morphology elements contribute little to the inhibition of platelet function by AR agonists.

1. Introduction

Activation of blood platelets plays a crucial role in the initiation and development of arterial thrombotic diseases, which are the leading cause of morbidity and mortality in Western countries. Therefore, antiplatelet therapy is the 'first choice' in the management and treatment of arterial thrombotic disorders [1]. Despite the fact that many anti-platelet agents are currently available and some are widely applied as drugs, the clinical problem of the effective treatment of arterial thrombosis has not been fully solved [2–4]. Therefore, there is still a need to develop novel platelet inhibitors with better efficacy and safety or use combined therapy based on currently-available agents.

Adenosine diphosphate (ADP) is a crucial mediator of both physiological haemostasis and thrombosis. Although ADP is regarded as a weak agonist of blood platelets, it is nonetheless an important factor promoting platelet activation induced by other activators (thrombin, collagen); these activators themselves promote ADP release from intraplatelet storage pools, such as dense granules, where it is present in molar concentrations. It results in a positive feedback that enhances platelet aggregation and platelet plug formation. Additionally, ADP acts synergistically to other platelet agonists, even the weak ones, such as serotonin, adrenaline or chemokines [5].

* Corresponding author at: Department of Haemostasis and Haemostatic Disorders, Chair of Biomedical Sciences, Medical University of Lodz, ul. Mazowiecka 6/8, 92-215 Lodz, Poland.
E-mail address: magdalena.boncler@umed.lodz.pl (M. Boncler).
[1] Both authors contributed equally to this manuscript.

https://doi.org/10.1016/j.vph.2018.11.005
Received 24 July 2018; Received in revised form 1 October 2018; Accepted 18 November 2018
Available online 22 November 2018

Platelets express two receptors for ADP: the $P2Y_1$ receptor, which initiates platelet aggregation, and the $P2Y_{12}$ receptor, which enhances this process, leading to thrombus formation. $P2Y_{12}$ has become a more attractive therapeutic target than $P2Y_1$ for preventing arterial thrombotic disorders [6]. The major clinically approved $P2Y_{12}$ inhibitors include the thienopyridines (ticlopidine, clopidogrel and prasugrel), the ATP analogue cangrelor and the cyclopentyl-triazolo-pyrimidine (CPTP) derivative-ticagrelor [3,6]. Thienopyridines are prodrugs that are converted to short-living active metabolites that irreversibly inactivate the receptor and consequently inhibit ADP-induced platelet activation. Cangrelor is the first intravenous $P2Y_{12}$ receptor inhibitor, which reversibly blocks ADP signaling in a non-competitive manner. Ticagrelor is an allosteric antagonist of $P2Y_{12}$, acting directly *via* reversible binding to the $P2Y_{12}$ receptor, resulting in the non-competitive inhibition of ADP-induced $P2Y_{12}$ activation; it is therefore used in the prevention of thromboembolic events in patients with acute coronary syndromes [3,6,7]. Clopidogrel, prasugrel, and ticagrelor are the most frequently-used oral platelet $P2Y_{12}$ inhibitors in current clinical practice. Although all three agents have indications for use in acute coronary syndromes, current guidelines favour prasugrel and ticagrelor over clopidogrel because of their superior clinical benefits [8]. In turn, cangrelor, being the first intravenously-administered $P2Y_{12}$ inhibitor to be approved, seems to be the most promising option for percutaneous coronary interventions [9].

Adenosine serves not only as a component of nucleic acids and the most important energy carrier in the cell (ATP), but also as a signaling molecule which regulates tissue function [10,11]. Adenosine receptors (AR) are a subfamily of G-protein-coupled receptors, found in membranes of various human cells and playing various physiological functions. Of four known adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3), platelets express only A_{2A} and A_{2B}. Of the platelet AR receptors, A_{2A} is regarded as a high affinity receptor, and platelets have a significantly higher density of A_{2A} than A_{2B} [10,12,13]. Agonization of platelet AR results in an enhanced intracellular cAMP level and consequently leads to the inhibition of platelet activation and aggregation [11,14]. Therefore, the adenosine receptors A_{2A} and A_{2B} could act as targets for antiplatelet therapy, especially under circumstances when classical therapy of antagonizing the other purinergic receptor $P2Y_{12}$ is insufficient or problematic. Apart from the natural agonist, adenosine, a group of synthetic, longer-lasting agonists of A_{2A} and A_{2B} receptors also exists [11–13,15]. This group includes agonists with good selectivity for A_{2A} or A_{2B} receptors, as well as non-selective compounds that activate more than one type of adenosine receptor. Several A_{2A} and A_{2B} agonists have been tested clinically, and two of them, regadenoson and binodenoson, have even been approved for use in cardiac imaging procedures. Chemically, most A_{2A} and A_{2B} adenosine receptor agonists are adenosine analogues, with either adenine or ribose substituted by single or multiple substituents. However, a group of non-adenosine derivative agonists has also been described [12,13,15,16]. One of the oldest known adenosine analogues which has been reported to possess anti-platelet effects is 2-chloroadenosine [17]. UK 432097, MRE 0094, PSB 0777 and BAY 60-6583 form a group of relatively new AR agonists possessing anti-inflammatory properties, however their antiplatelet effects have not been studied so far. The structure, binding affinities and physiological effects of those compounds are presented in Table 1.

The aim of this study was to evaluate and compare the anti-platelet action of new commercially-available AR agonists, and verify whether the inhibition of platelet function by selected $P2Y_{12}$ antagonists can be further enhanced by the application of AR agonists exhibiting an antiplatelet profile. The findings indicate that both non-selective (2-chloroadenosine) and A_{2A}-selective AR agonists (UK 432097, MRE 0094 and PSB 0777) were effective in enhancing platelet function inhibition *in vitro* by cangrelor and prasugrel metabolite.

2. Materials and methods

2.1. Chemicals

Adenosine receptor agonists were purchased from Tocris Bioscience (Bristol, UK) (BAY 60-6583, PSB 0777 and 2-chloroadenosine), Toronto Research Chemicals (Toronto, Canada) (MRE 0094) or Axon Medchem (Reston, VA, USA) (UK 432097). Cangrelor (AR-C69931MX) was from Cayman Chemical (Ann Arbor, MI, USA). Prasugrel metabolite (R-138727) was obtained from BOC Sciences (Shirley, NY, USA). Calcein AM was obtained from Molecular Probes (Eugene, OR, USA). Monoclonal antibodies anti-human CD61/PerCP, CD62/PE, PAC-1/FITC, mouse IgG1/PE isotype control, mouse IgG1/FITC isotype control, Cellfix, buffered sodium citrate was obtained from Becton-Dickinson (San Diego, CA, USA). Phosphate buffered saline (PBS) was obtained from Biomed Lublin (Lublin, Poland). Dimethyl sulfoxide (DMSO), adenosine diphosphate (ADP) were obtained from Sigma (St. Louis, MO, USA). All other chemicals, unless otherwise stated, were purchased from POCH (Gliwice, Poland).

2.2. Subjects

Human blood was obtained from healthy donors, who gave their written informed consent (35 men and 65 women; mean age 29 ± 10 years). All individuals confirmed not having taken medications known to influence platelet function (e. g. non-steroidal anti-inflammatory drugs, NSAIDs) for at least two weeks prior to the study. The study was approved by the Human Studies Committee of the Medical University of Lodz (Poland) and was conducted in accordance with the guidelines established by the Declaration of Helsinki.

2.3. Collection and processing of blood samples

Whole blood was collected into the polypropylene tubes with a blood to citrate ratio of 9:1 (vol:vol). Platelet-rich plasma (PRP) was prepared by 12-min of 190 × *g* centrifugation of a withdrawn blood at 37 °C. Platelet-poor plasma (PPP) was obtained by centrifuging PRP samples at 2000 x g for 15 min at 37 °C. Platelets in plasma were counted using photometry [38] and their count was adjusted to 2×10^8 cells/ml. To ensure that the washing procedure did not artefactually activate the platelets, the expression of P-selectin and active form of GPIIbIIIa in PRP was randomly monitored with the use of flow cytometry (CD62P/PE-positive and PAC-1/FITC objects within CD61/PerCP-gated objects) (FACSCanto II, Becton-Dickinson, Franklin Lakes, NJ, USA) [39].

2.4. Preparation of the solutions of AR agonists and $2PY_{12}$ antagonists

The 100 mM stock solutions and working solutions of PSB 0777, cangrelor or prasugrel metabolite were prepared in distilled water. The 100 mM stock solutions of BAY 60-6583, 2-chloroadenosine, MRE 0094 or UK 432097 were prepared in 100% DMSO. Stock solution of 2-chloroadenosine (100 mM in DMSO) was diluted with PBS to 5 mM working solution (5% DMSO). Stock solutions (100 mM in DMSO) of BAY 60–6583, MRE 0094 and UK 432097 were directly added to the target biological material (blood or PRP); the dilution factor was at least 1000-fold for the highest concentration of the AR agonist which yielded the maximal concentration of DMSO 0.1%. Generally, in all the assays, the final concentration of DMSO in the sample never exceeded 0.1%.

The antiplatelet activity of AR agonists was assessed at the concentration range of 0.1–100 μM; working concentration points of 0.1, 1, 10, 50 and 100 μM were chosen based on earlier reports [40,41]. The $P2Y_{12}$ antagonist concentrations were also chosen based on previous results [42,43]: 1–1000 nM for cangrelor and 0.025–20 μM for prasugrel metabolite. The inhibitory effects of AR agonists in the presence of

Table 1
Overall biological effects and affinities of the examined AR agonists towards adenosine receptors expressed in platelets.

Agonist name (selectivity)	Affinity		AR-mediated effects/Clinical trials	References
	A_{2A}	A_{2B}		
UK 432097 (A_{2A}-selective)	4 nM[a]	ND	Anti-inflammatory, anti-atherogenic Clinical Trials: Safety And Efficacy Of UK-432,097 In Chronic Obstructive Pulmonary Disease (NCT00430300)[c]	[18–20]
2-chloroadenosine (non-selective)	180 nM[a]	ND	Anti-platelet, cerebrovasodilatory, proapoptotic/anti-cancer, anti-convulsant, immunomodulatory, anti-proliferative/anti-vasoocclusive Clinical Trials: ND	[18,21–26]
MRE 0094 (A_{2A}-selective)	490 nM[a]	10,000 nM[b]	Wound repair, angiogenic Clinical Trials: Safety and Efficacy of MRE 0094 to treat Chronic, Neuropatic, Diabetic Foot Ulcers (NCT00312364, NCT00318214)[d]	[18,27,28]
PSB 0777 (A_{2A}-selective)	360 nM[a]	> 10,000 nM[b]	Anti-inflammatory Clinical Trials: ND	[29]
BAY 60-6583 (A_{2B}-selective)	> 10,000 nM[b]	3 nM[b]	Cardiopriotective, protective in ischaemic postconditioning, anti-inflammatory, protective in diabetic nephropathy, protumor activity, modulation of glucose homeostasis, vasorelaxant Clinical Trials: ND	[30–37]

Affinities were determined based on [a]binding data (K_i) or [b]functional assays (EC_{50}).
[c]Data were taken from https://pubchem.ncbi.nlm.nih.gov. [d]Data were taken from https://clinicaltrials.gov.

$P2Y_{12}$ antagonists were examined at the AR agonists concentrations corresponding to their IC_{50} values. When possible, the IC_{50} values for AR agonists were calculated based on the preliminary experimental data collected at the established concentrations (0.1–100 μM). In other cases, the IC_{50} values were determined after performing additional experiments with the use of additional concentration points within the range of 0.005 to 1000 μM, depending on the AR agonist.

2.5. Estimation of AR agonist solubility

The solubility of poorly water-soluble AR agonists in media with different concentrations of DMSO was evaluated using microscopic observation. Briefly, 100 μl of 5 mM working solutions of 2-chloroadenosine, MRE 0094, UK 432097 and BAY 60-6583 containing 5%–100% DMSO were mixed vigorously for 10 min at 37 °C. The solutions were then transferred into the wells of flat bottomed 96-well ELISA plates and mixed again for 10 min. Directly after mixing, the samples were observed under the AxioVert inverted microscope at ×20 magnification using an Axiocam digital camera with Zen 2.3 software (Carl Zeiss, Oberkochen, Germany).

2.6. Determination of platelet viability

Platelet viability in the presence of AR agonist was assessed in resting and activated platelets (20 μg/ml collagen, five minutes, RT) according to Rywaniak et al. [44]. The choice of collagen as platelet agonist was based on our observations indicating that stimulation of platelets with collagen, but not ADP, leads to a significantly increased fraction of calcein-negative cells (dead cells). Briefly, samples preincubated for 15 min at RT with a 100 μM AR agonist or 1% paraformaldehyde (PFA; a positive control) were diluted 10-fold with PBS pH 7.4, labeled with anti-CD61-PerCP antibodies (30 min, RT) and stained with 0.1 μM calcein AM (15 min, 37 °C). The percentage of calcein-negative platelets was measured immediately after staining using flow cytometry. In each sample 5000 events (CD61/PerCP-positive objects) were analysed with a FACSCanto II flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

2.7. Turbidimetric aggregometry

Platelet aggregation in PRP preincubated with AR agonists at concentrations of 0.1–100 μM (three minutes, RT) or with saline was determined using a dual-channel optical aggregometer (Chrono-Log Corp., Hovertown, PA). Platelet aggregation was monitored for 10 min in response to 10 μM ADP. Maximal aggregation was assessed with the use of the Platelet Aggregation Monitoring and Analysis software package [45]. Maximum aggregation (A_{max}) and AUC were the readout parameters included in the analysis.

2.8. Impedance aggregometry

Impedance aggregometry was used to assess the anti-platelet effect of AR agonists and $2PY_{12}$ antagonists in whole blood. The selected AR agonists were examined alone or in combination with $P2Y_{12}$ antagonists (cangrelor or prasugrel metabolite). The measurement procedure was performed following the instructions of the manufacturer. Briefly, 300 μl of blood preincubated with an AR agonist (at the concentrations within the range equivalent to half IC_{50}, IC_{50} and twice IC_{50}) alone (three minutes, RT) or simultaneously in combination with cangrelor (three minutes, RT) or prasugrel metabolite (15 min, RT) was added to 300 μl saline (0.9%) and preheated to 37 °C in the test cell for three minutes. Then, 10 μM ADP was added and platelet aggregation was recorded continuously for 10 min using a Multiplate analyzer (Dynabyte, Munich, Germany). The anti-aggregatory action of AR agonists was first evaluated over a specified concentration range (0.1–100 μM; experiments without $P2Y_{12}$ antagonists) and then at the concentrations equivalent to half IC_{50}, IC_{50} and twice IC_{50} (tests with $P2Y_{12}$ antagonists). Blood incubated with saline instead of AR agonists/$P2Y_{12}$ antagonists and further supplemented with 10 μM ADP to trigger platelet aggregation served as a control, and was performed independently in each series of measurements. Maximum aggregation (A_{max}) and area under the aggregation curve (AUC) were the readout parameters included in the analysis. All measurements were completed within three hours after blood collection.

2.9. Data analysis

The results are expressed as arithmetic mean ± SD or median with interquartile range (Me; LQ–UQ), depending on normality of data distributions and homogeneity of variances. The Shapiro-Wilk test and Levene's test were used to test the assumption of normal data distribution and homogeneity of variances. Sphericity was verified with

Mauchley's test. The effect of tested compounds on platelet function was estimated with the pairwise Student's *t*-test, Wilcoxon's signed ranks test, two-way ANOVA with repeated measures or Friedman's test and the *post hoc* Dunnett's or Bonferroni's multiple comparisons test. Compounds demonstrating a significant anti-platelet effect were tested across an expanded concentration range and a nonlinear regression curve fit to determine their potency (IC_{50}).

All the analysed compounds were subjected to four-parametric dose response (inhibition) curves, with the estimation of bottom, top, slope and IC_{50}. The significance of the differences in IC_{50} values between PRP and whole blood was estimated with the F-test. Regression analysis was used to determine the relationship between compound concentration and cell response. The coefficient of determination (R square) and standard deviation of the residuals were used to determine the goodness of fit of the non-linear regression curves for AR agonists and $P2Y_{12}$ antagonists analysed alone.

Overall assessment of AR agonists was performed by summing up the normalized values (the van der Waerden score method) calculated for three examined variables: (i) the concentration of AR agonist required for 50% platelet inhibition (in practice, we used the inverse IC_{50} value; $1/IC_{50}$), (ii) maximum inhibition at 100 μM (percent) and (iii) solubility (the maximum possible water content (%) in 5 mM working solution of AR agonist, which did not contain crystals). In addition, due to the relatively small sample sizes and the low statistical power of numerous estimated inferences and associations, the resampling bootstrap technique (1000–10,000 iterations) was used to ensure that the revealed differences and associations were not observed due to pure chance. The statistical analysis was performed using the following software packages: Statistica v.13, StatsDirect v.2.8.0, GraphPad Prism v.5.

3. Results

3.1. Characteristics of AR agonists in terms of solubility

At the concentration range of 5%–100% DMSO, the compounds considerably differed in solubility. 2-chloroadenosine, which is structurally closest to adenosine, appears to be the second most soluble compound in water-based media (after the water-soluble PSB 0777, data not shown): it was fully soluble in 5% DMSO at the concentration of 5 mM. Otherwise, UK 432097 and BAY 60-6583, the most extensively modified adenosine derivatives, were the least soluble agents; they were fully dissolved only in solvents containing high DMSO concentrations, respectively 50% and 90% DMSO; precipitated crystals appeared in the working solutions prepared with the use of lower DMSO concentrations (Fig. 1).

3.2. Effects of AR agonists on cell viability

Platelet viability in the presence of AR agonists was measured to exclude any potential cytotoxic effects of the examined compounds, which might have affected the results of functional tests. Viability assay was performed on resting and agonized platelets and on AR agonists which exhibited anti-platelet potential (UK 432097, 2-chloroadenosine, MRE 0094 and PSB 0777). To study platelet activation, 20 μg/ml collagen was used to achieve maximal cell response. Results showed that, regardless of the experimental model, 1% PFA (positive control) considerably decreased platelet viability: the fraction of viable cells was decreased by over 74% in resting platelets ($P < .0001$) and by 72% in collagen-stimulated platelets ($P < .0001$). However, none of the AR agonists exerted cytotoxic effects on resting and collagen-stimulated platelets (Fig. 2).

3.3. Anti-aggregatory effect of AR agonists in platelet-rich plasma

The washing procedure for the preparation of PRP did not influence platelet activation as the levels of platelet activation markers in blood and PRP did not exceed 3% (n = 4). The average A_{max} and AUC values in PRP stimulated with 10 μM ADP were respectively 83 ± 10% and 720 ± 86 AU, n = 23. All examined AR agonists but BAY 60–6583 exhibited inhibitory properties on ADP-induced platelet aggregation measured in PRP (Supplementary Fig. 1). At the concentration range of 0.1–100 μM, UK 432097 and PSB 0777 were, respectively, the most and the least effective compounds at reducing platelet aggregation in response to 10 μM ADP. However, at 100 μM, a similar platelet response to UK 432097, 2-chloroadenosine and MRE 0094 was observed (AUC was decreased by 88.0 ± 6.5%, 89.0 ± 5.9% and 82.4 ± 8.8% respectively, $P < .001$, n = 5). Moreover, UK 432097, 2-chloroadenosine and MRE 0094 significantly decreased platelet aggregation at 10 μM, whereas PSB 0777 effectively inhibited platelet aggregation only at the highest-used concentration (decrease in AUC by 23.0 ± 15.5%, $P < .01$, n = 5) (Supplementary Fig. 1). The examples of aggregation curves is shown in Supplementary Fig. 2.

The extent of platelet response decreased with the increasing concentrations of AR agonists. Regarding AUC values, the coefficients of determination for the four-parametric dose response (inhibition) curves of UK 432097, 2-chloroadenosine, MRE 0094, PSB 0777 and BAY 60-6583 were: $R^2 = 0.732$, $R^2 = 0.927$, $R^2 = 0.612$, $R^2 = 0.634$ and $R^2 = 0.309$.

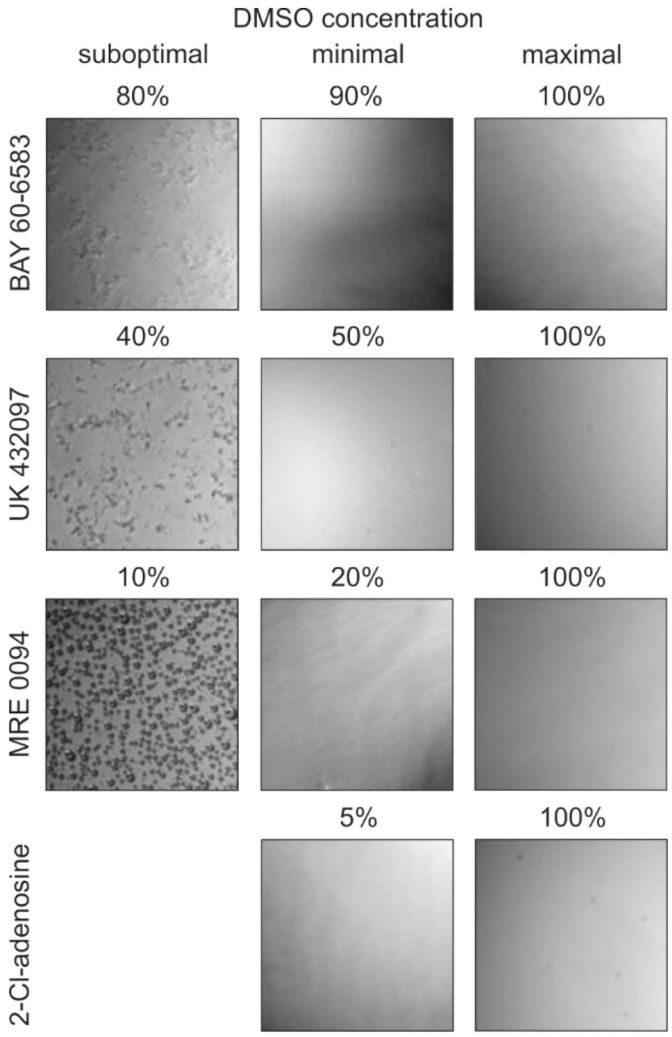

Fig. 1. Example images are presented for the tested compounds. Microscopic observation was conducted at 20-fold magnification in 5 mM working solutions of BAY 60-6583 UK 432097, MRE 0094 and 2-chloroadenosine containing 5%–100% DMSO.

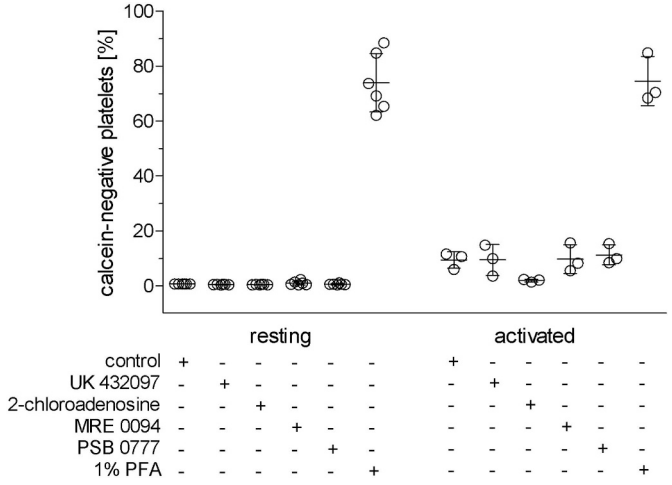

Fig. 2. Influence of AR agonists on platelet viability. Data are shown as mean ± SD (n = 3–6). Viability of resting and collagen-activated platelets following their incubation with 100 μM UK 432097, 2-chloroadenosine, MRE 0094 or PSB 0777 (15 min, RT) was assessed in whole blood using 0.1 μM calcein AM. Samples treated with 1% PFA served as positive controls. Significance of differences was estimated with ANOVA with repeated measures and the *post-hoc* Dunnett's multiple comparisons test: Resting platelets: $\mu_{control} \neq \mu_{1\%\ PFA}$, $P < .0001$.
Collagen-stimulated platelets: $\mu_{control} \neq \mu_{1\%\ PFA}$, $P < .0001$.

3.4. Anti-aggregatory effect of AR agonists and $P2Y_{12}$ antagonists in whole blood

The average A_{max} and AUC values in whole blood stimulated with 10 μM ADP were respectively 117 ± 23 AU and 136 ± 30 U, n = 23. Preincubation of whole blood with UK 432097, 2-chloroadenosine, MRE 0094 or PSB 0777 at the concentrations within the range of 0.1–100 μM led to the inhibition of ADP-stimulated platelet aggregation (Supplementary Fig. 1). UK exhibited the strongest anti-platelet properties, diminishing platelet aggregation between 37% at 1 μM and 79% at 100 μM (calculations based on the raw data of AUC, n = 5). For comparison, the maximum inhibitory effects of 2-chloroadenosine, MRE 0094 and PSB 0777 on platelet aggregation at 100 μM were respectively: 70%, 53% and 40% (calculations were performed on the raw data of AUC). BAY 60-6583, unlike the remaining compounds, stimulated ADP-induced platelet aggregation. Incubation of whole blood with 50 μM and 100 μM BAY 60-6583 did not result in significantly decreased ADP-induced platelet aggregation (Supplementary Fig. 1).

Platelet response depended on AR agonist concentration. Regarding AUC values, coefficients of determination for the four-parametric dose response (inhibition) curves of UK 432097, 2-chloroadenosine, MRE 0094, PSB 0777 and BAY 60-6583 were $R^2 = 0.686$, $R^2 = 0.926$, $R^2 = 0.359$, $R^2 = 0.597$ and $R^2 = 0.217$.

Preincubation of whole blood with 10 nM cangrelor or with 3 μM prasugrel metabolite led to the statistically-significant inhibition of platelet aggregation in response to 10 μM ADP. The concentration of cangrelor required for half maximal inhibition of ADP-stimulated platelet aggregation was calculated to be 17.8 nM for A_{max} and 16.1 nM for AUC (n = 5), whereas the IC_{50} value of prasugrel metabolite was estimated to be 1.3 μM (for both A_{max} and AUC, n = 7) (Fig. 3). Based on AUC values, the coefficients of determination for cangrelor and prasugrel metabolite were respectively, $R^2 = 0.941$ and $R^2 = 0.868$.

3.5. Comparison of AR agonist effects on ADP-induced platelet aggregation measured in whole blood and plasma

Effectiveness of AR agonists in the inhibition of ADP-induced platelet aggregation, depending on whether measured in whole blood or PRP, was evaluated by comparing the relevant IC_{50} values. To calculate the IC_{50} values for each AR agonist, they were tested at concentrations ranging from 0.005 to 1000 μM, depending on the compound. The IC_{50} of BAY 60-6583 was not determined, since this compound did not exert any distinct anti-platelet properties. As shown in Fig. 4, the IC_{50} values of the examined AR agonists in whole blood varied from 0.4–fold to 4.5-fold in comparison to PRP; however, no significant differences were revealed between the groups.

3.6. Influence of AR agonist on $P2Y_{12}$ antagonist-mediated inhibition of platelet aggregation

At their IC_{50} concentrations, cangrelor (17 nM) and prasugrel metabolite (1.3 μM) reduced platelet aggregation in whole blood on average respectively by 29%–56% and 27%–43%, depending on the AR agonist involved (Fig. 5). Adenosine receptor agonists used alone, at concentrations in the range equivalent to half IC_{50}, IC_{50} and twice IC_{50}, inhibited platelet aggregation by 42%–53% (UK 432097), 17%–32% (2-chloroadenosine), 18%–39% (MRE 0094) and 23%–33% (PSB 0777). All the tested AR agonists significantly enhanced platelet response to cangrelor and/or prasugrel metabolite. Depending on the used AR agonist concentration, the inhibition of platelet aggregation caused by cangrelor was augmented by 67%–81% (UK 432097, $P < .01$ or less), 33%–42% (2-chloroadenosine, $P < .01$), 45%–53% (MRE 0094, $P < .01$ or less) and 31%–35% (PSB 0777, $P < .05$). UK 432097, 2-chloroadenosine, MRE 0094 and PSB 0777 further deepened the prasugrel metabolite-induced reduction of platelet aggregation respectively by: 42%–60% ($P < .01$ or less), 29–35% ($P < .05$), 11%–18% (NS) and 46%–51% ($P < .001$) (Fig. 5).

3.7. Overall assessment of AR agonists

The overall evaluation of AR agonists included three examined parameters: the inverse IC_{50} value ($1/IC_{50}$; values obtained in whole blood aggregation for AUC parameter), the inhibition estimated at the highest tested concentration (100 μM) and solubility (the maximum possible water content in 5 mM working solutions of AR agonists). Based on the overall score, 2-chloroadenosine and MRE 0094 were found to be, respectively, the most and least powerful AR agonists in terms of IC_{50}, anti-platelet potential at 100 μM and agent solubility (Table 2).

4. Discussion

Targeting multiple platelet activation pathways is a promising strategy to develop effective anti-platelet therapy. In this study we investigated whether the inhibition of platelet function mediated by $P2Y_{12}$ antagonists can be potentiated by adenosine receptor (AR) agonists. The effect of AR agonists on platelet function is also evaluated in relation to their toxicity, specificity towards adenosine receptor subtypes, structure and solubility. The main message of the study is that both non-selective (2-chloroadenosine) and A_{2A}-selective AR agonists (UK 432097, MRE 0094 and PSB 0777) are effective in the enhancement of the inhibition of platelet function by $P2Y_{12}$ antagonists, such as cangrelor and prasugrel metabolite and that, at the same time, they remain non-cytotoxic to the cells.

Activation of adenosine receptors is known for its cytoprotective role in tissues. It may produce bradycardia, mediate vasodilation and ischemic preconditioning, inhibit neurotransmitter release, suppress apoptosis and reduce activation of platelets and neutrophils, thus preventing initiation and further development of thrombosis [15]. In this *in vitro* aggregation-based study, we examined the anti-platelet effects of five commercially-available AR agonists used alone and in a combination with $P2Y_{12}$ antagonists (cangrelor or prasugrel metabolite). Since only two subtypes of adenosine receptors, A_{2A} and A_{2B}, are expressed on human platelets, the chosen compounds were either non-

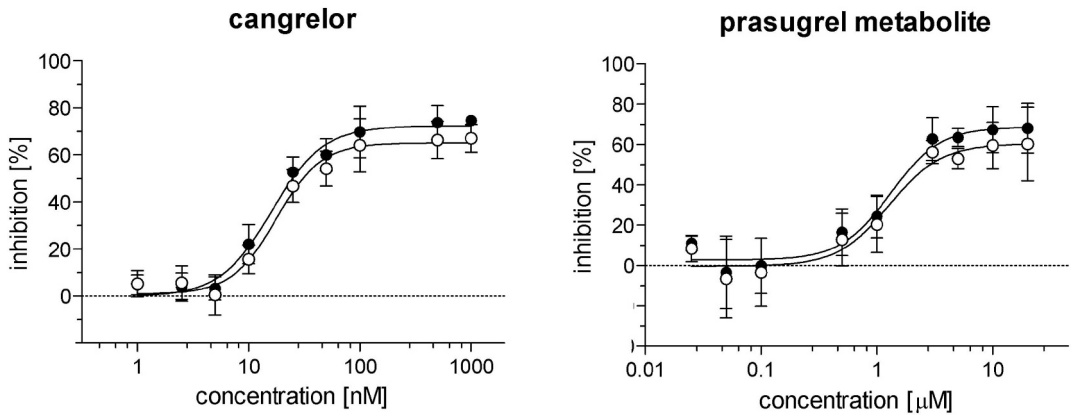

Fig. 3. Inhibition of whole blood platelet aggregation by $P2Y_{12}$ antagonists. Data are shown as mean ± SD (n = 5–7). Changes in platelet aggregation were measured in whole blood in response to 10 μM ADP after preincubation of whole blood with cangrelor (1–1000 nM) or prasugrel metabolite (0.025–20 μM). The IC_{50} values were calculated from dose-response plots based on the values of Amax (○) or AUC (●) using non-linear regression analysis.

selective (2-chloroadenosine), A_{2A}-selective (UK 432097, MRE 0094, PSB 0777) or A_{2B}-selective (BAY 60-6583) AR agonists. Within the concentration range of 0.1–100 μM, only non-selective and A_{2A}-selective AR agonists were effective in the inhibition of ADP-induced platelet aggregation, and the platelet response to those AR agonists was concentration dependent. Changes of platelet aggregation in the presence of the A_{2B}-selective AR agonist BAY 60-6583, which has been described as a specific and high-affinity (EC_{50} of 3 nM) A_{2B} agonist [32], were noticeable at 50–100 μM (a slight increase in platelet aggregation in whole blood and a small decrease in platelet aggregation in PRP), but these changes remained not significant. Lack of platelet response to BAY 60-6583 could be explained in part by the earlier overestimation of BAY 60-6583 affinity to A_{2B} receptors, as previously suggested [46]. Furthermore, even if A_{2A} and A_{2B} adenosine receptors mediate the same effects in platelets, the latter is regarded as a low affinity receptor and therefore could be important under states of high AR agonist concentration or in the case of increased A_{2B} AR density occurring upon systemic inflammation or vascular injury [13]. Noteworthy, *in vivo* platelets have a significantly smaller density of A_{2B} AR compared to A_{2A} AR [12].

The observation of blocking of platelet aggregation by 2-chloroadenosine was not surprising, because it is one of the oldest known adenosine analogs, the anti-platelet activity of which has been widely described [22,47]. Apart from anti-platelet potential, the purine analogs 2-chloroadenosine and 2-chlorodeoxyadenosine can induce apoptosis in human cells [48–50], and perhaps this is one of the reasons why 2-chloroadenosine is not considered to be of potential interest as an anti-platelet agent.

UK 432097, MRE 0094, PSB 0777 and BAY 60-6583 form a group of relatively new AR agonists with anti-inflammatory abilities and, contrary to 2-chloroadenosine, their effects on platelet function have not been elucidated so far. In addition, some of them, like UK 432097 and MRE 0094, have been tested in clinical trials (Table 1) [19,29,33,51]. Within the concentration range of 1–100 μM, UK 432097 was found to be the most effective examined agent in the inhibiting of the ADP-induced platelet aggregation in both PRP and whole blood (Supplementary Fig. 1). Furthermore, UK 432097 demonstrated the highest anti-platelet activity, which corresponded to its high receptor affinity, estimated at 4 nM (Table 1). 2-chloroadenosine, possessing lower affinity to adenosine receptors (180 nM), appeared a less potent AR agonist than UK 432097, because it did not suppress ADP-dependent platelet aggregation at lower concentrations. On the other hand, 2-chloroadenosine had much better solubility in water than UK 432097 and overall, in terms of the ranking of anti-platelet activity and solubility, it turned out the most powerful AR agonist, *i.e.* with the highest overall score (Table 2). Our experiments with AR agonists in whole blood and PRP showed indirectly that the examined AR agonists do not undergo rapid or excessive uptake and metabolism by erythrocytes as is the case for endogenous adenosine [52]: UK 432097, MRE 0094, PSB 0777, together with 2-chloroadenosine demonstrated similar abilities to prevent platelet aggregation, in either PRP or whole blood. It could be also inferred that red and white blood cells have rather little impact on the effectiveness of AR agonists in hampering platelet aggregation since the IC_{50} values obtained for a given AR agonist by turbidimetric and impedance assay did not differ significantly from each other. Recently, the anti-platelet activity of some other AR agonists has been demonstrated. A series of novel PSB derivatives (PSB 15826, PSB 16301 and PSB 12404; A_{2A}-selective) upregulated cAMP levels in platelets and exhibited strong inhibition of ADP-induced platelet activation and platelet aggregation in response to ADP, TRAP-6 or convulxin. Importantly, their effects were established in human platelet-rich plasma or in suspensions of isolated platelets, but not in whole blood [41].

In our observations, UK 432097, 2-chloroadenosine, MRE 0094 and PSB 0777 were effective in preventing platelet aggregation when used alone or in a combination with cangrelor or prasugrel metabolite (Fig. 5). The latter proved for the first time the adjunctive role of AR agonists in anti-platelet therapy with the use of $P2Y_{12}$ receptor inhibitors. In 2012, Pan et al. investigated the separate effects of adenosine derivative – BF066 (which inhibits platelet function *via* adenosine A_{2A} receptor) and clopidogrel on thrombus formation in mouse mesenteric arterioles *in vivo* [2]. It has been shown that BF066, administered intravenously to mice at a dose of 29 mg/kg (a single bolus), had similar anti-thrombotic efficacy as clopidogrel administered *per os* at a dose of 30 mg/kg (once daily for two days) [2].

Given dual anti-platelet therapy and considering the short half-life of endogenous adenosine in circulation, due to its rapid cellular uptake, two more reports deserve mention. It has been found that some anti-platelet agents, such as ticagrelor, may have a dual mode of action: $P2Y_{12}$ antagonism and inhibition of adenosine uptake by erythrocytes and other cells. With regard to the latter, ticagrelor most likely inhibits the adenosine transporter ENT1 expressed on blood cells resulting in increased plasma adenosine level and inhibition of platelet function. The dual mechanism of action by ticagrelor seems to give it a huge advantage over other anti-platelet agents because its action is not restricted to platelets, but also concerns the adenosine-mediated effects, like coronary vasodilation [53,54]. The above results encourage further investigations with the use of AR agonists, not only in the field of cellular uptake, but also in terms of absorption, metabolism, distribution and elimination. Such studies are important in the drug discovery process – in conjunction with biological efficacy they may identify compounds biologically active, selective with favorable pharmacokinetic and toxicological profile.

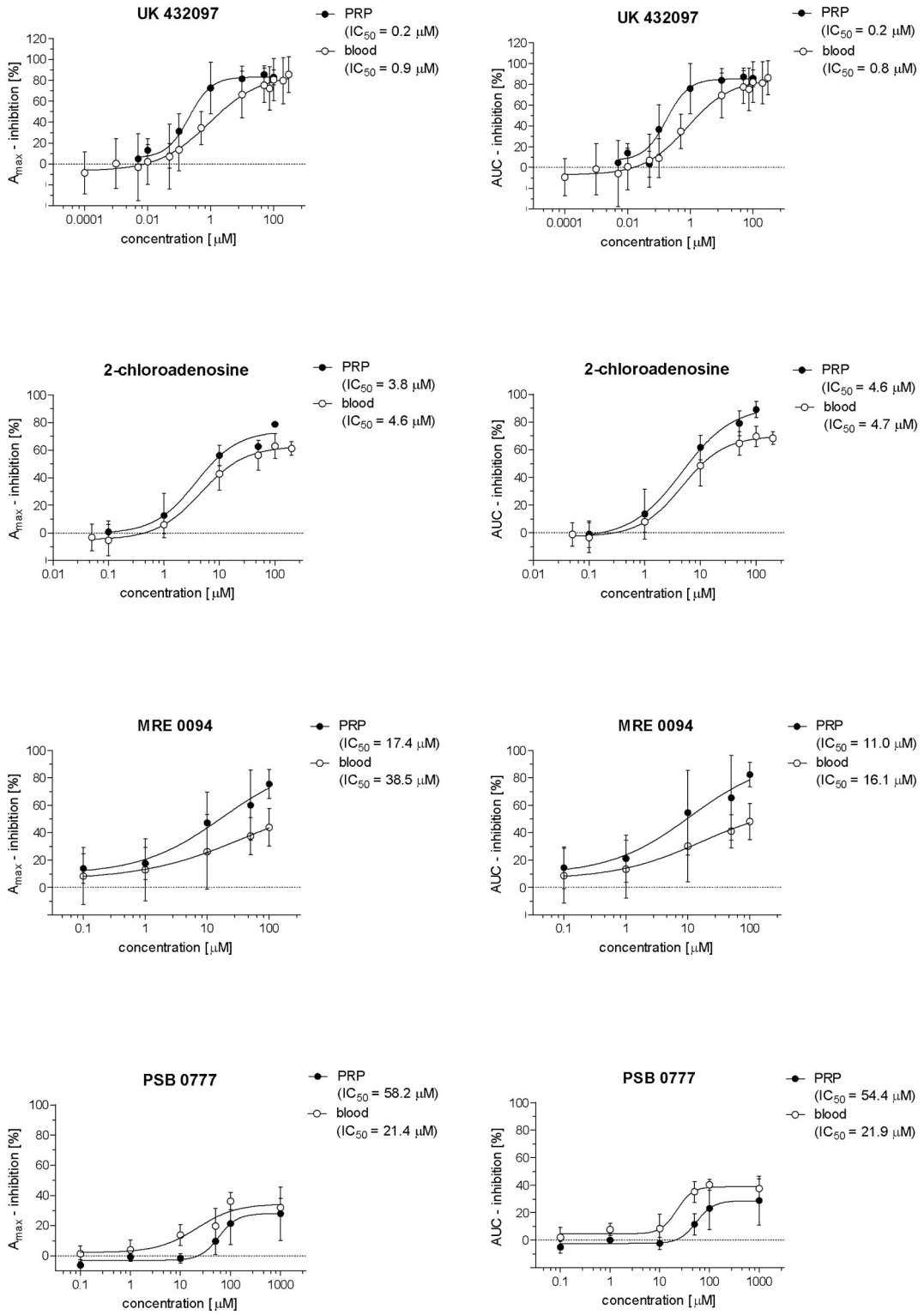

Fig. 4. Comparison of inhibition curves produced by AR agonists. Data are shown as mean ± SD (n = 5–9). Changes in ADP-induced platelet aggregation were measured in PRP (●) or whole blood (○) after preincubation with UK 432097, 2-chloroadenosine, MRE 0094 or PSB 0777. The range of concentrations was from 0.005 to 1000 μM, depending on compound. The IC_{50} values were calculated from dose-response plots based on the values of Amax (the left column) or AUC (the right column) using non-linear regression analysis. Statistical differences between PRP and whole blood in IC_{50} values estimated by F-test were not observed.

5. Final remarks and conclusions

Platelet aggregation monitored in the presence of AR agonists was described with two parameters: maximum aggregation and area under curve (AUC). However, the study is based mainly on AUC value because this is considered the best parameter to reflect overall platelet aggregation (as it is affected by both velocity and maximum aggregation). Furthermore, irrespective of the parameter used for evaluation of platelet aggregation, we observed high variation in data obtained in the experiments with AR agonists. At first, we thought that such a high

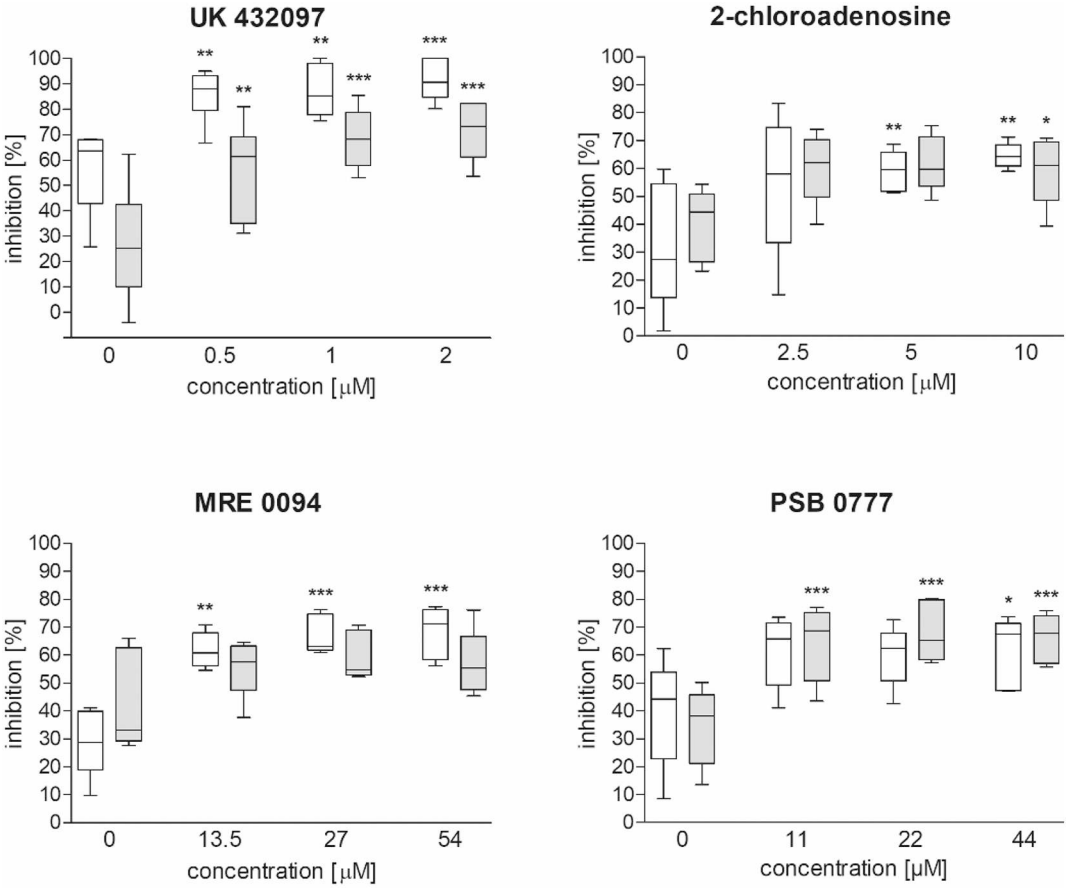

Fig. 5. Effect of AR agonists on the inhibition of ADP-induced platelet aggregation mediated by $P2Y_{12}$ antagonists. Data are given as median, interquartile range and minimum and maximum values (n = 5–6). Platelet aggregation was measured in whole blood stimulated with 10 μM ADP after incubation with cangrelor (white boxes) or prasugrel metabolite (grey boxes) alone or with combination with AR agonist at the concentrations equivalent to half of IC_{50}, IC_{50} and doubled IC_{50}. Significance of differences was estimated for AUC with ANOVA with repeated measures and or Friedman's test and the *post-hoc* Dunnett's or Bonferroni's multiple comparisons test:
Cangrelor (white boxes) - UK 432097: $\mu_{control} \neq \mu_{0.5-2\ \mu M}$ ($P < .01$ or lower); 2-chloroadenosine: $\mu_{control} \neq \mu_{5-10\ \mu M}$ ($P < .01$); MRE 0094: $\mu_{control} \neq \mu_{13.5-54\ \mu M}$ ($P < .01$ or less); PSB 0777: $\mu_{control} \neq \mu_{44\ \mu M}$ ($P < .05$).
Prasugrel metabolite (grey boxes) - UK 432097: $\mu_{control} \neq \mu_{0.5-2\ \mu M}$ ($P < .01$ or lower); 2-chloroadenosine: $\mu_{control} \neq \mu_{10\ \mu M}$ ($P < .05$); MRE 0094: $\mu_{control} \neq \mu_{13.5-54\ \mu M}$ (NS); PSB 0777: $\mu_{control} \neq \mu_{11-44\ \mu M}$ ($P < .001$).

variation could be a result of the presence of DMSO in the system, but it does not seem likely, because in the study with PSB 0777, which was the only AR agonist fully soluble in water, we achieved variation comparable with the remaining AR agonists (data not shown). Another, far more likely, possibility is that the high variation in platelet response to AR agonists may be associated with nature of AR-ligand interactions, *i.e.* with activation of adenosine receptors (unlike $P2Y_{12}$ antagonists), leading to inhibition of platelet function. The latter could also be related to individual AR density on platelet surface and/or a relatively short time of incubation of platelets with AR agonists.

The majority of known AR agonists are poorly water soluble, thus the work with these compounds requires a detailed analysis of solubility in order to achieve real solutions. At such low concentrations, corresponding to the basal level of adenosine in the extracellular space

Table 2
Ranking of AR agonists according to 1/IC50, maximal inhibition at 100 μM and solubility in water.

AR agonist	$1/IC_{50}$ [μM^{-1}]	max inhibition [%]	solubility [%]	comprehensive score
2-chloroadenosine	0.213 (0.176; 0.234)	70.1 (67.8; 72.7)	96.2 ± 1.8	1.420 (1.342; 1.479)
PSB 0777	0.045 (0.044; 0.047)	40.7 (38.5; 43.1)	100.0 + 2.0	−0.188 (−9273; 0.175)
UK 432097	0.115 (−0.677; 3.309)	74.6 (69.3; 95.7)	52.4 ± 1.9	−0.134 (−0.894; 0.128)
MRE 0094	0.038 (−0.022; 0.168)	47.6 (39.0; 68.7)	82.3 ± 2.1	−0.953 (−1.212; −0.921)

Data presented as mean + SD or median with interquartile range (Me; LQ [25%]; UQ [75%]); n = 10. Normal scores were calculated raw or Box-Cox-transformed data using van der Waerden algorithm (see 'Materials and methods') and summarized to give the overall comprehensive normal scores. Significance of differences estimated with the bootstrap-boosted one-way ANOVA or the bootstrap-boosted Kruskal-Wallis test, followed resp. by Tukey's test or Conover-Inman test for post-hoc multiple comparisons:
$1/IC_{50}$: $P = .0206$ by bootstrap-boosted Kruskal-Wallis test; n.s., $m_{2\text{-chloroadenosine}} = m_{UK\ 432097} = m_{MRE\ 0094} = m_{PSB\ 0777}$.
max inhibition: $P < .0001$ by bootstrap-boosted Kruskal-Wallis test; $P < .0005$, $m_{UK\ 432097} > m_{2\text{-chloroadenosine}} = m_{MRE\ 0094} = m_{PSB\ 0777}$.
solubility: $P < .0001$ by bootstrap-boosted one-way ANOVA; $P < .0005$, $m_{PSB\ 0777} > m_{2\text{-chloroadenosine}} > m_{MRE\ 0094} > m_{UK\ 432097}$.
comprehensive score: $P < .0001$ by bootstrap-boosted Kruskal-Wallis test; $P < .0001$, $m_{2\text{-chloroadenosine}} > m_{UK\ 432097} = m_{PSB\ 0777} > m_{MRE\ 0094}$.

(30–200 nM), only one of all the examined AR agonists, UK 432097, was able to significantly affect platelet function.

Adenosine receptors, in particular A_{2A} receptors, are highly expressed on various body cells, so that they have been suggested as potential targets in a wide variety of pathophysiological conditions, including inflammation, arrhythmia, ischaemia, angiogenesis, cancer [10]. However, the systemic use of A_{2A} adenosine receptor agonists as anti-inflammatory drugs is limited by their potent hypotensive activity caused by the activation of A_{2A} adenosine receptors expressed in heart and blood vessels. At present, a local therapy at sites of inflammation with the use of A_{2A} AR agonists seems to be a promising therapeutic approach; it could reduce the inflammation without undesirable cardiovascular effects like hypotension [29,51].

All the examined AR agonists, with the exception of BAY 60–6583, were more or less effective in the potentiation of the inhibition of platelet function by $P2Y_{12}$ antagonists. The A_{2A} AR agonist UK 432097 has been identified as the most potent anti-aggregatory agent, able to inhibit platelet function at nanomolar concentrations. Our results support the idea of dual anti-platelet therapy; they indicate the importance of platelet adenosine receptors as therapeutic targets and the potential benefits of therapeutic use of combined therapy of $P2Y_{12}$ antagonist and AR agonist in cardioprotection.

Acknowledgements

The authors dedicate the work to the memory of Professor Gustav V.R. Born (1921–2018), the prominent pharmacologist, who invented and developed the technique of light transmission aggregometry. The authors wish to thank M. Jóźwiak (MSc) for his initial participation in the protocol setting and his contribution to the experimental work. This work was supported by the project “Dual purinoreceptor-dependent approach to prevent thromboembolic events dependent on blood platelets and endothelium – approaches based on animal and cellular models” carried out within the TEAM programme (TEAM/2016-1/8) of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund. This work was supported by a grant from Medical University funds: 502-03/6-020-01/502-64-127-18.

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2018.11.005.

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