DOCTORAL THESIS

Adenosine receptor agonists and $P2Y_{12}$ receptor antagonists exhibit combined inhibitory effect on blood platelet function

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Łódź, 2020

Acknowledgements

This work was funded by TEAM/2016-1/8 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 of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund.

This work was also supported by ETIUDA7 doctoral scholarship “Adenosine receptor agonists and $P2Y_{12}$ receptor antagonists exhibit combined inhibitory effect on blood platelet function” funded by National Science Centre, Poland (2019/32/T/NZ3/00333), and Medical University of Lodz grant for young scientists (502-03/6-020-01/502-64-125-18).

The research presented in this dissertation is a result of a collaboration. I am immensely grateful for the help and genuine kindness of many of my colleagues and co-workers. I am especially thankful that not only could I contribute to scientific knowledge under the guidance of more experienced colleagues, but also for the privilege of working alongside them. I was very lucky to collaborate with a wonderful group of PhD students and post-docs who made my research both productive and enjoyable.

I would like to especially thank my supervisor Marcin Różalski, PhD for his patience, guidance and support. I would also like to thank Bogusława Luzak, PhD and Tomasz Przygodzki, PhD for their sympathetic ear, invaluable advice and generosity.

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List of abbreviations

ADP	Adenosine Diphosphate
AR	Adenosine Receptor
cAMP	Cyclic Adenosine Monophosphate
HE-NECA	2-Hexynyl-50-(N-ethylcarboxamido)adenosine
IC_{50}	Half maximal inhibitory concentration
NECA	50-(N-ethylcarboxamido) adenosine
PRP	Platelet-rich plasma

I. Introduction

Blood platelet activation is a crucial element of natural blood clotting mechanisms, and plays a pivotal role in the pathogenesis of arterial thrombotic diseases, like coronary heart disease, myocardial infarction, and stroke. Thrombosis related conditions, especially heart diseases, are currently the primary cause of mortality in developed countries. Antiplatelet therapy is one of the most important tools in the treatment of arterial thrombotic disorders (Kaplan and Jackson, 2011), especially as novel research points to the increasing possibility of antiplatelet treatment combating thrombosis without damaging the functioning of the coagulation cascade (Nickel *et al.*, 2015; Vara *et al.*, 2020).

Although multiple antiplatelet drugs are now routinely used in clinical settings, the effective therapy of arterial thrombosis still poses a problem. The available treatments are burdened with a high incidence of severe side effects, such as gastrointestinal bleeding (observed in over 10% of patients treated with oral antiplatelet drugs, and many of those patients requiring recurrent hospitalizations (Delaney *et al.*, 2016)) or significant risk of intracranial and intracerebral haemorrhage (Khan *et al.*, 2017). On the other hand, for some patients, the applied antiplatelet therapy is insufficient and fails to provide a protective effect.

The problem described above stems mainly from the fact that currently used antiplatelet agents either interfere with only one of several pathways of platelet activation, therefore being insufficient to effectively block platelet activation, or robustly blocks the final common step of platelet aggregation, such as fibrinogen binding (blockers of fibrinogen receptor), causing a high risk of bleeding (Patrono *et al.*, 2017). A compounding problem is the fact that individual variability of the dose-response resulting from both environmental and genetic factors, especially in case of prodrugs is very high in case of antiplatelet interventions (Rozalski *et al.*, 2005a). Altogether, there still is a high demand for novel platelet inhibitors with improved efficacy and safety profiles, or alternatively, a combined therapy based on the application of currently-available agents.

Platelets express two ADP receptors: the $P2Y_1$ receptor (initiates platelet aggregation) and the $P2Y_{12}$ receptor (enhances this process, finally leading to thrombus formation; full activation is required for sustained platelet aggregation) (Rozalski *et al.*, 2005b). $P2Y_{12}$ receptor is almost exclusively expressed in the platelets while $P2Y_1$ receptor mRNA

is detectable in most human tissues (Janssens *et al.*, 1996). $P2Y_{12}$ has therefore become the major therapeutic target. Most clinically approved $P2Y_{12}$ inhibitors are the thienopyridine-class inhibitors (ticlopidine, clopidogrel, and prasugrel) or the ATP analogues (cangrelor, ticagrelor) (Patrono *et al.*, 2017; Kupka and Sibbing, 2018). Thienopyridines are prodrugs converted to short-lived active metabolites - which irreversibly inhibit this receptor and prevent ADP-induced platelet activation. Cangrelor is the first reversible intravenous $P2Y_{12}$ receptor inhibitor that non-competitively blocks ADP signalling. Ticagrelor acts directly via reversible binding to the $P2Y_{12}$ receptor, causing non-competitive inhibition of ADP-induced activation. It is used in the treatment of patients with acute coronary syndrome to prevent recurrent thromboembolic events (Patrono *et al.*, 2017; Kupka and Sibbing, 2018). Clopidogrel, prasugrel, and ticagrelor are the most frequently used oral platelet $P2Y_{12}$ inhibitors in the modern medical practice; the use of numerous previously accepted drugs (e.g. ticlopidine) has been abandoned. Clopidogrel is the only oral $P2Y_{12}$ inhibitor currently recommended for the treatment of stable coronary artery disease. These three drugs have been indicated for use in acute coronary syndromes but the application of prasugrel and ticagrelor is preferred over clopidogrel, because of their superior clinical benefits - including improved efficacy, lower individual variation in response, and less frequent and severe side effects (Angiolillo *et al.*, 2017). Cangrelor, approved in 2015, is the most promising in percutaneous coronary interventions (Tyler *et al.*, 2016).

Adenosine is an important purine metabolite, not only a component of nucleic acids and ATP - the most important energy carrier in the cell, but also a signalling molecule (Fredholm, 2010; Chen *et al.*, 2013b). Adenosine receptors (AR) constitute a subfamily of highly-conserved G-protein coupled receptors expressed intracellularly and on the membranes of most types of human cells and performing various physiological functions. Blood platelets express two (A_{2A} and A_{2B}) of the four known adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3; the subtype distribution being highly tissue-specific). A_{2A} receptor is believed to be expressed on platelets at a much higher density than A_{2B} receptor (Koupenova and Ravid, 2018), even though only one study has estimated the gene expression profile for A_{2A} and A_{2B} receptors in human platelets, in fact finding comparable mRNA expression levels for A_{2A} and A_{2B} AR (Amisten *et al.*, 2008). No further evidence exists regarding protein levels present on the platelet surface, however, two independent studies (Burkhart *et al.*, 2012; Wijten *et al.*, 2013) have failed to quantify A_{2A} AR in platelet proteome,

while easily identifying other receptors, such as $P2Y_{12}$ (known to be expressed on healthy platelets at around 450–1,000 copy number (Ohlmann *et al.*, 2013)), therefore indirectly indicating an overall low copy number of these type of receptors present on the platelet surface.

It is important to note that the activation of A_{2A} and A_{2B} receptors has known systemic outcomes, such as coronary artery vasodilatation, decreased dopaminergic activity in the central nervous system, inhibition of central neuron excitation, and bronchospasm (Ongini and Fredholm, 1996; Cristalli *et al.*, 2003; Fredholm, 2010; Federico and Spalluto, 2012), therefore all adenosine receptor ligands should be used with the utmost caution (Chen *et al.*, 2013a), despite some having already been approved for human use (Murray *et al.*, 2009; Townsend *et al.*, 2017).

A_{2A} AR was identified as a mediator of adenosine inhibition of platelet aggregation, and therefore is a receptor crucial for proper platelet function maintenance (Müller and Scior, 1993). It acts through the inhibition of internal calcium stores mobilization and influx of external calcium, both linked to activation of adenylate cyclase and increase of cAMP concentration (and therefore platelet activation inhibition) (Paul *et al.*, 1990). Cyclic nucleotides also strongly inhibit the release of calcium ions into the cytosol, necessary for many events in platelet activation. The activation of A_{2A} AR by specific agonists was alsoreported to reduce P-selectin surface expression in thromboxane A2 or ADP stimulated platelets (Sheth *et al.*, 2014). The role of A_{2B} AR in platelets remains to be fully elucidated. It was proposed that it may activate signal transduction pathways other than adenylate cyclase related ones (Livingston *et al.*, 2004).

The half-life of adenosine in circulation is extremely short (approximately 1 s, no longer than 10 s), as in the bloodstream it is rapidly converted to inosine by adenosine deaminases, and subsequently phosphorylated to 5’-AMP, or alternatively eliminated due to the activity of nucleoside transporters (Müller and Scior, 1993). Therefore, finding longer-lasting synthetic ligands, that differentiate between receptor subtypes, was necessary for both biochemical studies and prospective intervention. It was achieved mainly by adding substituents to the molecule of adenosine in the hope of improving a receptor-ligand binding, or by utilizing molecules of other chemical structure, to a varying degree of success, resulting in a selection of commercially available AR agonists of differing receptor affinities and physiochemical properties. Several adenosine receptor agonists were reported to have

an antiplatelet effect (reviewed in one of the publications comprising this thesis: Wolska, N., Rozalski M. 2019. *Blood Platelet Adenosine Receptors as Potential Targets for Anti-Platelet Therapy*, International Journal of Molecular Sciences, 2019), however, the concept of dual antiplatelet therapy with the use of AR agonists and $P2Y_{12}$ receptor inhibitors is a novel one.

A dual therapy targeting multiple platelet activation pathways is a concept known and currently used in medical practice. For example, acetylsalicylic acid (an inhibitor of thromboxane A2 formation) and clopidogrel (an inhibitor of the $P2Y_{12}$ receptor) combination is the standard of care for prevention of thrombosis in several high-risk patient groups. Unfortunately, such treatment is still plagued by the problem of drug resistance, especially among patients with type 2 diabetes, for whom an effective antithrombotic intervention is crucial (Watala *et al.*, 2006; Serebruany *et al.*, 2014; Wang *et al.*, 2019). Generally, the driving idea behind dual treatment is to maintain the dose of both agents low enough to avoid or moderate adverse effects while providing an effective treatment.

This work presents the AR agonists' potential to overcome resistance to $P2Y_{12}$ blockers. The dual approach can comprehensively block multiple stages of platelet activation, therefore presenting an attractive subject of further research, which may result in a clinical approach avoiding the harmful side effects of antiplatelet treatment associated with high-dose $P2Y_{12}$ inhibitors and providing adequate and consistent antithrombotic protection, regardless of individual responses to low-dose $P2Y_{12}$ antagonist.

II. Aims of the study

Our research group has recently proposed a novel, previously uninvestigated, approach of simultaneous use of two antiplatelet agents: a $P2Y_{12}$ antagonist and an AR agonist.

This doctoral research project aims to characterize the dual inhibitory potential of adenosine receptor agonists and antagonists of the $P2Y_{12}$ receptor on blood platelet function. The objective is to provide a proof of concept that adenosine receptor agonists significantly enhance the antiplatelet effect of $P2Y_{12}$ antagonists, despite possessing various selectivity profiles and antiplatelet activities, and investigate the molecular mechanism of platelet activation impacted by this treatment.

III. Materials and methods

This research project was conducted using human blood collected from healthy volunteers. All experiments were approved by the Ethics of Research in Human Experimentation Committee at the Medical University of Lodz (approval number RNN/43/17/KE). All volunteers provided written consent and stated that they had not taken medications known to influence platelet function (e.g. acetylsalicylic acid or non-steroidal anti-inflammatory drugs) for at least two weeks prior to the study.

This study utilizes both specific platelet-oriented methods such as aggregometry and thrombus formation under flow studies, and general biochemical methods, such as cytometry, to assess surface activation markers or intracellular calcium flux, and ELISA methods.

Aggregation methods based on either light transmittance or impedance (effective resistance) measurement were employed to perform dose-dependence experiments and subsequent non-linear regression analysis and determine half maximal inhibitory concentrations (IC_{50}) of AR agonists and $P2Y_{12}$ inhibitors. Those were applied in the following experiments to provide a suboptimal effective dosage. On this basis, further aggregometric experiments were performed to investigate the effectiveness of AR agonists, $P2Y_{12}$ inhibitors, and their pairings in the populations of experimentally determined groups of low- and high-responders to $P2Y_{12}$ antagonists. The physiological environment-simulating experiments under shear stress using collagen-coated biochambers were performed to prove the effectiveness of such an approach in more comprehensive *in vitro* model. The effect that the proposed approach has on platelet signalling was tested using a panel of *in vitro* methods - flow cytometry measurements of platelet viability, P-selectin expression, GPIIb-IIIa activation, fibrinogen binding, calcium ion mobilization, and VASP (vasodilator-stimulated phosphoprotein) phosphorylation, and cAMP level determined by ELISA.

As platelet function deteriorates quickly after a blood draw, all presented results were obtained by using samples within a 4 h window from the blood draw.

The AR agonists are, in majority, water-insoluble and were therefore prepared using DMSO as a solvent. All tests were run in parallel with the appropriately matched controls while maintaining DMSO concentration equal or below 0.1%.

IV. Results

Doctoral thesis “Adenosine receptor agonists and $P2Y_{12}$ receptor antagonists exhibit combined inhibitory effect on blood platelet function” aims to verify the concept of the simultaneous use of compounds that interact with two different purinergic platelet receptors in order to achieve enhanced and stable inhibition of platelet function.

The literature concerning adenosine receptor activation as an antiplatelet intervention and known properties of the commercially available adenosine receptor agonists was comprehensively reviewed in the paper entitled “Blood Platelet Adenosine Receptors as Potential Targets for Anti-Platelet Therapy”, published in the International Journal of Molecular Sciences (Wolska and Rozalski, 2019).

The results required to achieve the aims of the doctoral project were published in two peer-reviewed journals.

The first of the original papers included in this dissertation entitled “Adenosine Receptor Agonists Exhibit Anti-Platelet Effects and the Potential to Overcome Resistance to $P2Y_{12}$ Receptor Antagonists” was published in the journal Molecules (Wolska *et al.*, 2019), presents data from aggregometric, cytometric, and under flow experiments utilizing whole blood from healthy, consenting volunteers.

We firstly proved a lack of cytotoxic effect of AR agonists on blood platelets, and then proceeded with the aggregation experiments. We presented dose-dependency aggregation inhibition curves for agonists NECA and regadenoson, and demonstrated an overall lack of effect of the AR agonist LUF5835. We did however find a statistically significant improvement in the dual system (AR agonist + $P2Y_{12}$ antagonist - cangrelor or prasugrel metabolite R-138727, prasugrel being a pro-drug) over treatment with only $P2Y_{12}$ antagonist for all tested groups, including LUF5834 combined with both $P2Y_{12}$ inhibitors applied alone, in aggregometric experiments. The donors were subsequently assessed for their response to subclinical doses of $P2Y_{12}$ inhibitors and split into two groups: high- and low-responders. We found that in dual systems the responses generally reached a high, consistent level in both groups, as AR supplementation does not prominently elevate aggregation inhibition for donors in the high-responder group but provides a steep increase in inhibition for those in the low-responder group. We have also provided an additional insight into the signalling

events governing this response by testing GPIIb-IIIa activation and P-selectin expression, finding a more moderate, but statistically significant, effect of AR agonists. The experiments conducted under flow mimicking physiological conditions (using type I collagen-coated flow chamber biochips) demonstrated considerably higher ability of AR agonists to prevent thrombotic events. The major conclusion of this publication is the indication of the potential benefit of the proposed approach to improve the safety and efficiency of antiplatelet therapy.

In the second of the original papers included in this dissertation entitled "Adenosine Receptor Agonists Increase the Inhibition of Platelet Function by $P2Y_{12}$ Antagonists in a cAMP- and Calcium-Dependent Manner" published in the journal Pharmaceuticals (Wolska *et al.*, 2020) we used a panel of nine AR agonists (PSB0777, CGS21680, MRE0094, 2-chloroadenosine, CV1808, HE-NECA, NECA, regadenoson, and UK423,097) and two $P2Y_{12}$ antagonists (cangrelor and prasugrel metabolite R-138727) to investigate the influence of these compounds and their combinations on platelets function.

After confirming a lack of cytotoxic effect of AR agonists on platelets, we conducted a series of biochemical, cytometric and ELISA experiments to establish which platelet signalling events are susceptible to this treatment. We found that the AR agonists affected the platelet signalling pathways to varying degrees. AR agonists and $P2Y_{12}$ antagonists lowered the expression of P-selectin and the activation of GPIIb-IIIa, and the combined systems (AR agonist + $P2Y_{12}$ antagonist) demonstrated stronger effects. The exogenous fibrinogen binding and calcium mobilization were considerably more inhibited, especially when AR agonists were combined with the $P2Y_{12}$ antagonists. The cAMP levels in the resting and ADP-activated platelets were increased by AR agonists and significantly elevated in the case of a dual treatment in comparison to a variant when only $P2Y_{12}$ antagonists were applied. Therefore, we concluded that the methods detecting early activation events are more suitable for assessing the antiplatelet action of fast-acting compounds, and that the exogenous fibrinogen binding, calcium mobilisation and cAMP level are appropriately sensitive markers for detecting the inhibition caused by such treatment.

We therefore reason that comprehensive *in vitro* evidence of platelet inhibition on many pivotal points of the activation cascade provides a strong indication of a possible clinically beneficial antiplatelet approach based on combining the inhibition of $P2Y_{12}$ receptor supplemented by the activation of AR receptors.

Overall, the studies conducted during the completion of this thesis provide a rationale to study the multiple purinergic receptor-based strategies of antiplatelet intervention further, in the hope of establishing a future treatment protocol for patients at risk of developing thrombosis, especially those for whom current strategies are problematic or insufficient.

V. Major Observation and Conclusions

1. Adenosine receptor agonists have a moderate inhibitory effect on multiple stages of platelet activation, from early events (cAMP formation, calcium mobilization), through the activation markers (GPIIb-IIIa receptor activation, fibrinogen binding, P-selectin surface expression) to platelet aggregation.
2. The antiplatelet effect of AR agonists is of variable strength, and not strictly correlated to their selectivity or binding affinity to the A_{2a} AR.
3. AR agonist antiplatelet activity is more pronounced under flow mimicking physiological setting than in the static conditions.
4. Suboptimal $P2Y_{12}$ inhibitor dosage affects whole-blood aggregation in healthy donors to varying degrees – coefficient of variation up to over 50%, with some subjects having low- to non-response.
5. The platelet activity can be lowered through the simultaneous inhibition of $P2Y_{12}$ receptor and activation of adenosine receptors to the further extend than by inhibition of $P2Y_{12}$ receptor alone.
6. Use of AR agonists in combination with $P2Y_{12}$ receptor inhibitors brings the low level of responses to $P2Y_{12}$ inhibitors to the level observed in highly responding individuals – it suggests that patients burdened with drug resistance to $P2Y_{12}$ inhibitors could potentially benefit from supplemental AR agonization treatment.
7. Quantification of cAMP formation, calcium ion mobilization and exogenous fibrinogen binding are sensitive methods to detect platelet activation inhibition.
8. AR agonists increase the $P2Y_{12}$ antagonists' platelet activation inhibition in a cAMP- and calcium mobilization-dependent manner.
9. *In vitro* evidence indicates that the application of AR agonists as compounds of dual antiplatelet therapy together with a lower dosage of $P2Y_{12}$ receptor inhibitors is a promising approach to improved prevention of thrombotic events.

VI. Abstract

Existing antiplatelet therapies are frequently associated with a risk of bleeding, while on the other hand, some patients respond insufficiently to the antiplatelet treatment. Large inter-individual variation in platelet response to endogenous agonists and pharmacological agents, including resistance to antiplatelet therapy, prompts a search for novel platelet inhibitors and development of new antithrombotic strategies. Recently, a dual experimental strategy for lowering platelet activity has been proposed that involves a low-dose inhibition of $P2Y_{12}$ receptor with a simultaneous activation of adenosine receptors (AR). This novel approach could result in the sufficient rate of platelet inhibition while minimizing the adverse effects.

Blood platelets express two (A_{2a} and A_{2b}) of the four known AR subtypes. Activation of these receptors results in an enhanced intracellular cAMP level and leads to the inhibition of platelet aggregation. This work aims to provide the proof of concept for a supporting role of AR agonists in $P2Y_{12}$-based antiplatelet therapy.

I tested the antiplatelet potential of AR agonists in combination with the standard strategy of antagonizing $P2Y_{12}$ receptor with inhibitors currently in clinical use (cangrelor and prasugrel metabolite R-138727, prasugrel being a pro-drug). Various hallmarks of platelet activation were monitored to assess the effects of AR agonists on platelet function with the use of platelet aggregation assays, flow cytometry, cAMP measurements, and under flow clot formation studies with the use of whole blood or isolated platelets obtained from healthy volunteers.

I evaluated *in vitro* the effects of three AR agonists: regadenoson, LUF5835 and NECA, of varying selectivity for platelet adenosine receptors. The major finding was the conclusion that AR agonists while much less effective under static conditions than $P2Y_{12}$ antagonists, demonstrated similar antiplatelet activity under flow mimicking the physiological environment. AR agonists significantly improved the antiplatelet effect of $P2Y_{12}$ antagonists, regardless of their selectivity profiles and antiplatelet activities. Moreover, inhibitory effects in combination with $P2Y_{12}$ antagonists were similar in high- and low-responders to $P2Y_{12}$ inhibitors, indicating that a combination of such antiplatelet agents represents a promise of overcoming the drug resistance problem and offers hope of a more predictable and stable antiplatelet intervention in the population.

A broad panel of AR agonists (PSB0777, CGS21680, MRE0094, 2-chloroadenosine, CV1808, HE-NECA, NECA, regadenoson, and UK423,097) in combinations with $P2Y_{12}$ receptor inhibitors has been investigated providing a methodologically consistent report of their respective effects on multiple stages of platelet function (platelet viability, P-selectin expression, GPIIb-IIIa activation, fibrinogen binding, calcium ion mobilization, VASP-P level, and cAMP formation). I found that the antiplatelet effect potentiation in the combined system is a robust phenomenon across all the tested methods. It was also established that the methods assessing early activation events are better suited to assess the antiplatelet action in a case of the proposed treatment, as the most sensitive markers among the tested ones were calcium mobilization and cAMP level measurements.

In conclusion, a strategy focused on a purinergic pathway and involving a suboptimal inhibition of classical purinergic ADP receptors (P2Y) with a simultaneous activation of adenosine receptors presents a novel, promising approach to prevent thrombotic events of improved safety and efficacy.

VII. Streszczenie

Obecnie stosowane leczenie przeciwpłytkowe często wiąże się z ryzykiem krwawienia, z drugiej strony znaczna grupa pacjentów odpowiada na terapię w niewystarczającym stopniu. Duża zmienność osobnicza w odpowiedzi płytek krwi na agonistów i stosowane leki przeciwpłytkowe, w tym oporność na terapię przeciwpłytkową obserwowana w niektórych grupach pacjentów skłaniają do poszukiwań nowych inhibitorów aktywacji płytek krwi i/lub nowych strategii terapeutycznych. Niedawno postawiona przez naszą grupę badawczą hipoteza sugeruje, że skojarzona terapia eksperymentalna za pomocą agonistów receptorów adenozynowych (AR) i antagonistów receptora $P2Y_{12}$ może skutkować wystarczającym hamowaniem funkcji płytek krwi przy jednoczesnym zminimalizowaniu skutków ubocznych.

Płytki krwi wykazują ekspresję dwóch (A_{2a} i A_{2b}) z czterech znanych podtypów AR. Aktywacja tych receptorów skutkuje wzrostem wewnątrzkomórkowego stężenia cAMP I prowadzi do zahamowania aktywacji i agregacji płytek krwi. Celem poniższej pracy jest udowodnienie słuszności koncepcji wspierającej roli agonistów AR w eksperymentalnej terapii przeciwpłytkowej opartej na jednoczesnej aktywacji AR i inhibicji receptora $P2Y_{12}$.

Potencjał przeciwpłytkowy agonistów AR w połączeniu z klasyczną strategią blokowania receptora $P2Y_{12}$ współcześnie stosowanymi inhibitorami (kangrelor i metabolit R-138727 prasugrelu (prasugrel jest prolekiem)) został zbadany korzystając z szeregu klasycznych metod biochemicznych i metod specyficznych dla oznaczania funkcji płytek krewi. Oznaczono wpływ agonistów AR na funkcję płytek krwi z wykorzystaniem testów agregometrycznych, cytometrii przepływowej, testu ELISA oraz badań tworzenia skrzepów w warunkach przepływu, z użyciem pełnej krwi lub izolowanych płytek krwi pobranych od zdrowych ochotników.

Oceniłam działanie przeciwpłytkowe trzech agonistów AR: regadenozonu, LUF5835 i NECA, o różnej selektywności w stosunku do płytkowych receptorów adenozynowych. Wykazałam, że agoniści AR pomimo, że działają znacznie mniej skutecznie w warunkach statycznych niż antagoniści $P2Y_{12}$, wykazują podobną aktywność przeciwpłytkową w środowisku przepływu naśladującym warunki fizjologiczne. Agoniści AR znacząco nasilali działanie przeciwpłytkowe antagonistów $P2Y_{12}$, niezależnie od ich profilu selektywności i aktywności przeciwpłytkowej. Co więcej, działanie hamujące w połączeniu z antagonistami $P2Y_{12}$ osiągnęło porównywalny poziom u pacjentów u których obserwowano silną i słabszą

odpowiedź na inhibitory $P2Y_{12}$ co wskazuje, że takie połączenie leków przeciwpłytkowych może przyczynić się do przezwyciężenia problemu lekooporności i daje nadzieję na bardziej przewidywalną i stabilną interwencję przeciwpłytkową.

Przebadałam szeroki panel agonistów AR (PSB0777, CGS21680, MRE0094, 2-chloroadenozyna, CV1808, HE-NECA, NECA, regadenoson i UK423,097) w połączeniu z inhibitorami receptora $P2Y_{12}$, koncentrując się na ich wpływie na różne parametry funkcji płytek krwi (żywotność płytek, ekspresja P-selektyny, aktywacja GPIIb-IIIa, wiązanie fibrynogenu, mobilizacja jonów wapnia, poziom VASP-P i tworzenie cAMP). Dowiodłam, że wzrost efektu przeciwpłytkowego w modelu skojarzonym jest zjawiskiem zachodzącym na wszystkich testowanych poziomach aktywacji płytki. Ustaliłam również, że metody badania wczesnych epizodów aktywacji płytek są bardziej odpowiednie do oceny działania przeciwpłytkowego w przypadku proponowanej interwencji, gdyż najbardziej czułymi markerami spośród badanych były pomiary mobilizacji wapnia i stężenia cAMP.

Podsumowując, strategia przeciwpłytkowa skoncentrowana na szlaku purynergicznym i polegająca na suboptymalnym hamowaniu receptora ADP ($P2Y_{12}$) z jednoczesną aktywacją receptorów adenozynowych stanowi nowe, obiecujące podejście do zapobiegania zdarzeniom zakrzepowym, o zwiększonym bezpieczeństwie i skuteczności.

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IX. List of publications constituting the doctoral dissertation

Wolska, N., Rozalski, M. (2019). Blood Platelet Adenosine Receptors as Potential Targets for Anti-Platelet Therapy. Int J Mol Sci, 20(21): 5474.
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(IF 2019 = 4.556, 140 MNiSW points)

Wolska, N., Boncler, M., Polak, D., Wzorek, J., Przygodzki, T., Gapinska, M., Watala, C., Rozalski, M. (2019). Adenosine Receptor Agonists Exhibit Anti-Platelet Effects and the Potential to Overcome Resistance to $P2Y_{12}$ Receptor Antagonists. Molecules, 25(1):130.
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(IF 2019 = 3.267, 100 MNiSW points)

Wolska, N., Kassassir, H., Luzak, B., Watala, C., & Rozalski, M. (2020). Adenosine Receptor Agonists Increase the Inhibition of Platelet Function by $P2Y_{12}$ Antagonists in a cAMP- and Calcium-Dependent Manner. Pharmaceuticals, 13(8): E177.
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Summarized impact factor = 12.109
Summarized MNiSW points = 340

V. List of other publications and achievements

Publications in peer-reviewed journals:

1. Popielarski, M., Ponamarczuk, H., Stasiak, M., Gdula, A., Bednarek, R., Wolska, N., Swiatkowska M. (2020). $P2Y_{12}$ receptor antagonists and AR receptor agonists regulates Protein Disulfide Isomerase secretion from platelets and endothelial cells. Biochem Biophys Res Commun, 526(3), 756-763.
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2. Boncler, M., Wzorek, J., Wolska, N., Polak, D., Watala, C., & Rozalski, M. (2019). Adenosine receptor agonists deepen the inhibition of platelet aggregation by $P2Y_{12}$ antagonists. Vascul Pharmacol, 113, 47-56.
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3. Przygodzki, T., Wolska, N., Talar, M., Polak, D., Gapinska, M., & Watala, C. (2018). Comparison of different microscopy approaches to quantification of inhibitory effect on thrombus formation under flow conditions by the example of adenosine receptor agonist HE-NECA. J Pharmacol Toxicol Methods, 94(Pt 1), 94-104.
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4. Bagavant, H., Dunkleberger, M. L., Wolska, N., Sroka, M., Rasmussen, A., Adrianto, I., Montgomery, C., Sivils, K., Guthridge, J. M., James, J. A., Merrill, J. T., Deshmukh, U. S. (2019). Antibodies to periodontogenic bacteria are associated with higher disease activity in lupus patients. Clin Exp Rheumatol, 37(1), 106-111
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   (IF 2016 = 6.400, 200 MNiSW points)

6. Szczerba, B. M., Kaplonek, P., Wolska, N., Podsiadlowska, A., Rybakowska, P. D., Dey, P., Rasmussen, A., Grundahl, K., Hefner, K. S., Stone, D. U., Young, S., Lewis, D. M., Radfar, L., Scofield, R. H., Sivils, K. L., Bagavant, H., Deshmukh, U. S. (2016). Interaction between innate immunity and Ro52-induced antibody causes Sjogren's syndrome-like disorder in mice. Ann Rheum Dis, 75(3), 617-622.
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7. Maier-Moore, J. S., Koelsch, K. A., Smith, K., Lessard, C. J., Radfar, L., Lewis, D., Kurien, B. T., Wolska, N., Deshmukh, U., Rasmussen, A., Sivils, K. L., James, J. A., Farris, A. D., Scofield, R. H. (2014). Antibody-secreting cell specificity in labial salivary glands reflects the clinical presentation and serology in patients with Sjogren's syndrome. Arthritis Rheumatol, 66(12), 3445-3456.
   (IF 2014 = 6.910, 200 MNiSW points)

Conference communications:

1. Wolska, N., Boncler, M., Watala, C., Rozalski, M. (2019) Standard anti-platelet therapy with $P2Y_{12}$ blockers can be improved by including adenosine receptor agonists to achieve stronger antithrombotic effect. Presented at the European Congress on Thrombosis and Haemostasis. Abstract P-188

2. Wolska, N., Boncler, M., Luzak, B., Kassassir, H., Watala, C., Rozalski, M. (2019) Adenosine receptor agonists and $P2Y_{12}$ receptor inhibitors exhibit combined inhibitory effect on blood platelet function. Presented at the Congress of the Croatian Society of Biochemistry and Molecular Biology HDBMB2019 "Crossroads in Life Science".

3. Wolska, N., Przygodzki, T., Luzak, B., Watala, C., & Rozalski, M. (2019). The combined action of adenosine receptor agonists and $P2Y_{12}$ antagonists increases the inhibition of platelet function. Presented at the First Platelet Society Meeting. Abstract PC17

4. Piechocka, I.K., Wolska, N., Luzak, B. (2019). The role of shear flow in glycosylated fibrin clot response. Presented at the 3rd Interdisciplinary FNP Conference. Abstract 104

5. Piechocka, I.K., Wolska, N., Luzak, B. (2019). Glycation of fibrinogen affects FXIII-induced crosslinking and shear flow response of fibrin networks. Presented at the 44th FEBS Congres. Abstract 34645

6. Piechocka, I.K., Wolska, N., Luzak, B. (2019). Shear flow promotes isotropic redistribution of fibrin fibers insides glycosylated fibrin networks. Presented at Joint 12th EBSA Congress and 10th ICBP-IUPAP Congress. Abstract P-082

7. Kassassir, H., Polak, D., Wzorek, J., Wolska, N., Przygodzki, T., Gapinska, M., Watala, C. (2019). Total Thrombus-Formation Analysis System (T-TAS) as a potential method of monitoring the dual antiplatelet effect of AR agonist in combination with $P2Y_{12}$ antagonist. Presented at the First Platelet Society Meeting. Abstract PC1

8. Wolska, N., Przygodzki, T., Talar, M., Gapinska, M., Watala, C., Rozalski M. (2018). Comparison of the use of wide-field and confocal microscopy for the quantitative evaluation of thrombi formation under *in vitro* flow conditions. Presented at the Platelet Society Early Career Research Meeting, oral presentation (runner-up for best oral presentation)

9. Wolska, N., Przygodzki, T., Talar, M., Gapinska, M., Watala, C., Rozalski, M. (2018). Inhibitory effect of adenosine receptor agonist HE-NECA on thrombi formation on collagen under *in vitro* flow conditions – comparison of different microscopy approaches to quantification of anti-platelet activity. Presented at the 1st ESM/EVBO Summer School in Dresden. Abstract #22

10. Piechocka, I.K., Wolska, N., Luzak, B., Kowalewski, T.A. (2018). The role of in vitro fibrinogen glycation on FXIII-induced crosslinking and shear flow clot response. Presented at Jülich Soft Matter Days. Abstract BIP-07.

11. Bagavant, H., Dunkleberger, M. L., Wolska, N., Rybakowska, P., Sroka, M., Rasmussen, A., Adrianto, I., Montogomery, C., Sivils, K., Guthridge, J. M., James, J. A., Merrill, J. T., Deshmukh, U. S. (2018). Periodontal pathogen exposure facilitates disease activity in Systemic Lupus Erythematosus. Presented at the Annual Meeting of the American-Association-of-Immunologists (AAI). Abstract 45.7

12. Siewiera, K., Labieniec-Watala, M., Kassassir, H., Polak, D., Wolska, N., and Watala, C. (2017). Altered blood platelet activation is not related to elevated mitochondrial respiration in rat model of streptozotocin-induced diabetes. Presented at the 1st Italian-UK Platelet Meeting. Abstract #28

13. Wolska, N., Polak, D., Stanczyk, L., T. Przygodzki, T., Rozalski, M., Lesnikowski, Z., Watala, C. (2017). Adenosine receptor agonists as anti-platelet agents. Presented at the 1st Italian-UK Platelet Meeting. Abstract #37

14. Siewiera, K., Labieniec-Watala, M., Wolska, N., Polak, D., Kassassir, H., Watala C., (2017). Blood platelets as bioenergetic markers of brain damage in STZ-diabetes. Presented at the MiPschool Obergurgl.

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17. Bagavant, H., Wolska, N., Rybakowska, P., Kamp, S., Guthridge, J., James, J. A., Merrill, J., Deshmukh, U. (2016). A potential link between immune response to a periodontal bacterium, Aggregatibacter actinomycetemcomintans and Systemic Lupus Erythematosus. Presented at the Annual Meeting of the American-Association-of-Immunologists (AAI).

18. Wolska, N., Rybakowska, P., Klopocki, A., Brown, M., Montgomery, C. G., Grundahl, K., Rasmussen, A., Scofield, R. H., Radfar, L., Stone, D. U., Anaya, J. M., Ice, J., Lessard, C., Lewis, D. M., Rhodus, N. L., Gopalakrishnan, R., Huang, A., Hughes, P. J., Rohrer, M. D., Weismann, M., Venuturupalli, S., Guthridge, J. M., James, J. A., Sivils, K. L., Bagavant, H., Deshmukh, U. S. (2015). Co-Occurence of Anti-Ro52/TRIM21 and Anti-TRIM38 Autoantibodies Is Associated with Higher Severity of Dry Eye in Sjogren's Syndrome. Presented at the Arthritis & Rheumatology Meeting. Abstract 2802

19. Kaplonek, P., Szczerba, B., Wolska, N., Rybakowska, P., Klopocki, A., Dey, P., Rasmussen, A., Hefner, K., Young, S., Stone, D. U., Lewis, D. M., Radfar, L., Scofield, R. H., Sivils, K. M., Bagavant, H., Deshmukh, U. (2014). Interaction Between Innate Immunity and Anti-Ro52 Antibodies is Critical for the Induction of Sjogren's Syndrome-like Disease in Mice. Presented at the Arthritis & Rheumatology Meeting. Abstract 1798

20. Bagavant, H., Wolska, N., Kamp, S., Guthridge, J., James, J. A., Merrill, J., Deshmukh, U. (2015). Antibody Response to Periodontogenic Bacterium Aggregatibacter Actinomycetemcomitans and Lupus. Presented at the ACR/ARHP Annual Meeting. Abstract 811

21. Rybakowska, P., Wolska, N., Klopocki, A., Sivils, K., James, J., Bagavant, H., Deshmukh, U. (2014). Multiple TRIM proteins are targets of autoimmune response in lupus and Sjogren's syndrome. Presented at the Annual Meeting of the American-Association-of-Immunologists (AAI). Abstract HUM7P.308

Other scientific achievements:

- Laureate of the ETIUDA7 doctoral scholarship “Adenosine receptor agonists and $P2Y_{12}$ receptor antagonists exhibit combined inhibitory effect on blood platelet function” funded by National Science Centre, Poland (2019/32/T/NZ3/00333)
- Runner-up for best oral presentation: Wolska, N., Przygodzki, T., Talar, M., Gapinska, M., Watala, C., Rozalski M. (2018). “Comparison of the use of wide-field and confocal microscopy for the quantitative evaluation of thrombi formation under *in vitro* flow conditions”, presented at the Platelet Society Early Career Research Meeting
- Principal Investigator in the Medical University of Lodz grant for young scientists (502-03/6-020-01/502-64-125-18)
- Stipend for best doctoral students, for academic year 2019/2020
- Stipend for best doctoral students, for academic year 2018/2019
- Stipend for best doctoral students, for academic year 2017/2018

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Summarized impact factor for all peer-reviewed publications = 48.635

Summarized MNiSW points for all peer-reviewed publications = 1310

Sum of times cited for all peer-reviewed publications (without-self citations) = 52

h-index = 4

VI. Publications constituting the doctoral dissertation and co-author's contribution statements

International Journal of
Molecular Sciences

MDPI

Review

Blood Platelet Adenosine Receptors as Potential Targets for Anti-Platelet Therapy

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Received: 30 September 2019; Accepted: 1 November 2019; Published: 3 November 2019

Abstract: Adenosine receptors are a subfamily of highly-conserved G-protein coupled receptors. They are found in the membranes of various human cells and play many physiological functions. Blood platelets express two (A_{2A} and A_{2B}) of the four known adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3). Agonization of these receptors results in an enhanced intracellular cAMP and the inhibition of platelet activation and aggregation. Therefore, adenosine receptors A_{2A} and A_{2B} could be targets for anti-platelet therapy, especially under circumstances when classic therapy based on antagonizing the purinergic receptor $P2Y_{12}$ is insufficient or problematic. Apart from adenosine, there is a group of synthetic, selective, longer-lasting agonists of A_{2A} and A_{2B} receptors reported in the literature. 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. Chemically, most A_{2A} and A_{2B} adenosine receptor agonists are adenosine analogues, with either adenine or ribose substituted by single or multiple foreign substituents. However, a group of non-adenosine derivative agonists has also been described. This review aims to systematically describe known agonists of A_{2A} and A_{2B} receptors and review the available literature data on their effects on platelet function.

Keywords: adenosine; adenosine receptor; adenosine receptor agonist; platelet; AR; therapy

1. Introduction

Activation of blood platelets plays a critical role in the pathogenesis of arterial thrombotic diseases, such as coronary heart disease, myocardial infarction, and stroke, which are the primary cause of mortality in developed countries. Therefore, anti-platelet therapy is one of the most important tools in the treatment of arterial thrombotic disorders [1].

Platelets express two receptors for ADP: The $P2Y_1$ receptor, which initiates platelet aggregation, and the $P2Y_{12}$ receptor, which enhances this process, finally leading to thrombus formation. In contrast to the $P2Y_1$ receptor, the $P2Y_{12}$ receptor is almost exclusively expressed in the platelet plasma membrane. Therefore, $P2Y_{12}$ has become a major therapeutic target to prevent arterial thrombotic disorders instead of adenosine receptors [2]. In general, the major clinically approved $P2Y_{12}$ inhibitors include the thienopyridine-class inhibitors (ticlopidine, clopidogrel, and prasugrel), the ATP analogue—cangrelor, and the cyclo-pentyl-triazolo-pyrimidine (CPTP)—ticagrelor [2,3]. Thienopyridines are prodrugs that are converted to short-living active metabolites; these irreversibly inactivate the receptor and consequently inhibit ADP-induced platelet activation. Cangrelor is the first intravenous $P2Y_{12}$ receptor inhibitor to reversibly block 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, which leads to the non-competitive inhibition of ADP-induced $P2Y_{12}$ activation and is used for the prevention of thromboembolic events in patients with acute coronary syndromes [2–5]. As regards current clinical practice, clopidogrel, prasugrel, and ticagrelor are the most frequently used oral platelet

$P2Y_{12}$ inhibitors; the use of ticlopidine has been abandoned. Clopidogrel is the only oral $P2Y_{12}$ inhibitor recommended for the treatment of patients with stable coronary artery disease. Although all three agents have an indication for use in acute coronary syndromes, current guidelines suggest the preferential use of prasugrel and ticagrelor over clopidogrel because of their superior clinical benefits, i.e., the improved efficacy, lowered individual variation in response, and less frequent and severe side effects [6]. Cangrelor, in turn, as the recently approved, first $P2Y_{12}$ inhibitor administered intravenously, seems to be the most promising in percutaneous coronary interventions [7].

Although these anti-platelet agents are now commonly used as clinically approved drugs, effective therapy of arterial thrombosis still presents a problem. For example, gastrointestinal bleeding is a common adverse event observed in 5 to over 10% of patients treated with oral anti-platelet drugs. Many of the patients with this complication require recurrent hospitalization [8]. Another severe and relatively common side effect of anti-platelet therapy is a higher risk of intracranial and intracerebral hemorrhage [9]. On the other hand, for some patients, the applied anti-platelet therapy appears insufficient and does not prevent excessive clotting. This can be explained by the fact that anti-platelet agents either interfere with only one out of several pathways of platelet activation or, even if they block effectively a final common step of platelet aggregation, such as fibrinogen binding (blockers of fibrinogen receptor), their use is associated with a risk of bleeding [3]. Another problem affecting the efficiency of many anti-platelet drugs stems from the individual variability of the response to these drugs resulting from both environmental and genetic factors, especially in case of prodrugs [10]. Altogether, there still is a need for the development of novel platelet inhibitors with better efficacy and safety, or using a combined therapy based on various sets of currently-available agents.

Adenosine is an important purine metabolite, serving not only as a component of nucleic acids and the most important energy carrier in the cell—ATP—but also as a signaling molecule regulating tissue function [11,12]. Adenosine receptors (AR) are present in membranes of many types of human cells and play various physiological functions. Blood platelets express two (A_{2A} and A_{2B}) of the four known adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3). As regards platelet AR receptors, A_{2A} is characterized by the higher affinity to adenosine in comparison with A_{2A}; furthermore, platelets have a significantly lower density of A_{2B} [13,14]. Activation of platelet AR results in an enhanced intracellular cAMP level and consequently leads to the inhibition of platelet activation and aggregation [15,16]. Therefore, adenosine receptors A_{2A} and A_{2B} could be considered as targets for anti-platelet therapy, especially under circumstances when classic therapy based on antagonizing the $P2Y_{12}$ purinergic receptor is insufficient or problematic.

The aim of this review is to systematically present current knowledge of the impact of synthetic, selective, longer-lasting agonists of A_{2A} and A_{2B} receptors on platelet function inhibition, and evaluate their potential as anti-platelet therapeutics.

2. The Classification, Distribution, and Signaling of Adenosine Receptors

Adenosine receptors (AR) represent a subfamily of highly-conserved G-protein coupled receptors. They are found in membranes of various human cells and play a plethora of physiological functions. Four AR subtypes are known: A_1, A_{2A}, A_{2B}, and A_3. The A_1 and A_3 receptors preferentially couple to G_i protein to inhibit adenylate cyclase and, consequently, the production of cyclic AMP (cAMP). The A_{2A} and A_{2B} subtypes stimulate the production of cAMP by coupling to G_s or G_o protein [17]; they are therefore classified as adenylyl cyclase inhibiting (A_1 and A_3) or adenylyl cyclase activating (A_{2A} and A_{2B}) [18].

AR subtypes are characterized by high resemblance in terms of amino acid sequence: The human A_1 and A_3 ARs are identical in 49%, whereas human A_{2A} and A_{2B} AR sequences are identical in 59%. In general, an AR molecule consists of a single polypeptide chain that transverses the membrane from the extracellular side, beginning at the N terminus and forming seven transmembrane helices [15]. AR receptors are commonly expressed in many tissues and cells types; however, the distribution of subtypes is highly tissue-specific (Table 1).

Table 1. Adenosine receptors (AR) receptor distribution and expression in different tissue types.

Receptor Subtype	High Expression	Intermediary Expression	Low Expression
A_1 [19]	brain (cortex, hippocampus, cerebellum); spinal cord; adrenal gland; atria; eyes	brain (excluding cortex, hippocampus, and cerebellum); skeletal muscles; adipose tissue; liver; kidneys	lungs; pancreas
A_{2A} [20]	blood platelets; leukocytes; spleen; thymus	heart; lungs; blood vessels; peripheral nerves	brain
A_{2B} [21–23]	cecum; bladder	lungs; blood vessels; mast cells; eyes	brain; adipose tissue; blood platelets; adrenal gland; kidneys
A_3 [19]	testis; mast cells	brain (hippocampus, cerebellum)	brain (excluding hippocampus and cerebellum); heart; thyroid; adrenal gland; spleen; liver; kidneys

Adenosine receptors play multiple functions. A_{2A} receptor agonization is known to cause coronary artery vasodilatation, decreased dopaminergic activity in central nervous system, and inhibition of central neuron excitation, whereas A_{2B} receptor activation may cause bronchospasm [12,24–26]. Therefore, all adenosine receptor ligands should be used only with the utmost caution [11], despite some having already been approved for human use (one of them—regadenoson—is discussed further in this article). An interesting insight into adenosine receptor overstimulation may be gained from the study of adenosine deaminase deficiency—a rare, autosomal metabolic disorder that causes severe combined immunodeficiency [27]. In this syndrome, platelet dysfunction has been described, as well as severe thrombocytopenia [28,29]. It is, however, important to remember that there is no exact parallel between patients with this syndrome and an anti-platelet therapy with the use of AR agonists. The dose of synthetic adenosine agonist equivalent to adenosine would be much lower and, most importantly, it would be applied in adults.

As it has been already mentioned, blood platelets express two subtypes of AR receptors (A_{2A} and A_{2B}); however, the expression (the number of receptor copies in the plasma membrane) of the two receptor types has not been established. A_{2A} receptor is believed to be expressed on platelets in higher density as compared to A_{2A} [18]. Only one study has estimated the gene expression profile for A_{2A} and A_{2B} in human platelets. This report demonstrated comparable mRNA expression levels for A_{2A} and A_{2B} AR [22], but no further evidence exists regarding protein levels present on the platelet surface. Moreover, two studies [30,31] have not been able to quantify A_{2A} AR in platelet proteome, while have easily identified, for example, $P2Y_{12}$ receptor, expressed on healthy platelets in around 450–1000 copies [32].

A_{2A} AR was identified as an important receptor on platelets and a mediator of adenosine inhibition of platelet aggregation [33]. This is achieved through inhibition of mobilization of internal calcium stores and influx of external calcium, both associated with activation of adenylate cyclase and increase of cAMP concentration [34]. Cyclic nucleotides are also strong inhibitors of the release of calcium ions into the cytosol, which underpins many events in platelet activation. In addition to inhibiting platelet aggregation in human blood, the activation of A_{2A} AR by specific agonists leads to a reduction in P-selectin expression on the platelet cell surface, as a result of thromboxane A2 or ADP stimulation [15].

Phenotypically, counts of blood cell populations, including platelets, were found to be similar in A_{2A} AR knock-out mice and with wild-type mice [20]. In this knock-out model, the rate of ADP-induced platelet aggregation differed in both the genetic variants following the treatment with nonselective AR agonist, 5′-N-ethyl-carboxamidoadenosine (NECA). NECA administration led to inhibition of platelet aggregation in wild-type mice, but demonstrated no effect in A_{2A} AR-null mice [35].

The role of A_{2B} AR in platelets remains disputable. It was proposed that this AR subtype activates signal transduction pathways other than adenylate cyclase [36]. It was also proposed, based on a mouse knock-out study, that A_{2B} AR is upregulated under stress in vivo, and plays a significant role in regulating ADP receptor expression [23]. The same study also found that agonization of this

receptor inhibits agonist-induced platelet aggregation, but it should be noted that no specific agonist was used: A combination of a non-selective agonist and A_{2A} receptor inhibitor was applied.

The half-life of adenosine in circulation is extremely short (approximately 1 s), due to the action of enzymes like adenosine deaminase, which convert it to inosine, or adenosine kinase, which phosphorylates it to 5′-AMP, or due to uptake by nucleoside transporters [33]. Therefore, close study and pharmacological potential of ARs can be facilitated only by finding longer-lasting synthetic agonists and antagonists.

3. Adenosine Receptor Agonists—Structure, Chemical Properties, and Known Effects on Platelet Function

The purpose of synthesizing novel AR agonists is to achieve longer-lasting agonization and selectivity between receptor subtypes without compromising high affinity of binding to the receptor. This is accomplished, with varying success, either by introducing additional substituents to the molecule of adenosine in the hope of improving a receptor-ligand binding, or by utilizing molecules of other chemical structure. A nomenclature and the chemical structure of AR agonists is presented in Table 2.

Table 2. Nomenclature and chemical structure of AR agonists.

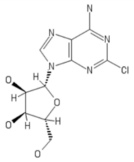
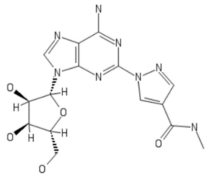
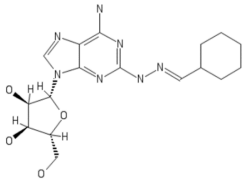
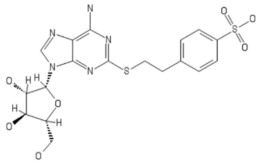
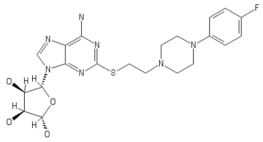
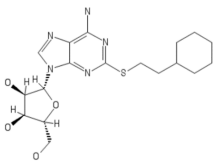
Name	Other Names	IUPAC Name	Structure
2-chloroadenosine	2-Chloro Adenosine, Cl-Ado, 2 ClAdo, 2-CADO	(2R,3R,4S,5R)-2-(6-amino-2-chloropurin-9-yl)-5- (hydroxymethyl) oxolane-3,4-diol	
Regadenoson	CVT 3146, CVT-3146, CVT3146, Lexiscan, Rapiscan	1-[6-amino-9-[(2R,3R,4S,5R)- 3,4-dihydroxy -5-(hydroxymethyl)oxolan-2-yl]purin-2-yl]-N-methylpyrazole-4-carboxamide	
Binodenoson	2-((Cyclohexylmethylene) hydrazino)adenosine	(2R,3R,4S,5R)-2-{6-amino-2-[(E)-2-(cyclohexylmethylidene) hydrazin-1-yl]-9H-purin-9-yl}-5-(hydroxymethyl)oxolane-3,4-diol	
PSB-0777	PSB0777	4-[2-[(6-Amino-9-b-D-ribofuranosyl-9H-purin-2-yl) thio]ethyl]benzenesulfonic acid ammonium salt	
PSB-15826	-	(2S,3S,4R,5R)-5-(6-Amino-2-((2-(4-(4-fluorophenyl)piperazin-1-yl) ethyl)thio)-9H-purin-9-yl) tetrahydrofuran-2,3,4-triol	
PSB-12404	-	(2R,3R,4S,5R)-2-(6-Amino-2-(2-cyclohexylethylthio)-9Hpurin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol	

Table 2. *Cont.*

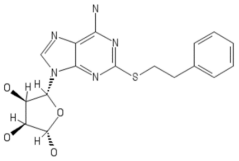
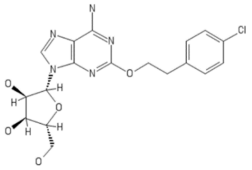
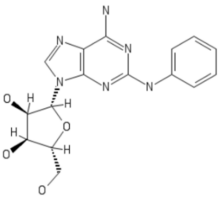
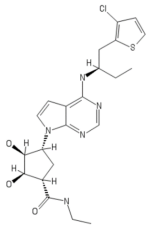
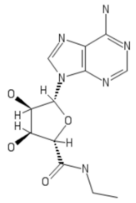
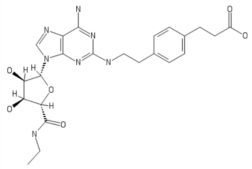
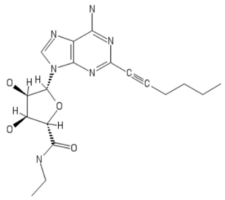
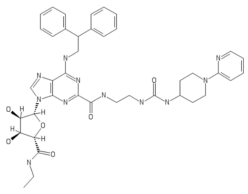
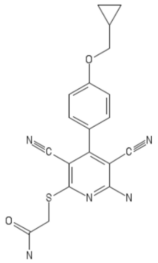
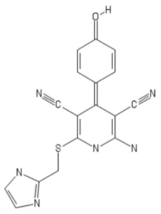
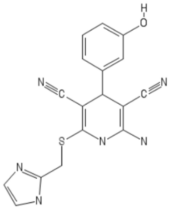
Name	Other Names	IUPAC Name	Structure
PSB-16301	-	(2S,3S,4R,5R)-5-(6-amino-2-(phenethylthio)-9H-purin-9-yl)tetrahydrofuran-2,3,4-triol	
MRE0094	Sonedenoson, 2-[2-(4-Chlorophenyl) ethoxy]adenosine	(2R,3R,4S,5R)-2-[6-amino-2-[2-(4-chlorophenyl)ethoxy]purin-9 -yl]-5-(hydroxymethyl)oxolane-3,4-diol	
CV1808	2-phenylaminoadenosine, CV-1808	(2R,3R,4S,5R)-2-(6-amino-2-anilinopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol	
AMP597	RPR 100579	(1S,2R,3S,4R)-4-(4-(((R)-1-(3-chlorothiophen-2-yl)butan-2-yl)amino)-7H-pyrrolo [2,3-d]pyrimidin-7-yl)-N-ethyl-2,3-dihydroxycyclopentane-1-carboxamide	
NECA	N-Ethyl-5′-Carboxamido Adenosine, 5′-ethylcarboxamidoadenosine	(2S,3S,4R,5R)-5-(6-aminopurin-9-yl) -N-ethyl-3,4-dihydroxyoxolane-2-carboxamide	
CGS21680	CGS-21680, Cgs 21680, 2-(4-(2-carboxyethyl) phenethylamino)-5′-N-ethylcarboxamidoadenosine	3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxyoxolan-2-yl]purin-2-yl]amino]ethyl]phenyl] propanoic acid	
HE-NECA	HENECA, Heneca, 2-hexynyl-NECA, 2-hexynyladenosine-5′-N-ethylcarboxamide	(2S,3S,4R,5R)-5-(6-amino-2-hex-1-ynylpurin-9-yl)-N-ethyl-3,4-dihydroxyoxolane-2-carboxamide	
UK-432097	UK-432,097	6-(2,2-diphenylethylamino)-9-[(2R,3R,4S,5S) -5-(ethylcarbamoyl)-3,4-dihydroxyoxolan-2-yl] -N-[2-[(1-pyridin-2-ylpiperidin-4-yl) carbamoylamino]ethyl]purine-2-carboxamide	

Table 2. *Cont.*

Name	Other Names	IUPAC Name	Structure
BAY 60-6583	BAY-60-6583, BAY60-6583, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl) pyridin-2 yl) sulfanyl)acetamide	2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl] pyridin-2-yl]sulfanylacetamide	
LUF5834	LUF 5834, LUF-5834, 2-Amino-4-(4-hydroxy-phenyl) -6-(1H-imidazol-2-ylmethylsulfanyl) -pyridine-3,5-dicarbonitrile	2-amino-6-(1H-imidazol-2-ylmethylsulfanyl) -4-(4-oxocyclohexa-2,5-dien-1-ylidene) -1H-pyridine-3,5-dicarbonitrile	
LUF5835	LUF 5835, LUF-5835	2-amino-6-(1H-imidazol-2-ylmethylsulfanyl) –4-(3-hydroxy-phenyl) pyridine-3,5dicarbonitrile	

3.1. Adenosine Derivatives

Numerous adenosine-derived compounds have been tested for their affinity and selectivity for ARs. The most prevalent strategies for obtaining novel derivatives were substitutions of adenosine at the 2-position, usually with (thio)ethers, secondary amines, and alkynes, as well as at the N^6-position. The latter substitutions appear to increase affinity to the A_{2A} receptor subtype [37]. Below, we present an overview of adenosine derivatives classified according to the position of substituents. It is, however, important to point out that the citied study was performed using various protocols and utilizing nonhomogeneous materials, hence is not possible to make a direct comparison between available data, for example for IC_{50} or EC_{50} measured for various AR agonists.

3.1.1. Adenosine Derivatives with Substituents at C1 to C8 Positions

2-chloroadanosine

2-chloroadenosine is one of the first characterized AR agonists, first described in 1964 [38,39]. Conducted studies were predominantly concentrated on its effect on platelets, from the pioneering research into activation signaling [40,41], including recognition of AR subtypes [42], through examination of platelet disorders [43,44], to investigations of 2-chloroadenosine antiaggregatory effects [45].

IC_{50} of 2-chloroadenosine for human platelets was established at 1.6 μM (CI95% 0.61–4.4) (by photometric method in PRP (platelet rich plasma) with ADP), while its EC_{50} was found to be 1.7 μM in adenylate cyclase assay using human platelet membranes (CI95% 1.5–2.0) [46]. For aggregation in whole blood, IC_{50} was later measured to be 2.3 μM [47]. 2-chloroadenosine is a non-selective AR agonist, with high affinity, especially to A_1 and A_2 AR classes [48,49]. Nowadays, it is employed in numerous research areas as a stable adenosine analogue [50–53]. Its advantage over the adenosine arises from the fact that 2-chloroadenosine has a longer half-time and exerts more potent activating effect on AR (A_{2A} receptor biding affinity Ki = 180 nM), being only minimally different chemically [54].

Regadenoson

Regadenoson is also commonly denoted as CVT-3146 and known under its trade names Lexiscan or Rapiscan. It is a selective A_{2A} AR agonist of low affinity (Ki = 1095 nM). It also binds to A_1 receptor subtype (Ki > 16,460 nM), but has much higher affinity constants in the case of A_{2B} and A_3 receptor classes [55]. It was approved by the Food and Drug Administration (FDA) in 2008 for diagnostic purposes in radionuclide myocardial perfusion imaging, manufactured by Astellas Pharma and marketed by GE Healthcare. It is administered intravenously in bolus as a 0.08 mg/mL solution. Regadenoson rapidly increases coronary blood flow to over twice the baseline value in 30 s and decreases to below twice the baseline value in 10 min, and is removed from the human body (58% through renal excretion) within two hours [56] (clinical studies: NCT01019486 (RABIT1D) and NCT00881218). The influence of regadenoson on platelet aggregation has not been reported in literature so far. However, our unpublished results (manuscript currently under review) have confirmed that regadenoson has an anti-platelet effect—in whole blood aggregation, the obtained maximal inhibition value was of 38.1 ± 3.2%, and IC_{50} of 1.2 μM.

Binodenoson

Binodenoson is another AR agonist currently approved for human use. It was firstly reported in the literature in 1996 under the name WRC-0470 as a short-acting A_{2A} agonist [57], then a year later, it was presented as a potential imaging tool [58]. Subsequently, it was tested specifically for induction of pharmacological stress as an adjunct to myocardial perfusion imaging. Its pharmacokinetics and safety profile were tested in clinical trials [59–61]. Binodenoson has successfully completed two phase III clinical trials (identifiers NCT00944294 and NCT00944970) and is currently used as a single bolus injection prior to myocardial perfusion imaging.

It is characterized with good selectivity for A_{2A} AR receptor over other AR receptors, and good binding affinity (Ki = 270 nM) [62]. Despite being well characterized concerning general safety, no data concerning platelets or its potential anti-platelet effect are available.

PSB Family

PSB-0777 was developed in PharmaCenter Bonn, and described in 2011 as a potential anti-inflammatory agent for a treatment of inflammatory bowel disease [63]. Despite it being a polar and water-soluble substance, it is not absorbable when administered *per os*, but suitable for parenteral application only. It was determined to be a full A_{2A} agonist, of high affinity (Ki = 44.4 nM) and high selectivity (over 225-fold) over other ARs. The compound exhibits affinity for both human and rat A_{2A}. In cAMP accumulation assay using CHO cells expressing the A_{2A} receptor, EC_{50} was established at 117 nM. PSB-0777 was successfully utilized as an A_{2A} receptor agonist in a study concerning activation of brown adipose tissue [64]. It has yet to be investigated in the context of blood platelets.

A recent study reported the anti-platelet effects of three other recently-synthesized compounds from this family: PSB-15826, PSB-12404, and PSB-16301 [65–68]. PSB-15826 was found to be the most potent agonist out of these three compounds, characterized by IC_{50} values of 0.32 ± 0.05 μM for inhibition of platelet aggregation, 0.062 ± 0.2 μM for inhibition of platelet activation, and 0.24 ± 0.01 μM for cAMP production, making it a stronger anti-platelet agent than adenosine. PSB-16301 has also effectively reduced ADP-induced platelet aggregation with relatively low IC_{50} of 5.5 ± 0.2 μM, as well as PSB-12404, though at higher concentration: IC_{50} of 66.8 ± 0.07 μM [68]. Other members of this family were also described in the literature. They are either very weak AR agonists, or even AR inhibitors.

There are no data available on the cytotoxicity of this group of compounds; however, success in identifying multiple members of this family with anti-platelet properties suggests a high chance for finding an analogue with good safety profile.

MRE0094

MRE0094 is also known and marketed under names Sonedenoson and 2-[2-(4-chlorophenyl) ethoxy]adenosine. It is 39,000-fold more selective for adenosine A_2 receptors than adenosine A_1 receptors, with Ki for A_2 AR subtype established at 490 nM [69]. It was successfully utilized as an A_{2A} selective compound in cell signaling research focused on varying topics [70–72], but most promising investigations concentrated on its use in promoting wound healing [73,74]. In early 2000, it was being developed by King Pharmaceuticals with hopes of becoming a novel topical drug.

MRE0094 was tested in two Phase II clinical trials concerning wound healing in chronic, neuropathic, diabetic foot ulcers, both of them sponsored by Pfizer. The first trial (ClinicalTrials.gov Identifier: NCT00312364) was completed in 2006; however, no results are available. The second study (ClinicalTrials.gov Identifier: NCT00318214) has been terminated due to poor enrolment of participants. According to King Pharmaceuticals, MRE0094 did not show expected improvement over selected reference for the clinical endpoints. MRE0094 has not been examined for its anti-platelet activity to date.

CV1808

CV1808 is also sometimes denoted as 2-phenylaminoadenosine. It is one of the first characterized AR agonists that was utilized in studies aiming to define AR subclasses [75–77]. CV1808 is a non-selective agonist, with Ki values of 560–1100 nM for A_1, and 190 nM for A_{2A} [78]. It was later reported that A_{2B} Ki is similarly low to that of A_{2A} AR subtype [79].

CV1808 is being used in various investigations, mainly concerning cardiovascular and immune research areas [80–84]. CV1808 has yet to be tested for its anti-platelet properties.

AMP597

AMP597 was first described by Smits et al. in 1998 as a novel cardioprotective A_1/A_2 agonist [85]. It has high affinity for the A_1 (Ki = 2 nM) and A_{2A} (Ki = 56 nM) receptor subtypes [86] and was later determined to be an A_{2B} agonist as well, based on the observation of its ability to induce phosphorylation of extracellular signal-regulated kinase and its protection against infarction in rabbit heart reperfusion studies [87]. It could be regarded, therefore, as a potent but non-selective AR agonist.

It has not been extensively studied; although it has been the subject of cardiac protection studies, a lack of publications since 2010 suggests that this line of research has been abandoned, despite the fact that it was undergoing clinical phase II studies in patients suffering acute myocardial infarction in 2000 [86]. Its effect on platelets remains unknown.

3.1.2. Adenosine Derivatives with Substituents at C1′ to C5′ Positions

NECA

5′-N-ethylcarboxamidoadenosine, commonly abbreviated as NECA, was first described in 1977 as a vasodilator, and then as a platelet function inhibitor in the 1980s [88]. NECA was employed in early radioligand studies to characterize AR platelet receptors, and was established to bind to two distinct binding sides at submicromolar concentrations [89,90].

NECA IC_{50} for platelet aggregation in human material was established at 0.36 μM (CI95% 0.35–0.38 μM) [91]. Ki for human AR subtypes were set at 560 nM (480–650 nM) for A_1, 620 nM (300–1300 nM) for A_{2A}, and 6.2 nM (5.1–7.5 nM) for A_3, showing a lack of selectivity between A_{2A} and A_1 receptor subtypes [92]. However, it was described as a suitable A_{2B} agonist, with IC_{50} of 3.1 μM (cAMP production in CHO cells) [93].

NECA has no current medical applications and has never been a subject of clinical testing. It is most commonly used in basic, platelet and vascular, and neurological research.

3.1.3. Compounds with Substituents at C1 to C8 and C1′ to C5′ Positions

CGS 21680

CGS 21,680 is one of the earliest synthesized adenosine analogue AR agonists. It was primarily used to elucidate the AR subclass division into A_{2A} and A_{2B} [76,94]. It is a strong, full agonist, selective towards A_{2A} (Ki A_{2A} = 27 nM, Ki A_{2B} is over1000 nM) [95]. It is probably the most commonly-employed AR A_{2A} agonist; it is used through a variety of research, especially in neurological studies. However, it has not been a subject of any clinical trials.

Its effect on platelets has been already established. Early studies reported IC_{50} 0.82 μM (CI95% 0.6–1.1) for human platelet aggregation, as measured by turbidimetry, and EC_{50} 0.083 ± 0.005 μM for stimulation of adenylate cyclase in human platelets [91]. Subsequently, CGS 21,680 has been used for further platelet research, including, but not limited to, studies on the association between depression and platelet signaling dysregulation [96], species-dependent platelet function [97], and neutrophil involvement and signaling in thrombosis [98].

HE-NECA

Another AR agonist extensively employed in a variety of research areas is the A_{2A} selective agonist HE-NECA, which was derived from non-selective AR agonist NECA. It has good selectivity between A_2 and A_1 receptor subclasses, but only slight selectivity between A_2 and A_3 subclasses (Ki A_2 = 130 nM; Ki A_2 = 2.2 nM; Ki A_3 = 24 nM) [99]. It was reported as an anti-platelet agent in 1994 [99], when its anti-aggregatory potency was estimated as three-fold stronger in comparison to NECA. The anti-platelet activity of HE-NECA was confirmed in an in vivo study in rabbits, in which the drug was administered at a dose of 10 μg/kg: Platelet accumulation in pulmonary microcirculation was found to fall by over 50% after challenge with ADP [100]. HE-NECA was also used in a Borea group study investigating the influence of caffeine (AR inhibitor) on platelet function. In this study, HE-NECA was found to increase cAMP levels with an EC_{50} of 59 ± 3 nM, and inhibit ADP-induced human platelet aggregation (measured by turbidimetric method) with an IC_{50} of 90 ± 6 nM [101,102]. Recently, HE-NECA was also used in work focused on a quantification of different imaging approaches to experiments carried out under flow conditions, where 10 μM HE-NECA was found to inhibit clot formation under flow in whole blood by 82%, based on the volumes of aggregates recorded by confocal microscopy [103].

HE-NECA is also employed in other research disciplines, including renal function investigation [104], and neurological [105,106] and immunological [107] research.

UK-432094

This AR agonist is usually known as UK-432,094 in the literature, but notations UK-432094 or UK432094 are also in use. UK-432,094 was tested by PFIZER in a phase II clinical trial (ClinicalTrials.gov Identifier: NCT00430300) as an inhalation agent for severe chronic obstructive pulmonary disease; however, the trial was terminated due to low treatment effectiveness. UK-432094 is a selective A_{2A} agonist (Ki of 4.75 nM) [108], with reported EC_{50} as low as 5.4 ±1.8 nM (cAMP level evaluation in CHO cells stably expressing human A_{2A} and A_{2B} receptors) [109]. Its anti-platelet effect has been recently assessed using multiple electric aggregometry in whole blood. Using this technique, IC_{50} was found to be below 1 μM, with an inhibition rate of 40% at this concentration. The agonist had an ability to practically abolish aggregation at higher concentrations (79% inhibition at 100 μM), while demonstrating no cytotoxic effect on platelets [110].

UK-432,094 has been prevalently used in basic research of the receptor–ligand binding mechanisms, providing insights into A_{2A} receptor structure [108], binding sight dynamics and agonist efficacy [111–113], and receptor interactions with other molecules [114].

3.2. Non-Adenosine Compounds

Apart from compounds based on the adenosine molecule modified chemically by introducing various substituents, AR agonists could also be found among substances of different chemical structure. The examples of such the AR agonists are given below.

3.2.1. BAY 60-6583

BAY 60-6583 was patented in 2001 as a highly-selective A_{2B} agonist (Ki A_{2A} is over10000 nM, Ki A_{2B} = 3–10 nM). Since then, it has been used in various research areas, including in vitro and in vivo immunological [115], cardiological [116,117], and oncological [118,119] research, lung disease and damage control studies [120,121], as well as the therapy of renal nephropathy [122]. So far, it is the only selective A_{2B} agonist in wide use. It has been reported to be a subject of pre-clinical studies to treat coronary artery disease and atherosclerosis [123], but it has not been registered for clinical trials.

The anti-platelet effects of BAY 60-6583 have not been studied in humans; however, Bot et al. report no decrease in aggregation (as measured by turbidimetry) or any change in platelet surface activation markers after treatment with BAY 60-6583 (50 µg/day) in $ApoE^{-/-}$ mice [124].

3.2.2. LUF5834 and LUF5835

A series of non-adenosine compounds were synthesized in 2004 as an attempt to generate an A_{2B} AR subtype selective agonist [125]. LUF5834—2-amino4-(4-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile was described as a partial agonist (EC_{50} of 12 nM for A_{2B} receptor) slightly selective between A_{2A} and A_{2B} (Ki of 28 ± 4 nM and 12 ± 2 nM, respectively) or A_1 receptor subtypes, but selective over the A_3 subtype. Its analogue, LUF5835 (2-amino4-(3-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile) is a full A_{2B} agonist with EC_{50} of 10 nM, with a similar selectivity profile. It was later reported that LUF5834 binds to a different receptor site of A_{2A} AR as compared to adenosine-based agonists, suggesting a distinct binding site for this class of agonists on AR receptors [126].

Both of these compounds have yet to be thoroughly characterized in literature (however, they both have been used in cardiac research [127,128]), and have not been proposed as anti-platelet agents.

4. Dual Therapy

Anti-platelet therapy is an obvious solution for the treatment and management of arterial thrombosis dependent on blood platelet hyperactivity, often resulting in cardiovascular disease and stroke—the leading causes of morbidity and mortality in developed countries. Several therapeutic options are currently available; however, the problem of efficient and safe therapy remains unsolved, and there is still a demand for novel platelet inhibitors and new therapeutic options.

In clinical practice, efficient anti-platelet treatment is often hindered by reduced sensitivity to many anti-platelet agents. High dosages of anti-platelet drugs, while preventing excessive clotting, frequently also lead to bleeding incidents and moderate to severe side effects. To avoid higher drug doses, combined therapy based on the administration of two or more drugs acting on different platelet activation pathways is often used as an alternative. An example of such an approach currently used in clinical practice is the combined administration of acetylsalicylic acid (an inhibitor of thromboxane A_2 formation) and clopidogrel (an inhibitor of the $P2Y_{12}$ receptor). The $P2Y_{12}$ receptor is the main therapeutic target in anti-platelet therapy, targeted at the ADP-dependent activation pathway [2]. Its agonization enhances the process of platelet aggregation initiated through the $P2Y_1$ receptor. Unfortunately, such treatment is still beset by the problem of resistance, especially among patients with type 2 diabetes, i.e., a group at higher risk of thromboembolic events [129–131].

Our research group has recently proposed a novel approach based on the simultaneous application of two anti-platelet agents, a $P2Y_{12}$ antagonist and an AR agonist, which has been found to deepen the action of $P2Y_{12}$ antagonist [110]. Based on this report, we believe that adenosine receptor agonists could

significantly enhance the anti-platelet effect of $P2Y_{12}$ antagonists, despite possessing different selectivity profiles and anti-platelet activities. A strategy focused on a purinergic pathway and involving low-dose inhibition of classical ($P2Y_{12}$) purinergic ADP receptors with the simultaneous activation of adenosine receptors may present a novel, promising approach to prevent thrombotic events, and should be further investigated.

5. Conclusions

Adenosine receptor agonists have been shown to have anti-platelet effect; however, not all of them are of the same magnitude, with some even presenting no discernible impact on aggregation. It is difficult to unambiguously give a simple answer as to whether this group of compounds stands a fair chance of becoming anti-platelet drugs in the foreseeable future. Too few known AR agonists have been evaluated specifically for modulation of platelet function, and as this topic was studied in the 1980s, some of the data require replication and confirmation using modern methodology. However a few AR agonists, like NECA, HE-NECA, CGS 21680, 2-chloroadenosine, and recently, PSB-15826, were confirmed to have platelet inhibiting properties, and the concept of employing them in preventing thrombus formation is re-emerging. More studies of different AR agonists focused specifically on anti-platelet properties are needed, as predictions based on physicochemical properties prove to be unreliable [68]; however, currently-available data suggests that such attempts should be focused on A_{2A} AR agonists, as agonization of A_{2B} AR has not been reliably reported to impact platelet aggregation or activation.

The use of AR agonists as anti-platelet medication appears feasible following further research focused explicitly on this goal, especially when applied in combination with other anti-platelet agents, to identify therapies demonstrating effective antithrombotic properties without risking severe side effects.

Funding: 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, and by a grant from Medical University funds: 502-03/6-020-01/502-64-125-18.

Conflicts of Interest: The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

ADP	Adenosine Diphosphate
AR	Adenosine Receptor
cAMP	Cyclic Adenosine Monophosphate
CHO	Chinese Hamster Ovary Cells
EC_{50}	Half Maximal Effective Concentration
IC_{50}	Half Maximal Inhibitory Concentration
KO	Knock-Out
FDA	Food and Drug Administration
HE-NECA	2-Hexynyl-5′-(N-ethylcarboxamido)adenosine
IUPAC	International Union of Pure and Applied Chemistry
NECA	5′-(N-ethylcarboxamido)adenosine
PRP	Platelet-Rich Plasma

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STATEMENT

I declare that my contribution to the article:

Wolska N, Rozalski M. 2019. Blood Platelet Adenosine Receptors as Potential Targets for Anti-Platelet Therapy, International Journal of Molecular Sciences, 20.
DOI: 10.3390/ijms20215475

consisted of participation in writing the original draft of the manuscript, the manuscript review and editing, and preparing the response to the reviewers.

I agree to use of the above publication by Nina Wolska in her doctoral dissertation.

OŚWIADCZENIE

Oświadczam, że mój wkład w artykuł:

Wolska N, Rozalski M. 2019. Blood Platelet Adenosine Receptors as Potential Targets for Anti-Platelet Therapy, International Journal of Molecular Sciences, 20.
DOI: 10.3390/ijms20215475

polegał na udziale w przygotowaniu i edycji manuskryptu oraz odpowiedzi na uwagi recenzentów.

Wyrażam zgodę na wykorzystanie powyższej publikacji przez Ninę Wolską w jej pracy doktorskiej.

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Article

Adenosine Receptor Agonists Exhibit Anti-Platelet Effects and the Potential to Overcome Resistance to $P2Y_{12}$ Receptor Antagonists

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Received: 30 September 2019; Accepted: 25 December 2019; Published: 28 December 2019

Abstract: Large inter-individual variation in platelet response to endogenous agonists and pharmacological agents, including resistance to antiplatelet therapy, prompts a search for novel platelet inhibitors and development new antithrombotic strategies. The present in vitro study evaluates the beneficial effects of three adenosine receptor (AR) agonists (regadenoson, LUF 5835 and NECA), different in terms of their selectivity for platelet adenosine receptors, when used alone and in combination with $P2Y_{12}$ inhibitors, such as cangrelor or prasugrel metabolite. The anti-platelet effects of AR agonists were evaluated in healthy subjects (in the whole group and after stratification of individuals into high- and low-responders to $P2Y_{12}$ inhibitors), using whole blood techniques, under flow (thrombus formation) and static conditions (study of platelet activation and aggregation). Compared to $P2Y_{12}$ antagonists, AR agonists were much less or not effective under static conditions, but demonstrated similar antiplatelet activity in flow. In most cases, AR agonists significantly enhanced the anti-platelet effect of $P2Y_{12}$ antagonists, despite possessing different selectivity profiles and antiplatelet activities. Importantly, their inhibitory effects in combination with $P2Y_{12}$ antagonists were similar in high- and low-responders to $P2Y_{12}$ inhibitors. In conclusion, a combination of anti-platelet agents acting via the P1 and P2 purinergic receptors represents a promising alternative to existing antithrombotic therapy.

Keywords: platelet; adenosine receptor; adenosine receptor agonist; $P2Y_{12}$ antagonist; anti-platelet therapy

1. Introduction

The leading causes of morbidity and mortality in developed countries are cardiovascular disease and stroke, resulting predominantly from arterial thrombosis dependent on blood platelet hyperreactivity. Anti-platelet therapy would appear to be an obvious solution for the treatment and management of such disorders [1,2]. Several therapeutic options are currently available; however, the problem of efficient and safe therapy is complicated by the frequent development of drug resistance stemming from high inter-individual variation among patients caused by genetic and environmental factors [3,4]. Therefore, there is still a demand for novel platelet inhibitors and new therapeutic options.

ADP is one of the key mediators of both physiological haemostasis and thrombosis, being not only a direct agonist of platelets, but also an important factor released from platelet intracellular structures, enhancing the platelet response initially induced by other activators. Platelets have two ADP receptors on their surface: the $P2Y_1$ receptor initiates platelet aggregation, while the $P2Y_{12}$ receptor enhances this process, eventually leading to the formation of a clot. Due to this fact, the $P2Y_{12}$ receptor is the main therapeutic target in anti-platelet therapy targeted at the ADP-dependent activation pathway [5]. Generally, the most commonly used clinically approved $P2Y_{12}$ inhibitors include the thienopyridine-class inhibitors (ticlopidine, clopidogrel and prasugrel), the ATP analogue cangrelor, and the cyclo-pentyl-triazolo-pyrimidine derivative ticagrelor [3,5]. Thienopyridines are prodrugs: their short-lived active metabolites irreversibly inactivate the receptor and consequently inhibit ADP-induced platelet activation. Cangrelor is the first (recently approved) intravenous $P2Y_{12}$ receptor inhibitor that reversibly and non-competitively blocks ADP signalling [6].

Adenosine is an important purine metabolite, serving not only as a component of nucleic acids and ATP, the most important energy carrier in the cell, but also as a signalling molecule regulating many cell processes [7,8]. Adenosine receptors (AR) are a subfamily of highly conserved G protein-coupled receptors located in the membranes of various cells and with different physiological functions. Of the four known AR subtypes (A_1, A_{2A}, A_{2B} and A_3) only A_{2A} and A_{2B} are expressed in platelets [7,9]. Activation of platelet AR results in the enhancement of intracellular cAMP levels and, consequently, the inhibition of platelet activation and aggregation [10].

Apart from the natural agonist adenosine, a group of synthetic, long-lasting agonists were developed [11]; of these, some display good selectivity for A_{2A} or A_{2B} receptors, and others are non-selective compounds activating more than one type of adenosine receptor. One of the oldest known anti-platelet adenosine analogues is 2-chloroadenosine [12]. Other AR agonists were also previously described in the literature as platelet aggregation blocking compounds [12–14].

In clinical practice, efficient anti-platelet treatment is often hindered by reduced sensitivity to many anti-platelet agents. To avoid higher drug doses, and therefore a higher risk of bleeding, combined therapy based on the administration of two or more drugs acting on different platelet activation pathways is often used as an alternative. An example of such an approach is the combined administration of acetylsalicylic acid (an inhibitor of thromboxane A2 formation) and clopidogrel (an inhibitor of the $P2Y_{12}$ receptor). Unfortunately, such treatment is still beset by the problem of resistance, especially among patients with type 2 diabetes, i.e., a group at higher risk of thromboembolic events [15–17]. We recently proposed a novel approach based on the simultaneous application of two anti-platelet agents, a $P2Y_{12}$ antagonist and an AR agonist, which was found to deepen the action of $P2Y_{12}$ antagonist [18].

The aim of this study is to further explore the potential of combined anti-platelet therapy consisting of simultaneous $P2Y_{12}$ inhibition and adenosine receptor agonization. The lowered dosage of $P2Y_{12}$ inhibitors could potentially reduce side-effects (e.g., excessive bleeding), while the addition of AR agonization would provide adequate anti-platelet effect, and therefore excessive clot formation prevention. We used in vitro methods (whole blood electrical aggregometry, flow cytometry measurement of P-selectin and active form GPIIbIIIa expression, and aggregation/adhesion under flow conditions) to evaluate anti-platelet effects of $P2Y_{12}$ inhibitors: cangrelor and prasugrel (active metabolite R-138727), as well as AR agonists: NECA (non-selective agonist activating both A_{2A} and A_{2B}, with strong antiplatelet effect), regadenoson (agonist selective for A_{2A}, with moderate antiplatelet effect), and LUF5835 (agonist preferentially activating A_{2B}, with a weak antiplatelet effect) in single (either $P2Y_{12}$ antagonist or AR agonist alone) and dual ($P2Y_{12}$ antagonist and AR agonist applied simultaneously) systems.

In the present study, we found that the use of AR agonists can lead to significantly higher inhibition of platelet function caused by $P2Y_{12}$ antagonists and effect was on the same level in high- and low-responders to $P2Y_{12}$ inhibitors, which suggests that AR agonists have the potential to overcome resistance to $P2Y_{12}$ blockers.

2. Results

2.1. Effects of AR Agonists on Platelet Viability

To ensure that AR agonists used in this work do not exhibit cytotoxic effects on blood platelets, which could influence the results of functional tests, platelet viability was measured. The assay was performed on resting platelets preincubated with NECA, regadenoson and LUF5835. Positive control (1% paraformaldehyde) decreased the fraction of viable cells by 81% on average ($p < 0.01$), while none of the AR agonists exhibited cytotoxic effect (viable cells fraction was not decreased in comparison to the control of non-treated platelets) (Figure S1). This ensures that the anti-platelet effect observed in further research is not due to decreasing fraction of viable platelets (this result should not be interpreted as an assessment of the AR agonists overall toxicity).

2.2. Effects of AR Agonists on Platelet Aggregation

The anti-aggregatory effects of AR agonists NECA, regadenoson, and LUF5835 were evaluated using whole blood stimulated with 10 μM ADP. Dose-response non-linear regression curves were plotted, where possible, to determine the half maximal inhibitory concentration (IC_{50}). NECA yielded a curve with the maximal inhibition value of 79.1 ± 4.0%, and IC_{50} of 0.5 μM (95% confidence interval: 0.33 to 0.86) with a coefficient of determination (R^2) equal to 0.876 (Figure 1A). Regadenoson curve had the maximal inhibition value of 38.1 ± 3.2%, IC_{50} of 1.2 μM (95% confidence interval: 0.43 to 3.68), and R^2 equal to 0.201 (Figure 1B). LUF5835, unlike the other AR agonists, did not influence platelet aggregation, even at high concentrations - incubation with 50 and 100 μM LUF5835 did not result in a significant inhibition of platelet aggregation (Figure 1C).

2.3. Combined Effect of AR Agonists and $P2Y_{12}$ Inhibitors on Platelet Aggregation in Whole Blood

AR agonists were used in a combination with two $P2Y_{12}$ receptor antagonists (one AR agonist + one $P2Y_{12}$ antagonist in each combination): cangrelor and prasugrel metabolite R-138727 (PM). Each compound was used in its IC_{50}, with the values taken from our previous work [18]: NECA 0.5 μM, regadenoson 1.2 μM, cangrelor 17 nM, and PM 1.3 μM. In the case of LUF5835, it was not possible to establish an inhibition curve or an IC_{50} value; therefore, a concentration of 100 μM was used.

Both $P2Y_{12}$ antagonists significantly reduced platelet aggregation: cangrelor by a mean value of 41% and PM by 46%; however, the percentage inhibition between subjects displayed high coefficients of variation: 54% and 38%, respectively, ($n = 15$). Among the AR agonists, NECA caused a statistically significant decrease in aggregation, whereas regadenoson and LUF5835 did not (Figure 2). Considerable coefficients of variation were also observed (Table S1).

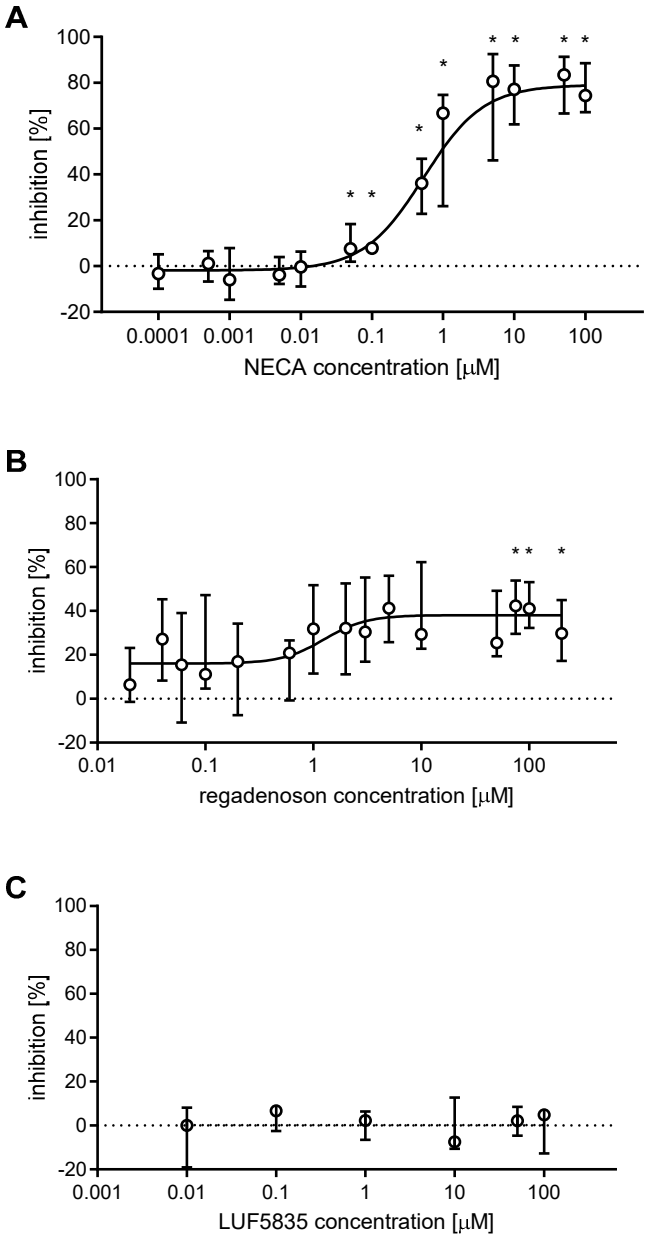

Figure 1. Inhibition of ADP-induced platelet aggregation by AR agonists. Data shown as median ± interquartile ranges, with dose-response plots based on the AUC values using non-linear regression analysis (NECA (**A**) $n = 5$, regadenoson (**B**) $n = 5$; LUF5834 (**C**) $n = 4$). Changes in platelet aggregation were measured in whole blood in response to 10 μM ADP after 3 min preincubation at 37 °C with AR agonist. Data was analysed for statistical significance using repeated measures ANOVA with Geisser-Greenhouse correction and Holm-Sidak's multiple comparisons test. * indicates statistical significance $p < 0.05$ or lower.

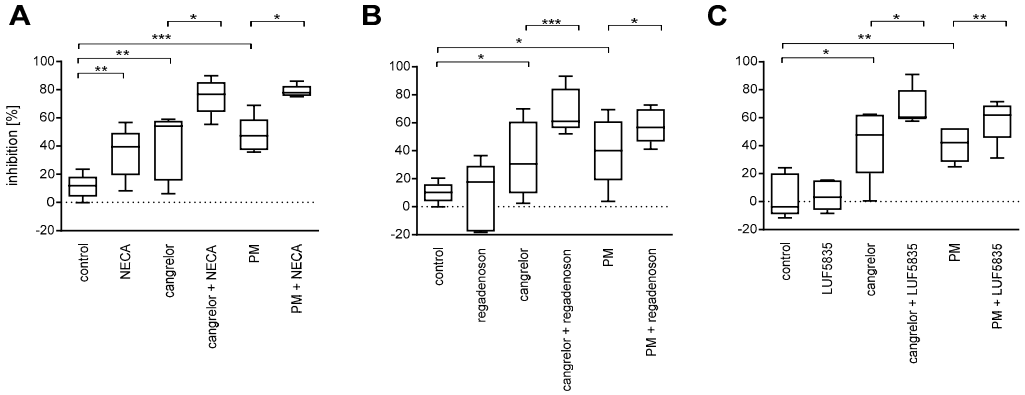

Figure 2. AR agonists intensify the anti-aggregatory effect of $P2Y_{12}$ antagonists: NECA (**A**), regadenoson (**B**), and LUF5835 (**C**). Data are presented as median, interquartile range and minimum and maximum values (n = 5 for each AR agonist; totally n = 15). Changes in platelet aggregation were measured in whole blood in response to 10 μM ADP after 3 min preincubation at 37 °C with AR agonist and cangrelor, or 15 min preincubation at 37 °C with PM. Statistical significance was estimated by repeated measures ANOVA with Bonferroni's multiple comparison test, or Friedman's test with Dunn's multiple comparison test depending on data distribution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Simultaneous application of an AR agonist was found to intensify the inhibitory effect of $P2Y_{12}$ on platelet aggregation for all (six) combinations; however, the coefficients of variation were also high (Table S1).

2.4. Identification of AR Agonist Effect in the Subpopulations of High- and Low-Responders to $P2Y_{12}$ Antagonists

Since platelet sensitivity to $P2Y_{12}$ antagonists was highly differentiated, the numbers of healthy subjects increased to n = 20 for each AR agonist and divided into two subpopulations: high-responders and low-responders to $P2Y_{12}$ receptor inhibitors, separately for cangrelor and PM. The cut-off values were medians of the inhibition rates (NECA: 51.6% for cangrelor, 44.7% for PM; regadenoson: 37.9% for cangrelor, 43.5% for PM; LUF5835: 41.9%, for cangrelor, 47.0% for PM). The anti-aggregatory effect of AR agonists on the $P2Y_{12}$ inhibited platelets was then evaluated in the established subpopulations.

Significant differences in the inhibition of platelet aggregation were found between the $P2Y_{12}$ antagonist + AR agonist group and the $P2Y_{12}$ antagonist group, for both low- and high-responder groups, for all (six) combinations (Figure 3). The inhibition increase factor, i.e., the number of times an anti-aggregatory effect is intensified by an AR agonist, indicated that the $P2Y_{12}$ antagonist low-responders demonstrated markedly higher inhibition of platelet response caused by AR agonists than the high-responders (Figure 3). Interestingly, combinations of stronger AR agonists (NECA and regadenoson) with $P2Y_{12}$ yielded comparable overall aggregation inhibition in both high- and low-responder groups.

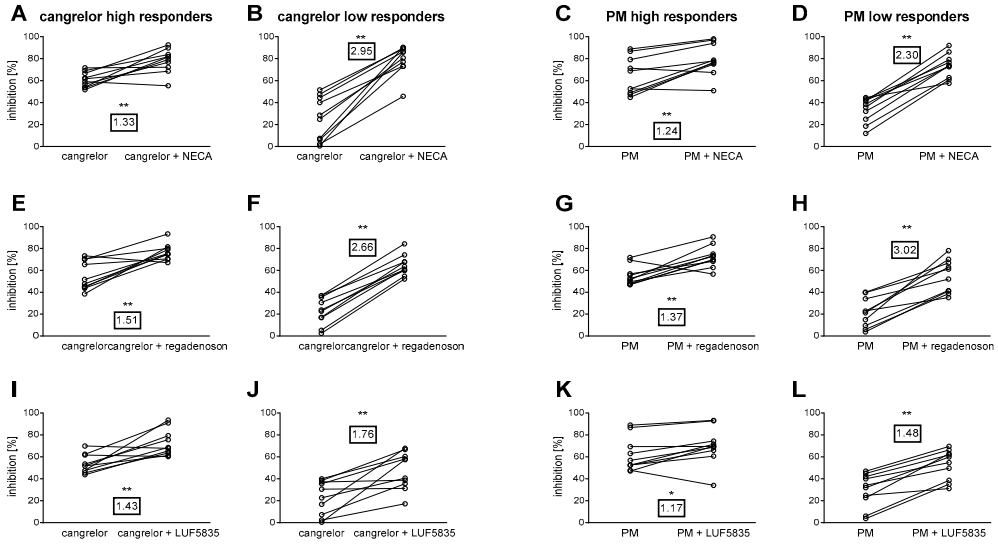

Figure 3. Anti-platelet effect of AR agonists in high- and low-responders to $P2Y_{12}$ antagonists. Combinations of cangrelor (**A**,**B**,**E**,**F**,**I**,**J**), and PM (**C**,**D**,**G**,**H**,**K**,**L**) with NECA (**A**–**D**) regadenoson (**E**–**H**), and LUF5835 (**I**–**L**) are shown. Framed numbers denote the mean inhibition increase factor (arithmetic mean of ratios calculated individually for each donor). Data are shown as pairs of data points (without and with AR agonist) for each blood donor (n = 10 in each group). Changes in platelet aggregation were measured in whole blood in response to 10 μM ADP after 3 min preincubation at 37 °C with AR agonist and cangrelor, or 15 min preincubation at 37 °C with AR agonist and PM. Statistical significance between $P2Y_{12}$ inhibitor alone and $P2Y_{12}$ inhibitor with AR agonist groups for each agonist-antagonist pairing estimated by two-tailed Wilcoxon signed rank test. * $p < 0.05$, ** $p < 0.01$.

2.5. Combined Effect of AR Agonists and $P2Y_{12}$ Inhibitors on Platelet Reactivity

AR agonists were used in combination with two $P2Y_{12}$ receptor antagonists: either cangrelor or prasugrel metabolite R-138727. NECA, regadenoson, cangrelor and PM were used at concentrations equal to previously established IC50 values, while LUF5835 was applied at 100 μM. Both $P2Y_{12}$ antagonists alone significantly reduced platelet activation: cangrelor by a mean value of 53% (P-selectin expression) and by 47% (PAC-1 binding); PM by 42% (P-selectin expression) and by 30% (PAC-1 binding). Observed coefficients of variation in the percentage inhibition by $P2Y_{12}$ antagonists or AR agonists between subjects ranged from 23% to 41% for P-selectin expression and from 14% to 35% for PAC-1 binding (Table S1). Among AR agonists, NECA showed a significant decrease of platelet reactivity (as shown by P-selectin expression), whereas regadenoson and LUF5835 did not significantly affect platelet reactivity in the applied experimental conditions.

The simultaneous application of a $P2Y_{12}$ antagonist and an AR agonist caused significantly greater inhibition of platelet reactivity for cangrelor pairings with NECA and regadenoson (P-selectin only) (Figure 4A)., and PM pairing with NECA (Figure 4A,B). Coefficients of variation were also high in those groups (Table S1 in the Supplementary Material).

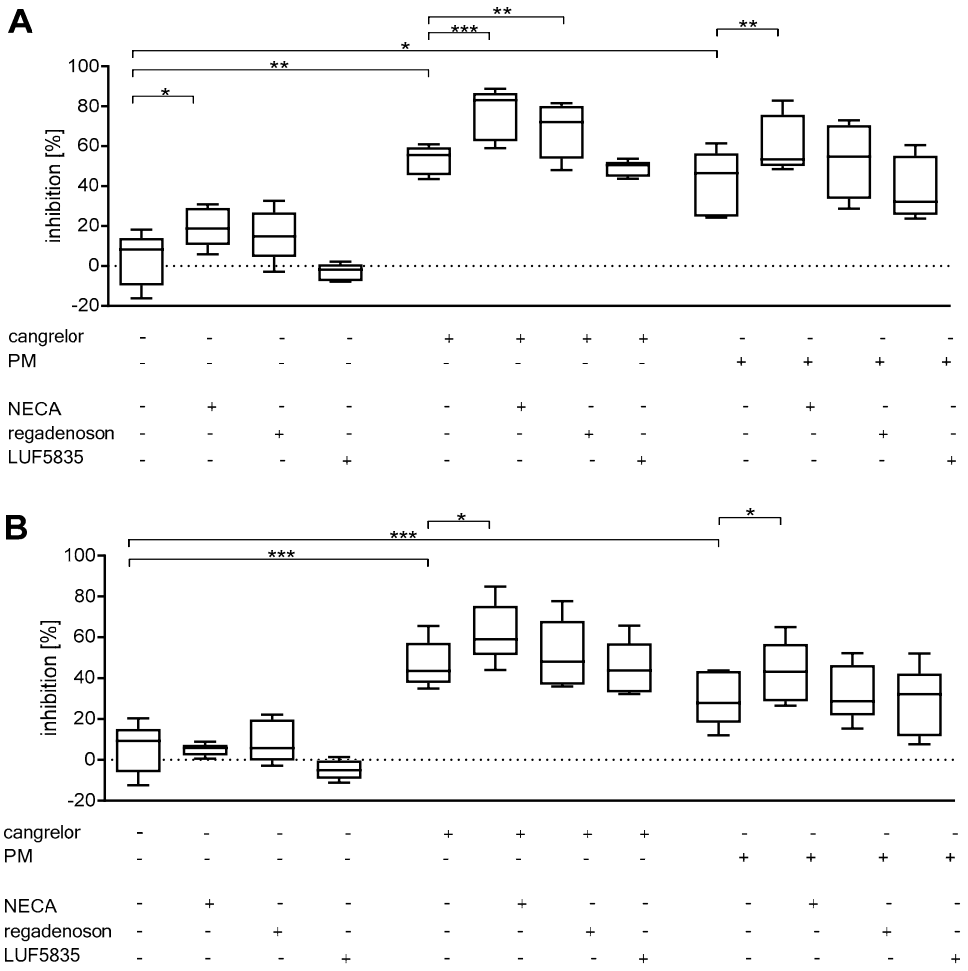

Figure 4. AR agonists deepen the $P2Y_{12}$ inhibitory effect on platelet reactivity as measured by P-selectin expression (**A**) and PAC-1 binding (**B**). Data are given as median, interquartile range, and minimum and maximum values ($n = 5$). Changes in platelet reactivity were measured in whole blood in response to 20 μM ADP after 3 min preincubation at 37 °C with AR agonist and cangrelor, or 15 min preincubation at 37 °C with PM. Statistical significance estimated by repeated measures ANOVA with Bonferroni's multiple comparison test or Friedman's test with Dunn's multiple comparison test depending on the data distribution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

2.6. Combined Effect of AR Agonists and $P2Y_{12}$ Inhibitors on Thrombus Formation Under Flow Conditions

In the experiments assessing the effects of AR agonists and $P2Y_{12}$ inhibitors on thrombus formation under flow conditions, all compounds were used in concentrations corresponding to the previously established IC_{50} for platelet aggregation, apart from LUF5835, which was applied at 100 μM.

Under applied conditions, AR agonists alone decreased clot formation by a mean value of NECA 83.5 ± 10.3%, regadenoson: 83.6 ± 2.0% and LUF5835 74.9 ± 18.9%. $P2Y_{12}$ antagonists alone decreased clot formation by cangrelor (by 68%) and PM (by 77%); however, the obtained variance coefficients for $P2Y_{12}$ inhibitors were high: 103% and 104% respectively. PM treatment did achieve significant inhibition (Figure 5). The mean clot formation decrease for AR agonists was found to be 16.5 ± 11.0% for NECA, 16.4 ± 22.3% for regadenoson, and 25.1 ± 20.0% for LUF5835, all with high CVs (Table S1).

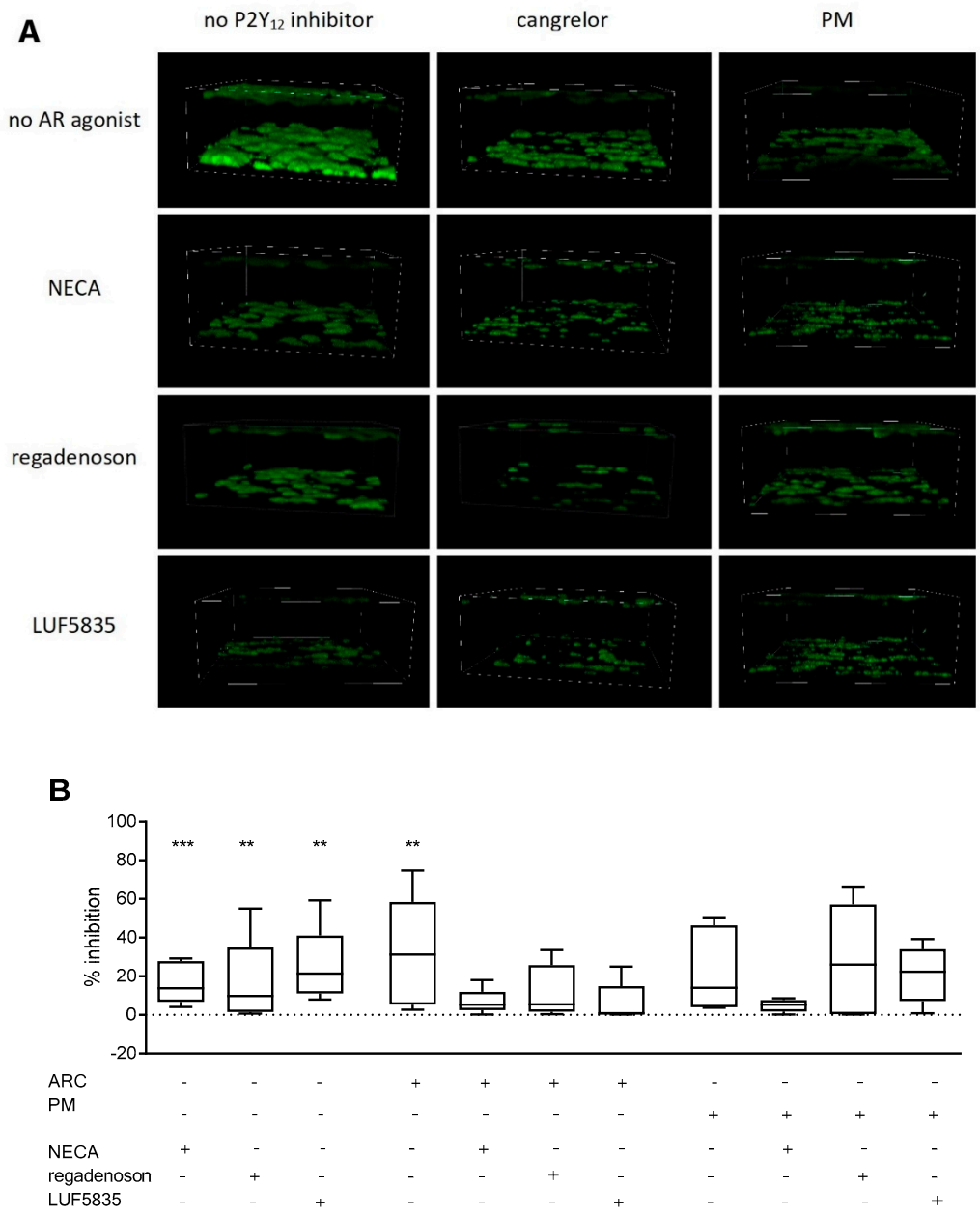

Figure 5. Anti-platelet effect of AR agonists and $P2Y_{12}$ antagonists on thrombus formation under flow conditions. Representative images (**A**) and clot formation inhibition rates (decrease of total clot volume) (**B**). Data are given as median, interquartile range, and minimum and maximum values ($n = 5$). Changes in thrombus formation were analysed in whole blood in response to 60 dyne/cm^2 flow after 3 min preincubation at 37 °C with an AR agonist and cangrelor, and 15 min preincubation at 37 °C with PM ($n = 5$). The samples were imaged by confocal microscopy and analysed using FIJI image analysis software to obtain summarized clot volumes (for details see Materials and methods). Statistical significance of differences between summarized clot volumes for various AR agonists, $P2Y_{12}$ antagonists and combinations thereof was estimated by the bootstrap-boosted unpaired Student's t-test. ** $p < 0.01$, *** $p < 0.005$.

Pairing AR agonists with $P2Y_{12}$ inhibitors did not significantly improve the anti-platelet effect. It should be noted that observed coefficients of variation were also very high in those groups: 151% for cangrelor + LUF5835 (Table S1).

3. Discussion

Adenosine receptor (AR) ligands, developed in recent decades, represent a group of agents with anti-inflammatory activity, which can be useful in the prevention and treatment of human diseases [19–21]. Promising prospects are emerging for the treatment of arrhythmias, cardiac and cerebral ischaemias, neurodegenerative diseases, inflammation, sleep disorders, pain, diabetes, cancer, renal failure and glaucoma [22]. Some investigated AR agonists were also shown to be potent anti-platelet agents [9,23]. Given the substantial inter-individual variability in response to platelet inhibitors observed in the general population, we hypothesized that the agonists of the A_{2A} and A_{2B} adenosine receptors, which are expressed in platelets, might play a supporting role for anti-platelet drugs, in particular in patients demonstrating poor responsiveness to conventional anti-platelet therapy. The potential advantage of this approach would lie in lowered dosage of traditional anti-platelet drugs, thus avoiding bleeding and other side-effects, while maintaining high protection from thromboembolic events. Therefore, the present in vitro study evaluates the beneficial effects of selected adenosine receptor agonists used in combination with newer $P2Y_{12}$ antagonists, which are administered intravenously (cangrelor) or given orally (prasugrel) during the anti-platelet therapy [24]. More precisely, it examines three AR agonists with molecular structures similar to or different from endogenous adenosine and which demonstrate different selectivity profiles toward A_{2A} and A_{2B} adenosine receptors [22].

Our findings demonstrate that the use of combination of a $P2Y_{12}$ antagonist (cangrelor or prasugrel metabolite) and an AR agonist (adenosine-based compounds: regadenoson, NECA; non-adenosine-based compound: LUF5835) leads to a greater inhibition of platelet function than the $P2Y_{12}$ antagonist alone. What is important is the effect was specific, as the examined AR agonists were not cytotoxic for the cells and their action was much more pronounced in individuals with poor response to $P2Y_{12}$ inhibitors. In a certain sense, this study supports and complements our recent in vitro work, where the anti-aggregatory activity of other AR agonists was examined in the absence or presence of the $P2Y_{12}$ antagonists given above [18].

However, while the two studies have similar aims and target populations, their experimental designs are quite different: they employ different AR agonists and use different methods to determine anti-platelet properties. In addition, the present work was extended with further analyses of the influence of $P2Y_{12}$ antagonists on the effects of the AR agonists, i.e., by comparing groups of high- and low-responders to $P2Y_{12}$ inhibitors. To more closely reflect the in vivo environment, the anti-platelet effects of AR agonists were evaluated using whole blood techniques, under flow (thrombus formation) and static conditions (study of platelet activation and aggregation). However, whole blood aggregometry was used to estimate the inhibitory strength of AR agonists and assess the half maximal inhibitory concentration (IC_{50}) values of examined compounds; they were also used to evaluate the anti-aggregatory effects of AR agonists in combination with $P2Y_{12}$ antagonists, which were monitored in a whole population of healthy individuals and in subpopulations of high- and low-responders to $P2Y_{12}$ inhibitors. It is important to note that all AR agonists chosen for the study were tested in a dual model with $P2Y_{12}$ inhibitors, irrespective of the degree of anti-aggregatory activity they exhibited in preliminary experiments. However, the concentration of the AR agonist used for testing with the $P2Y_{12}$ antagonist was chosen based on its anti-aggregatory activity: i.e., regardless of the parameter being estimated, the anti-aggregatory compound was applied at a concentration corresponding to its IC_{50} value. In addition, the AR agonist which did not display antiaggregatory properties was analysed further at the highest used concentration: 100 μM. Cangrelor and prasugrel, being strong $P2Y_{12}$ inhibitors, were used at the IC_{50} values, which were previously reported [18].

The anti-aggregatory potency of the tested compounds was examined at the beginning of the study. Apart from NECA, the anti-platelet effects of regadenoson and LUF5835 were not obvious. It is simply because the anti-platelet properties of regadenoson or LUF5835 have not been explored so far. Regadenoson (CVT-3146, Lexiscan) is a synthetic AR agonist currently approved for clinical use as a pharmacologic stress agent for myocardial perfusion imaging (MPI). It demonstrates non-inferiority

to adenosine for detecting reversible myocardial perfusion defects, although it is a moderately selective (K_i = 290 nM), short acting A_{2A} AR agonist with low affinity for the remaining adenosine receptors [22,25,26]. In addition to its diagnostic application, regadenoson was considered for the treatment of inflammation and sickle cell disease, and was involved in the development of brain tumor-targeted drug delivery systems [27–29]. In contrast, little is known about the biological activity of LUF5835, a synthetic, atypical non-ribose compound, although it was found to display interesting selectivity for adenosine receptors. On the one hand, LUF5835 has a strong ability to activate human A_{2B} adenosine receptor (EC_{50} = 10 nM) but, on the other, it can also interact with human A_1 (K_i = 4.4 nM), A_{2A} (K_i = 21 nM) and A_3 (K_i = 104 nM) adenosine receptors [30]. Contrary to LUF5835, NECA is a well-described adenosine analogue possessing high vasodilatory and anti-platelet activity. When compared to adenosine, this non-selective AR agonist, exhibiting high affinity at A_1 (K_i = 14 nM), A_{2A} (K_i = 20 nM) and A_3 (K_i = 25 nM) adenosine receptors and lower affinity at A_{2B} AR (EC_{50} = 140 nM), was shown to be over 20,000 times more potent as a vasodilator and 5-10 more effective as an inhibitor of platelet aggregation in response to ADP and adrenaline [22,31]. As a result, NECA is often used as a reference compound; in the present study, it was used as a point of comparison with regadenoson and LUF5835 [29,30,32].

As with NECA, but unlike LUF5835, regadenoson caused significant inhibition of platelet aggregation within the concentration range 1-100 µM. However, at a concentration of 1 µM or higher, NECA was approximately twice as effective as regadenoson in reducing platelet aggregation (Figure S1). Assuming that the anti-platelet potential of AR agonists corresponds with their respective values of binding constants (K_i) determined for the A_{2A} adenosine receptor (high affinity receptor), which is preferential over the A_{2B} AR (low affinity receptor) for adenosine [33], regadenoson should have exhibited weaker inhibition in the functional tests than NECA or LUF5835, whereas the anti-aggregatory activity of LUF5835 should be comparable to that observed for NECA. However, in contrast, LUF5835 alone was not found to exert any inhibitory effect. One possible explanation for this inconsistency is that the affinity in LUF5835 binding at A_{2A} adenosine receptor was overestimated. This may be possible, as while the selectivity profile of LUF5835 is only given in one report of Beukers et al. [30], many more reports exist on the binding affinities of NECA and regadenoson, all of which indicate that NECA demonstrates higher affinity for the A_{2A} adenosine receptor than regadenoson: the Ki values were calculated to be within the range of 2.2-60 nM for NECA [34–36] and 290 nM-1.7 µM for regadenoson [37–39]. Therefore, it is not surprising that NECA displayed greater inhibition of platelet aggregation than regadenoson (up to 80%; Figure S1), and was comparable to the high affinity A_{2A}-selective AR agonist UK 432097 [18]. Interestingly, a recent work by Fuentes et al. on another group of synthetic AR agonists (PSB family) anti-platelet properties also reported a discrepancy between Ki values and anti-platelet potential, concluding that Ki to A_{2A} receptor is not predictive of those effects [32].

In the light of these observations, further experiments were carried out with NECA and regadenoson at their IC_{50} values (determined in aggregation) and LUF5835 at 100 µM. At the indicated concentrations, the inhibitory effect of AR agonists on platelet activation (5%–19% inhibition) or aggregation (4%–35% inhibition) was considerably lower than observed in studies of platelet thrombus formation on collagen, which reported very high reduction of thrombus volume, i.e., by 75%–84%. NECA appeared to be the most effective AR agonist as it significantly diminished ADP-induced platelet activation, expressed by the fraction of CD62P-positive platelets, ADP-stimulated aggregation, and thrombus formation, compared to controls (DMSO). Regadenoson and LUF5835 only exerted significant inhibitory action against thrombus formation. These observations are mostly in agreement with the study of aggregation by shear stress that showed that some adenosine derivatives, including NECA, increased the blood flow and platelet cAMP level, reduced platelet activation and retention of white blood cells; however, the differences in platelet retention between samples and control were not statistically significant [40].

As regards $P2Y_{12}$ antagonists, all examined parameters were strongly decreased as compared to controls; the degree of inhibition of platelet activation, aggregation and thrombus formation by $P2Y_{12}$ inhibitors reached respectively 52%, 46% and 81%; insignificant changes were only observed in one case, i.e., formation of platelet thrombus in the presence of PM. Overall, the results indicate that compared to $P2Y_{12}$ antagonists, AR agonists were much less effective under static conditions (in models with exogenous ADP), but demonstrated similar anti-platelet activity in flow (without ADP).

Although it is possible that AR agonists may be able to inhibit platelet activation and aggregation more effectively at lower agonist concentration, the present study did not explore the agonist dose-response relationship for obvious reasons: our aim was to obtain high and reproducible cell reaction to ADP; the experimental protocol included five compounds, each of which was tested in various combinations, and more importantly, the samples contained from one to three platelet inhibitors (DMSO, $P2Y_{12}$ inhibitor, AR agonist), depending on the mode of treatment. A desirable cell response was obtained after incubation of whole blood with 10 μM ADP in the aggregation experiments and with 20 μM ADP when studying platelet activation. Such concentrations of ADP may appear high, but they are nevertheless used in studies of platelet function, particularly those concerning aggregation [41–44].

In most cases, the combination of AR agonists with $P2Y_{12}$ inhibitors led to further inhibition of platelet function. A statistically significant decrease was observed in aggregation experiments, where all compounds improved the action of $P2Y_{12}$ antagonists, and in platelet activation, where the platelet inhibition caused by $P2Y_{12}$ antagonists increased by NECA or regadenoson. These observations are consistent with previous findings indicating that the anti-aggregatory effect of $P2Y_{12}$ inhibitors was enhanced by AR agonists such as UK 432097, 2-Cl-adenosine, MRE 0094 or PSB 0777 [18]. In addition, NECA, regadenoson and LUF5835 were able to increase the inhibition of platelet function caused by the anti-thrombotic activity of $P2Y_{12}$ inhibitors (reduction of thrombus volume was up to 75% compared to $P2Y_{12}$ antagonist alone), although the changes did not reach statistical significance. There are at least two reasons why this might be the case: $P2Y_{12}$ antagonists significantly decreased thrombus volume when applied alone; in addition, very high inter-assay variation was observed. Indeed, the CV values for thrombus volume ranged from 62% to 184% and were substantially higher than the CVs obtained in the remaining assays. In comparison, Ranjit et al. report inter-assay CVs varying from 8% to 638% with regard to blood clot parameters measured by thromboelastography in whole blood, demonstrating the high variability between haemostatic parameters [45].

Taken together, our findings provide further support for the potential therapeutic use of AR agonists in dual anti-platelet therapy in combination with $P2Y_{12}$ receptor inhibitors. Adenosine receptor agonists seem to be an attractive alternative to GPIIbIIIa or $P2Y_1$ receptor antagonists, which were demonstrated to have favorable effects on platelet function in combined therapy with thienopyridines [46,47].

High degrees of inter-individual variability were previously observed for the platelet response to agonists (particularly ADP) and anti-platelet drugs [48–51]; this was also observed in the present study. Therefore, the effect of AR agonists on platelet aggregation was evaluated in high- and low-responders to $P2Y_{12}$ inhibitors using aggregometry: a simple method which allows rapid assessment of platelet response.

All three examined AR agonists increased the anti-aggregatory action of $P2Y_{12}$ inhibitors, allowing their effects to be analysed in the established subpopulations. All the agonists also significantly improved the observed anti-aggregatory effects of $P2Y_{12}$ inhibitors in both high- and low-responders, although the inhibition ratio indicated considerably higher inhibition of platelet aggregation by AR agonists among the $P2Y_{12}$ low-responders than the high-responders (Figure 3). Accordingly, no difference was observed between subpopulations with regard to mean platelet inhibition by cangrelor + AR agonist or prasugrel metabolite + AR agonist (with the exception of LUF5835). Hence, patients resistant to a $P2Y_{12}$ inhibitor may benefit from adjunctive therapy with non-selective or A_{2A}-selective AR agonists.

In conclusion, the pharmacological response to anti-platelet agents such as $P2Y_{12}$ receptor inhibitors displays high inter-individual variability, and this prompted the search for new anti-platelet strategies. Our findings show that adenosine receptor agonists may significantly enhance the anti-platelet effect of $P2Y_{12}$ antagonists, despite possessing different selectivity profiles and anti-platelet activities. Significantly, the combination of anti-platelet agents acting via the P_1 and P_2 purinergic receptors can be equally effective in both high- and low-responders to $P2Y_{12}$ inhibitors and therefore represents a promising alternative to existing anti-thrombotic therapy.

4. Materials and Methods

4.1. Chemicals

Adenosine receptor agonists were purchased from Sigma (St. Louis, MO, USA) (NECA (CAS № 35920-39-9)), and Cayman (Ann Arbor, MI, USA) (regadenoson (CAS № 313348-27-5)). LUF5835 (2-amino-6-(1H-imidazol-2-ylmethylsulfanyl)-4-(3-hydroxy-phenyl) pyridine-3,5dicarbonitrile) was synthesized at Laboratory of Molecular Virology and Biological Chemistry, Institute of Medical Biology, Polish Academy of Sciences, Lodz, Poland. 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). Antibodies anti-human CD61/PerCP, CD61/PE, CD62/PE, PAC-1/FITC, mouse IgG1/PE isotype control, mouse IgG1/FITC isotype control, Cellfix, buffered sodium citrate was purchased from Becton-Dickinson (San Diego, CA, USA). Phosphate buffered saline pH 7.4 (PBS) was obtained from Corning (New York, NY, USA). Dimethyl sulfoxide (DMSO), adenosine diphosphate (ADP), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). All other chemicals, unless otherwise stated, were purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland).

4.2. Chemicals Preparation

The stock and working solutions of cangrelor and prasugrel metabolite were prepared in distilled water. The 100 mM stock solutions of NECA, regadenoson and LUF5835 were prepared in DMSO. Stock solutions were then diluted with PBS to working concentrations not exhibiting precipitates, as noted by Boncler et al. [18], and added to the biological material. The dilution factor was chosen to yield the maximal concentration of DMSO 0.1%, thus the final concentration of DMSO in the biological sample never exceeded 0.1% in any of the assays.

4.3. Blood Donors

Experiments were approved by the Ethics of Research in Human Experimentation Committee at the Medical University of Lodz, approval number (RNN/43/17/KE). After having received written consent from volunteers, blood was collected from healthy donors (30 men and 62 women; mean age 29.5 ± 8.8 years) into a vacuum tube containing 0.105 mol/L buffered sodium citrate, with a the final citrate: blood ratio of 1:9 *v*/*v*. All individuals stated that they had not taken medications known to influence platelet function for at least two weeks prior to the study.

4.4. Platelet Viability Assay

Platelet viability in the presence of AR agonist and $P2Y_{12}$ inhibitors was assessed in resting platelets according to Rywaniak et al. [52]. Samples were preincubated with AR agonists for 3 min at 37 °C. Positive control (assumed to result in a low platelet viability) was blood preincubated in the presence of 1% paraformaldehyde (PFA) for 15 min at 37 °C. Samples were then diluted 10-fold with PBS pH 7.4, labelled with anti-CD61/PE antibodies (15 min, RT) and subsequently 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, gathering 5000 events (CD61/PE-positive objects), using FACSCanto II flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

4.5. Platelet Aggregometry Measured in Whole Blood

The measurements was performed according to the manufacturer's instructions. Briefly, whole blood was preincubated with an AR agonist and/or $P2Y_{12}$ inhibitors for 3 (AR agonists and cangrelor) or 15 (prasugrel metabolite) minutes at 37 °C, then 300 µL of blood was transferred into the measurement cell and diluted with 300 µL saline (0.9%) and preheated to 37 °C for another 3 min. Then, 10 µM ADP (final concentration) was added and platelet aggregation was recorded continuously for 10 min using a Multiplate analyser (Hoffmann-La Roche, Basel, Switzerland). Area under the curve (AUC) was analysed. All measurements were completed within three hours of blood collection.

4.6. Platelet Reactivity Measured by Flow Cytometry

Whole blood was preincubated with an AR agonist and/or a $P2Y_{12}$ inhibitor for 3 (AR agonists and cangrelor) or 15 (prasugrel metabolite) minutes at 37 °C then platelets were activated with 20 µM ADP (final concentration). Subsequently, a sample was diluted 10-fold with PBS, and labelled with anti-CD61/PerCP, anti-CD62P/PE and PAC-1/FITC antibodies (15 min, RT), then fixed with CellFix for 1 h at RT. Directly before measurement, the samples were diluted 1:1 with PBS and the assay was performed, gathering 10,000 events (CD61/PerCP-positive objects), using FACSCanto II flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

4.7. Thrombus Formation Under Flow Conditions

The effect of the AR agonists and $P2Y_{12}$ inhibitors on thrombus formation was assayed with the use of the Venaflux system (Cellix, Dublin, Ireland) according to the protocol based on studies published elsewhere [53] using Vena8 Fluoro+ biochips. The channels were coated with type I collagen (20 µg/mL) overnight at 4 °C and blocked with 0.1% BSA for 1 h at 4 °C. The biochip was mounted on the stage of an inverted AxioVert microscope thermostatically controlled throughout the experiment to maintain a constant temperature of 37 °C (Carl Zeiss, Oberkochen, Germany). Whole blood, supplemented with D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) as a thrombin inhibitor (final concentration 0.05 mM), was preincubated with an AR agonist and/or a $P2Y_{12}$ inhibitor for three (AR agonists and cangrelor) or 15 (prasugrel metabolite) minutes at 37 °C. Samples were recalcified with $CaCl_2$ (final concentration 1 mM) immediately before measurement. The samples were then perfused through the channels of the chip using a shear force of 60 dyne/cm^2 for 4 min. The thrombi were stained in channels by washing with 10 µg/mL fluorescein dissolved in PBS for two minutes at 5 dyne/cm^2. Following this, the samples were perfused with CellFix for 4 min at 5 dyne/cm^2. Such prepared channels were imaged by confocal microscopy.

4.8. Imaging and Image Analysis

Confocal imaging of thrombi was performed with a Leica TCS SP8 confocal microscope with LAS X 2.0.2.15022 software (Leica Microsystems, Wetzlar, Germany) using the objective HC PL IR APO 40×/1.10 (water immersion). The 488 nm supercontinuum white light laser (WLL) (12% intensity) was used to excite the fluorescein-stained thrombi. The emission was collected by a photomultiplier tube detector in the range of 492–564 nm. Confocal Z-stack scans were performed at a rate of 400 Hz, zoom 1.0, pinhole 1.0 and line averaging set at 3 to improve image quality. In each field of a view 70 focal planes were acquired (logical size format X/Y/Z 512/512/70) [54]. For the analysis of images, FIJI software was used [55], according to our previously established protocol [56]. Briefly, the thresholding procedure was performed with the use of 'Auto Local Threshold' function (Bernsen method) with a radius value set at 5. To quantify the identified objects, the '3D Object Counter' tool was applied, with a threshold set at 255, and a cut-off set at 20 µm^3 (to exclude the objects too small to qualify them as thrombi). Volumes of separated thrombi were acquired for further analysis, and subsequently summarized to obtain total clot volume per sample.

4.9. Statistical Analysis

The results are expressed as median with interquartile range. The Shapiro-Wilk test and Mauchley's test were used to test the normality of data distribution and sphericity of variances, respectively. Normally distributed data was analysed with the pairwise Student t-test or two-way analysis of variance for repeated measures with the post hoc Bonferroni's multiple comparisons test or repeated measures ANOVA with Geisser-Greenhouse correction and Holm-Sidak's corrections for multiple comparisons test. Data departing from normality, variance sphericity and/or variance homogeneity were assessed with the Wilcoxon's signed ranks test or Friedman's test with Dunn's correction for multiple comparisons. Coefficient of variation (CV) was used to compare the variability across the variables. Variables with extremely high values of CV (thrombus volume) were bootstrapped to ensure that the revealed differences between groups were not observed due to pure chance. The statistical analysis was performed using the following software packages: Statistica v.13 (Dell Software, Round Rock, TX, USA), StatsDirect v.2.8.0 (StatsDirect Ltd., Merseyside, UK) and GraphPad Prism v.5. (GraphPad Software, San Diego, CA, USA)

Supplementary Materials: The following are available online, Table S1: Coefficients of variation obtained in aggregometric, cytometric, and thrombus formation under flow conditions experiments carried out in single (either $P2Y_{12}$ antagonist or AR agonist alone) and dual ($P2Y_{12}$ antagonist and AR agonist applied simultaneously) systems (n = 5 per experimental method).

Author Contributions: Conceptualization, N.W., M.B., C.W. and M.R.; Data curation, N.W., M.B., D.P., J.W., T.P. and M.R.; Formal analysis, N.W., M.B., D.P., T.P., M.G. and M.R.; Funding acquisition, C.W.; Investigation, N.W., D.P. and J.W.; Methodology, N.W., M.B., T.P., M.G. and M.R.; Project administration, M.R.; Statistical analysis, M.B., C.W. Supervision, M.B., T.P., M.G., C.W. and M.R.; Visualization, M.G.; Writing—original draft, N.W. and M.B.; Writing—review & editing, M.B., C.W. and M.R. All authors have read and agreed to the published version of the manuscript.

Funding: 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 also supported by Medical University of Lodz Grant for Young Researchers (502-03/6-020-01/502-64-125-18).

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

ADP	Adenosine Diphosphate
AR	Adenosine Receptor
cAMP	Cyclic Adenosine Monophosphate
NECA	5′-(N-ethylcarboxamido) adenosine
IC_{50}	Half maximal inhibitory concentration

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Sample Availability: Samples of all the AR agonists and P2Y12 antagonists used in this study are available from the authors.

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STATEMENT

I declare that my contribution to the article:

Wolska, N., M. Boncler M., Polak D., Wzorek J., Przygodzki T., Gapinska M., Watala C. and Rozalski M. (2020). "*Adenosine Receptor Agonists Exhibit Anti-Platelet Effects and the Potential to Overcome Resistance to P2Y$_{12}$ Receptor Antagonists*." Molecules 25 (1).
DOI: 10.3390/molecules25010130

consisted of participation in a study design, data analysis, supervision of experiments, writing the original draft of manuscript, the manuscript review and editing.

I agree to use of the above publication by Nina Wolska, MSc. in her doctoral dissertation.

OŚWIADCZENIE

Oświadczam, że mój wkład w artykuł:

Wolska, N., M. Boncler M., Polak D., Wzorek J., Przygodzki T., Gapinska M., Watala C. and Rozalski M. (2020). "Adenosine Receptor Agonists Exhibit Anti-Platelet Effects and the Potential to Overcome Resistance to P2Y12 Receptor Antagonists." Molecules 25 (1).
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polegał na udziale w planowaniu eksperymentów, analizie danych, nadzorze nad wykonywaniem eksperymentów, przygotowaniu i edycji manuskryptu.

Wyrażam zgodę na wykorzystanie powyższej publikacji przez mgr inż. Ninę Wolską w jej pracy doktorskiej.

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consisted of participation in performing experiments using aggregometry method and studies on clot formation under flow conditions, imaging of samples obtained in clot formation under flow conditions experiments, as well as image analysis.

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polegał na udziale w prowadzeniu eksperymentów agregometrycznych i badaniach tworzenia skrzepów w warunkach przepływu, otrzymywaniu obrazów mikroskopowych uzyskiwanych w eksperymentach tworzenia skrzepu w warunkach przepływu, jak również analizie obrazu.

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consisted of planning experiments of clot formation under flow conditions, microscopic image analysis, data analysis, supervision of experiments.

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polegał na planowaniu eksperymentów tworzenia skrzepów w warunkach przepływu, analizie obrazów mikrospowych, analizie danych, oraz nadzorze nad wykonywaniem eksperymentów.

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consisted of expert assistance in sample visualization in confocal microscopy, performing visualization procedure and data processing.

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polegał na udzieleniu pomocy eksperckiej w zakresie wizualizacji próbek w mikroskopii konfokalnej, przeprowadzaniu procedury wizualizacji i przetwarzania danych.

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I agree to use of the above publication by Nina Wolska, MSc. in her doctoral dissertation.

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polegał na pozyskaniu funduszy na badania, planowaniu eksperymentów, analizie danych, nadzorze nad wykonywaniem eksperymentów, przygotowaniu i edycji manuskryptu.

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DOI: 10.3390/molecules25010130

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polegał na udziale w planowaniu eksperymentów, analizie danych, nadzorze nad wykonywaniem eksperymentów, przygotowaniu i edycji manuskryptu, odpowiedzi na uwagi recenzentów

Wyrażam zgodę na wykorzystanie powyższej publikacji przez mgr inż. Ninę Wolską w jej pracy doktorskiej.

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Adenosine Receptor Agonists Increase the Inhibition of Platelet Function by $P2Y_{12}$ Antagonists in a cAMP- and Calcium-Dependent Manner

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Received: 30 June 2020; Accepted: 30 July 2020; Published: 31 July 2020

Abstract: We have shown previously that platelet activity can be lowered through the simultaneous inhibition of $P2Y_{12}$ receptor and activation of adenosine receptors (AR). This work explores this concept by testing the antiplatelet potential of nine AR agonists in combination with $P2Y_{12}$ receptor antagonists—cangrelor and prasugrel metabolite. A panel of in vitro methods was used to assess platelet viability, P-selectin expression, GPIIb-IIIa activation, fibrinogen binding, calcium ion mobilization, VASP-P level, and cAMP formation, utilizing whole blood or isolated platelets from healthy volunteers. The AR agonists demonstrated anti-platelet effects, but stimulated signaling pathways to varying degrees. AR agonists and $P2Y_{12}$ antagonists reduced expression of both P-selectin and the activated form of GPIIb-IIIa on platelets; however, the combined systems (AR agonist + $P2Y_{12}$ antagonist) demonstrated stronger effects. The antiplatelet effects of AR when combined with $P2Y_{12}$ were more pronounced with regard to exogenous fibrinogen binding and calcium mobilization. The cAMP levels in both resting and ADPactivated platelets were increased by AR agonist treatment, and more so when combined with $P2Y_{12}$ inhibitor. In conclusion, as AR agonists are fast-acting compounds, the methods detecting early activation events are more suitable for assessing their antiplatelet action. The exogenous fibrinogen binding, calcium mobilisation and cAMP level turned out to be sensitive markers for detecting the inhibition caused by AR agonists alone or in combination with $P2Y_{12}$ receptor antagonists.

Keywords: platelet; adenosine receptor; adenosine receptor agonist; $P2Y_{12}$ antagonist; anti-platelet therapy

1. Introduction

The leading cause of death in Western countries, according to current World Health Organisation data, is cardiovascular disease that results primarily from arterial thrombosis dependent on blood platelet hyperactivity. Thromboembolic events can be prevented by anti-platelet therapy [1]. However, currently available therapeutic strategies often demonstrate unsatisfactory safety and efficiency, with one of the key issues being drug resistance stemming from high inter-individual variation among patients [2,3]. Novel platelet inhibitors and/or new therapeutic strategies are needed to provide safe and efficient treatments.

Currently, one of the major targets of antiplatelet drugs is the $P2Y_{12}$ receptor. Its inhibition blocks the ADP-dependent platelet activation pathway [4]—the enhancement of platelet aggregation initiated by another ADP receptor ($P2Y_1$), rendering the clot formation process ineffective. Additionally,

the inhibition of the third platelet receptor from the P2 class, ATP-gated ion channel receptor $P2X_1$, which activation does not directly induce the platelet aggregation but causes fast calcium mobilization and platelet shape change, was also suggested as a potential way of reducing thrombotic events [5]. The most commonly used clinically-approved $P2Y_{12}$ inhibitors are thienopyridines (ticlopidine, clopidogrel, and prasugrel—prodrugs whose short-lived active metabolites are irreversible $P2Y_{12}$ inhibitors), the ATP analogue cangrelor (the first intravenous, reversible, non-competitive $P2Y_{12}$ inhibitor) and the cyclopentyltriazolopyrimidine derivative ticagrelor [2,4].

Efficient anti-platelet treatment is often hindered in clinical practice by reduced sensitivity to many anti-platelet agents and by high inter-individual variation in response to treatment, resulting in bleeding and a high risk of failure. That problem is usually managed by a combined therapy—administering two or more drugs affecting various platelet activation pathways. For example, acetylsalicylic acid (inhibitor of thromboxane A_2 formation) is often combined with clopidogrel. This approach is however still burdened with a problem of drug resistance, especially in patients suffering from type 2 diabetes—a group with increased risk thromboembolic events [6–8].

Adenosine is an important purine metabolite; it is a signalling molecule regulating many cell processes that also serves as a component of nucleic acids and ATP [9,10]. Adenosine receptors (AR) are expressed in many cell types and are involved in a plethora of physiological functions. Structurally, they belong to G protein-coupled receptor family. AR subtypes A_{2A} and A_{2B} are expressed in platelets, while A_1, and A_3 are not [9,11]. Platelet AR activation decreases platelet activation and aggregation, mediated by an increase in intracellular cAMP (cyclic adenosine monophosphate) levels [12,13]. Adenosine is a natural AR agonist; however, as it is an extremely short-lasting one (less than 10 s in physiological conditions), there is a great need for synthetic, long-lasting agonists [13,14]. Among synthetic AR agonists, most do not differentiate between A_{2A} or A_{2B} subtypes, but some selective agonists have been identified. AR agonists are believed to block platelet aggregation [15–17], and hence interest in their antiplatelet property has been growing [13,18–20].

One possible treatment option involves the simultaneous inhibition of the $P2Y_{12}$ receptor and agonization of AR receptors expressed by platelets [13,19]. It appears to be a feasible option [19], and one that may solve the problem of drug resistance [21]. Such an approach could avoid the harmful side effects of anti-platelet treatment associated with high-dose $P2Y_{12}$ inhibitors and provide adequate and consistent antithrombotic protection, regardless of individual responses to low-dose $P2Y_{12}$ antagonists [21].

The aim of the present study is to determine the effectiveness of combined anti-platelet therapy based on a combination of $P2Y_{12}$ inhibitors and adenosine receptor agonists. A set of in vitro methods were used to assess platelet viability, P-selectin expression, GPIIb-IIIa activation, fibrinogen binding, calcium ion mobilisation, VASP [vasodilator-stimulated phosphoprotein] phosphorylation, and cAMP formation. The study examined the anti-platelet effects of two $P2Y_{12}$ inhibitors, cangrelor and prasugrel (active metabolite R-138727), and a panel of nine AR agonists (previously known to inhibit platelet aggregation [11]): PSB0777, CGS21680, MRE0094, 2-chloroadenosine, CV1808, HE-NECA, NECA, regadenoson, and UK423,097 both single (either $P2Y_{12}$ antagonist or AR agonist alone) and dual (simultaneous $P2Y_{12}$ antagonization and AR agonization) models were used (chemical structures of all the compounds tested are shown in Figure 1). It was found that a number of AR agonists are able to inhibit platelet function, as indicated by multiple markers of platelet activation; in addition, AR agonists consistently potentiated the anti-platelet effects of $P2Y_{12}$ inhibitors, including cAMP formation: the pivotal point of platelet signalling.

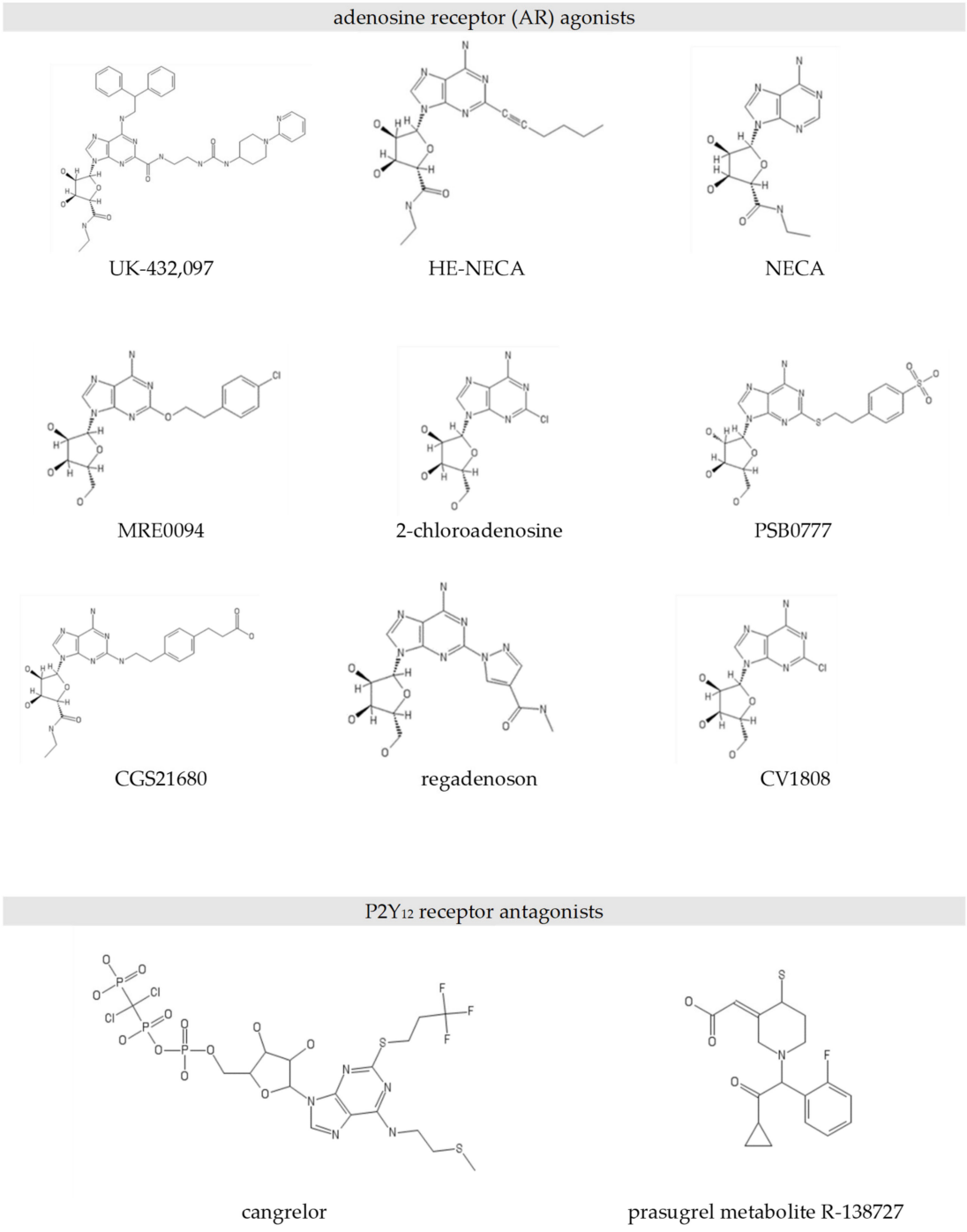

Figure 1. Chemical structures of selected adenosine receptor agonists and $P2Y_{12}$ antagonists.

2. Results

AR agonists were used in a combination with two types of $P2Y_{12}$ receptor antagonists: cangrelor (C) or prasugrel metabolite R-138727 (PM), the experimental set up was one AR agonist + one $P2Y_{12}$ antagonist. Each compound was used at its aggregation IC_{50} value [19,21]: PSB0777 23 μM, CGS21680 1 μM, MRE0094 26 μM, 2-chloroadenosine 5 μM, CV1808 25 μM, HE-NECA 0.2 μM, NECA 0.5 μM, regadenoson 1.2 μM, and UK423,097 1 μM, cangrelor 17 nM, and PM 1.3 μM, unless otherwise specified.

Before measuring platelet activity parameters, the potential cytotoxic effects of AR agonists on blood platelets were determined using calcein assay as described previously [22]. The cytotoxicity

assay was performed on resting platelets in whole blood preincubated with AR agonists. None of the AR agonists exhibited any cytotoxic effects, indicated by significant changes in cell viability, i.e., the fractions of calcein-negative platelets did not increase compared to untreated controls (Table S1). Significant increase (up to 79%) in the fraction of calcein-negative platelets was observed for the positive control (1% paraformaldehyde) ($p < 0.005$).

Representative cytometric dot-plots and histograms for results described in the Sections 2.1–2.3 are presented in Figure S1.

2.1. The Combined Effect of Adenosine Receptor Agonists and P2Y$_{12}$ Antagonists Increases the Inhibition of P-Selectin Expression

The ability of the tested compounds to decrease P-selectin expression, the main surface platelet activation marker, was measured following ADP stimulation. Both P2Y$_{12}$ antagonists used alone significantly reduced platelet activation. The AR agonists UK423,097, HE-NECA, NECA, MRE0094, 2-chloroadenosine, and CGS21680 demonstrated significant inhibition (Figure 2A). More pronounced effects were observed for the following combinations: cangrelor + UK423,097, cangrelor + HE-NECA, cangrelor + NECA (Figure 2B), PM + UK423,097, PM + HE-NECA, PM + NECA, PM + 2-chloroadenosine, and PM + CV1808 (Figure 2C).

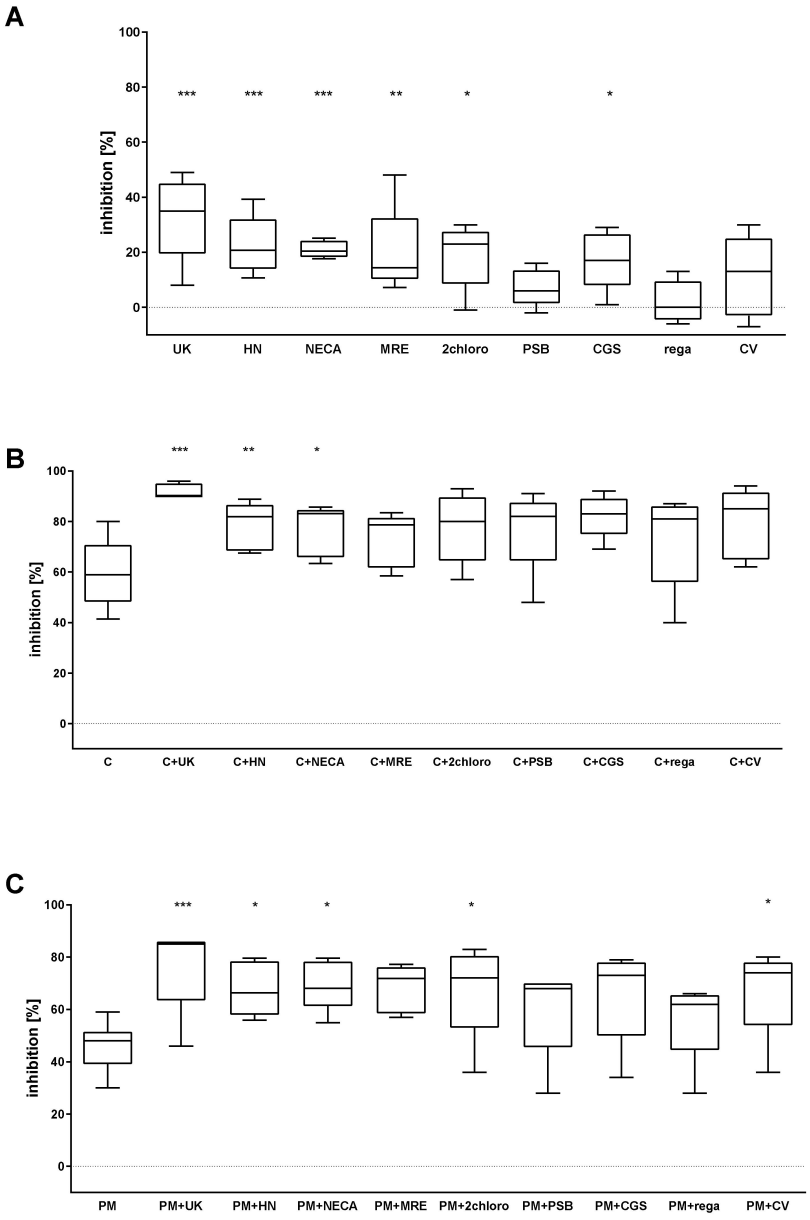

Figure 2. AR agonists intensify the inhibitory effect of $P2Y_{12}$ antagonists on platelet reactivity, as measured by P-selectin expression ($n = 5$). (**A**) Effect of AR agonist on platelets, (**B**) effect on platelets antagonised with the $P2Y_{12}$ inhibitor cangrelor, (**C**) effect on platelets antagonised with the $P2Y_{12}$ inhibitor prasugrel metabolite (PM). Data are presented as median, interquartile range (box), and minimum and maximum values (whiskers). Platelet reactivity was measured after activation with 20 μM ADP, in whole blood. Samples were preincubated at 37 °C for 3 min with AR agonist and cangrelor, or 15 min with PM, all in their previously determined IC_{50}. Statistical significance was estimated by one-way ANOVA for repeated measurements with the post hoc Bonferroni's multiple comparisons test or Friedman's test with Dunn's correction for multiple comparisons. Groups containing AR agonists are compared to control samples: untreated samples for AR agonists alone (**A**), $P2Y_{12}$ inhibitor-treated samples for combined systems (**B**,**C**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. UK—UK423,097, HN—HE-NECA, MRE—MRE0094, 2 chloro -2-chloroadenosine, PSB—PSB0777, CGS—CGS21680, rega—regadenoson, CV—CV1808, C—cangrelor, PM—prasugrel metabolite R-138727 IC_{50} values: UK423,097 1 μM, HE-NECA 0.2 μM, NECA 0.5 μM, MRE0094 26 μM, 2-chloroadenosine 5 μM, PSB0777 23 μM, CGS21680 1 μM, regadenoson 1.2 μM, CV1808 25 μM, and cangrelor 17 nM, and PM 1.3 μM.

2.2. The Combined Action of Adenosine Receptor Agonists and P2Y$_{12}$ Antagonists Increases the Inhibition of GPIIb-IIIa Activation and the Inhibition of Fibrinogen Binding

The ability of the tested compounds to decrease GPIIb-IIIa activation in platelets agonized with ADP was measured. Both P2Y$_{12}$ antagonists (Figure 3B,C) and the AR agonist UK 432,097 (Figure 3A) significantly reduced GPIIb-IIIa activation. More pronounced effects were observed for the combined systems cangrelor + UK423,097; cangrelor + HE-NECA (Figure 2B); PM + UK423,097; PM + HE-NECA; PM + NECA; PM + MRE0094; PM + 2-chloroadenosine; PM + CGS21680 and PM + CV (Figure 3B,C).

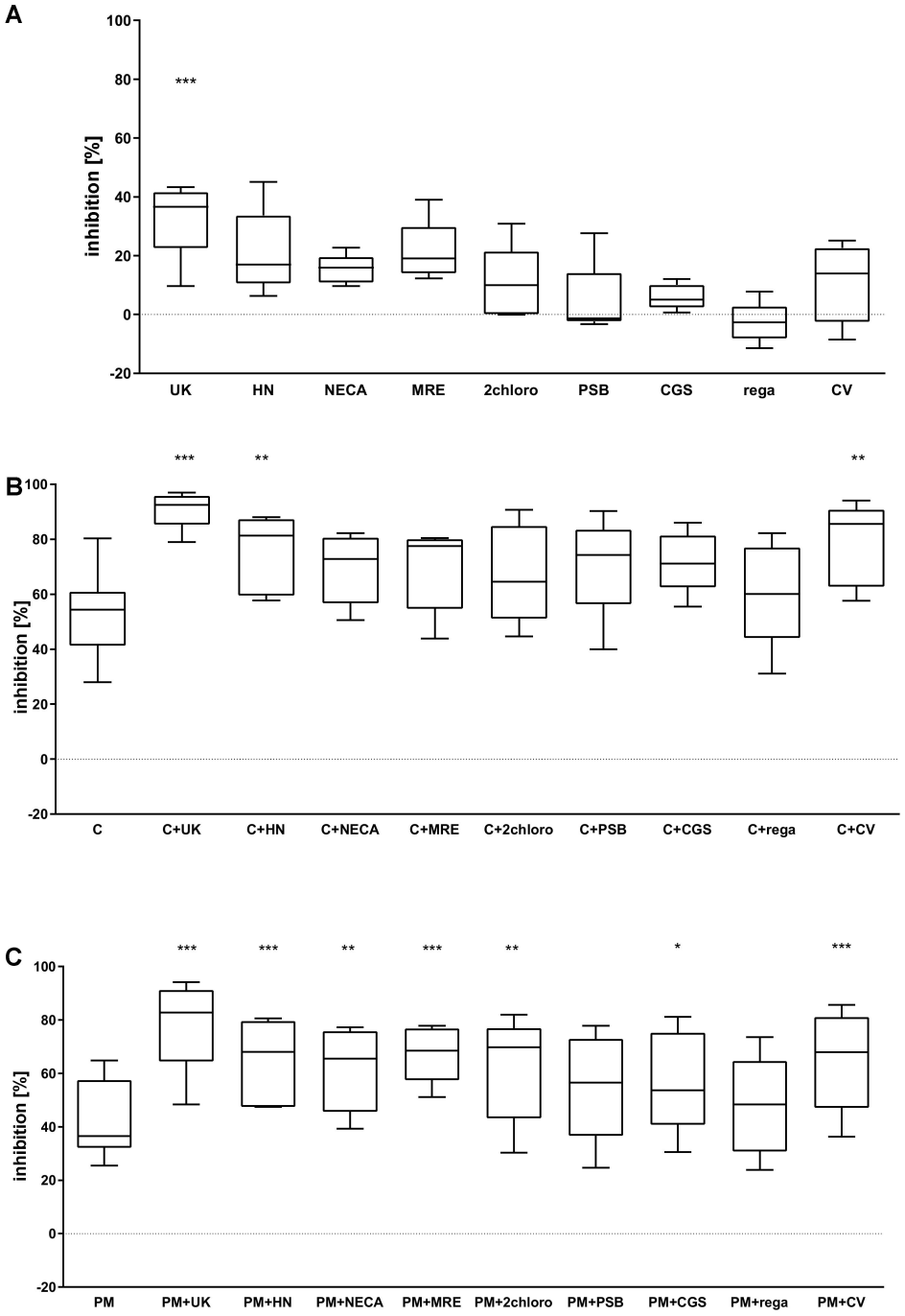

Figure 3. AR agonists intensify the inhibitory effect of P2Y$_{12}$ antagonists on platelet reactivity, as measured by expression of GPIIb-IIIa active form (PAC-1 antibody binding) (n = 5). (**A**) Effect of AR agonist on platelets, (**B**) effect on platelets antagonised with the P2Y$_{12}$ inhibitor cangrelor, (**C**) effect on

platelets antagonised with the $P2Y_{12}$ inhibitor prasugrel metabolite (PM). Data are shown as median, interquartile range (box), and minimum and maximum values (whiskers). Platelet reactivity was assessed after activation with 20 μM ADP, in whole blood. Samples were preincubated at 37 °C for 3 min with AR agonist and cangrelor, or 15 min with PM, all in their previously determined IC_{50}. Statistical significance estimated by one-way ANOVA for repeated measurements with the post hoc Bonferroni's multiple comparisons test or Friedman's test with Dunn's correction for multiple comparisons (groups containing AR agonist are compared to control samples: untreated sample for AR agonists alone (**A**) or $P2Y_{12}$ inhibitor-treated samples for combined version (**B**,**C**)). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. UK—UK423,097, HN—HE-NECA, MRE—MRE0094, 2 chloro—2-chloro- adenosine, PSB—PSB0777, CGS—CGS21680, rega—regadenoson, CV—CV1808, C—cangrelor, PM—prasugrel metabolite R-138727. IC_{50} values: UK423,097 1 μM, HE-NECA 0.2 μM, NECA 0.5 μM, MRE0094 26 μM, 2-chloroadenosine 5 μM, PSB0777 23 μM, CGS21680 1 μM, regadenoson 1.2 μM, CV1808 25 μM, and cangrelor 17 nM, and PM 1.3 μM.

Both $P2Y_{12}$ antagonists significantly reduced platelet activation, as measured by fibrinogen binding (Figure 4B,C). Significant inhibition was also observed for AR agonists: UK423,097, HE-NECA, NECA, MRE0094, 2-chloroadenosine, CGS21680, and CV1808 (Figure 4A).

The following combinations of AR agonists and $P2Y_{12}$ antagonists significantly inhibited the binding of exogenous fibrinogen: cangrelor + UK423,097; cangrelor + HE-NECA; cangrelor + NECA; cangrelor + MRE0094; cangrelor + 2-chloroadenosine; cangrelor + CGS21680 (Figure 4B); PM + UK423,097; PM + HE-NECA; PM + NECA; PM + MRE0094; PM + 2-chloroadenosine; PM + PSB0777; PM + CGS21680; PM + regadenoson; and PM + CV1808 (Figure 4C).

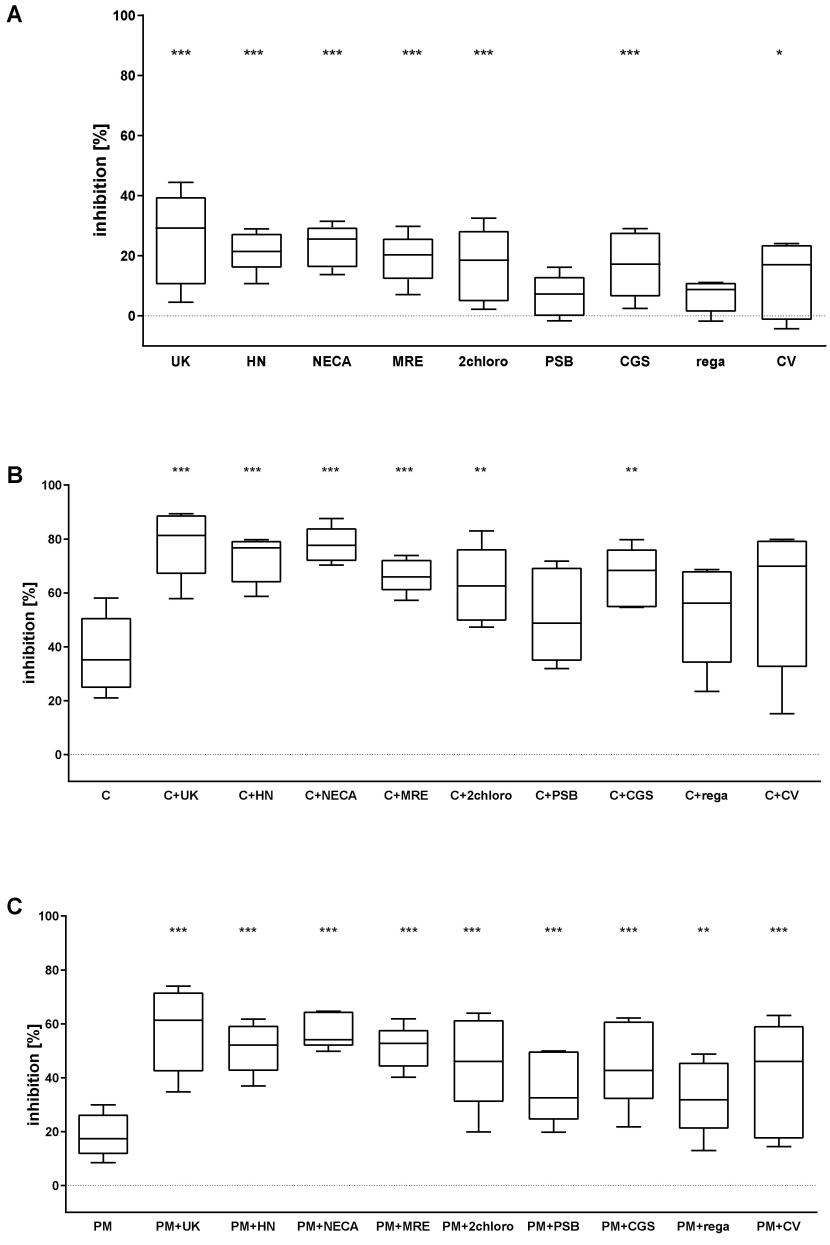

Figure 4. AR agonists intensify the inhibitory effect of $P2Y_{12}$ antagonists on platelet reactivity as measured by exogenous fibrinogen binding (n = 5). Panels present data on AR agonist impact on platelets (**A**), platelets antagonised with $P2Y_{12}$ inhibitor: cangrelor (**B**) or prasugrel metabolite (PM) (**C**). Data are shown as median, interquartile range (box), and minimum and maximum values (whiskers). Platelet reactivity was assessed after activation with 20 μM ADP, in whole blood. Samples were preincubated at 37 °C for 3 min with AR agonist and cangrelor or 15 min with PM, all in their previously determined IC_{50}. Statistical significance estimated by one-way ANOVA for repeated measurements with the post hoc Bonferroni's multiple comparisons test or Friedman's test with Dunn's correction for multiple comparisons (groups containing AR agonist are compared to control samples: untreated sample for AR agonists alone (**A**) or $P2Y_{12}$ inhibitor-treated samples for combined version (**B**,**C**)). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. UK—UK423,097, HN—HE-NECA, MRE—MRE0094, 2chloro—2-chloroadenosine, PSB—PSB0777, CGS—CGS21680, rega—regadenoson, CV—CV1808, C—cangrelor, PM—prasugrel metabolite R-138727. IC_{50} values: UK423,097 1 μM, HE-NECA 0.2 μM, NECA 0.5 μM, MRE0094 26 μM, 2-chloroadenosine 5 μM, PSB0777 23 μM, CGS21680 1 μM, regadenoson 1.2 μM, CV1808 25 μM, and cangrelor 17 nM, and PM 1.3 μM.

2.3. The Combined Action of Adenosine Receptor Agonists and P2Y$_{12}$ Antagonists Increases the Inhibition of Calcium Flux

A significant reduction in calcium ion mobilization was shown for cangrelor alone (but not PM) as well as for the samples incubated with AR agonists: UK423,097, HE-NECA, NECA, MRE0094, CGS21680, and CV1808 (Figure 5A). The following pairs demonstrated stronger anti-platelet effects: cangrelor + UK423,097, cangrelor + HE-NECA, cangrelor + NECA, cangrelor + MRE0094, cangrelor + PSB0777, cangrelor + CGS21680, cangrelor + CV1808 (Figure 5B), PM + UK423,097, PM + HE-NECA, PM + NECA, PM + MRE0094, PM + 2-chloroadenosine, PM + PSB0777, PM + CGS21680, PM + regadenoson, and PM + CV1808 (Figure 5C).

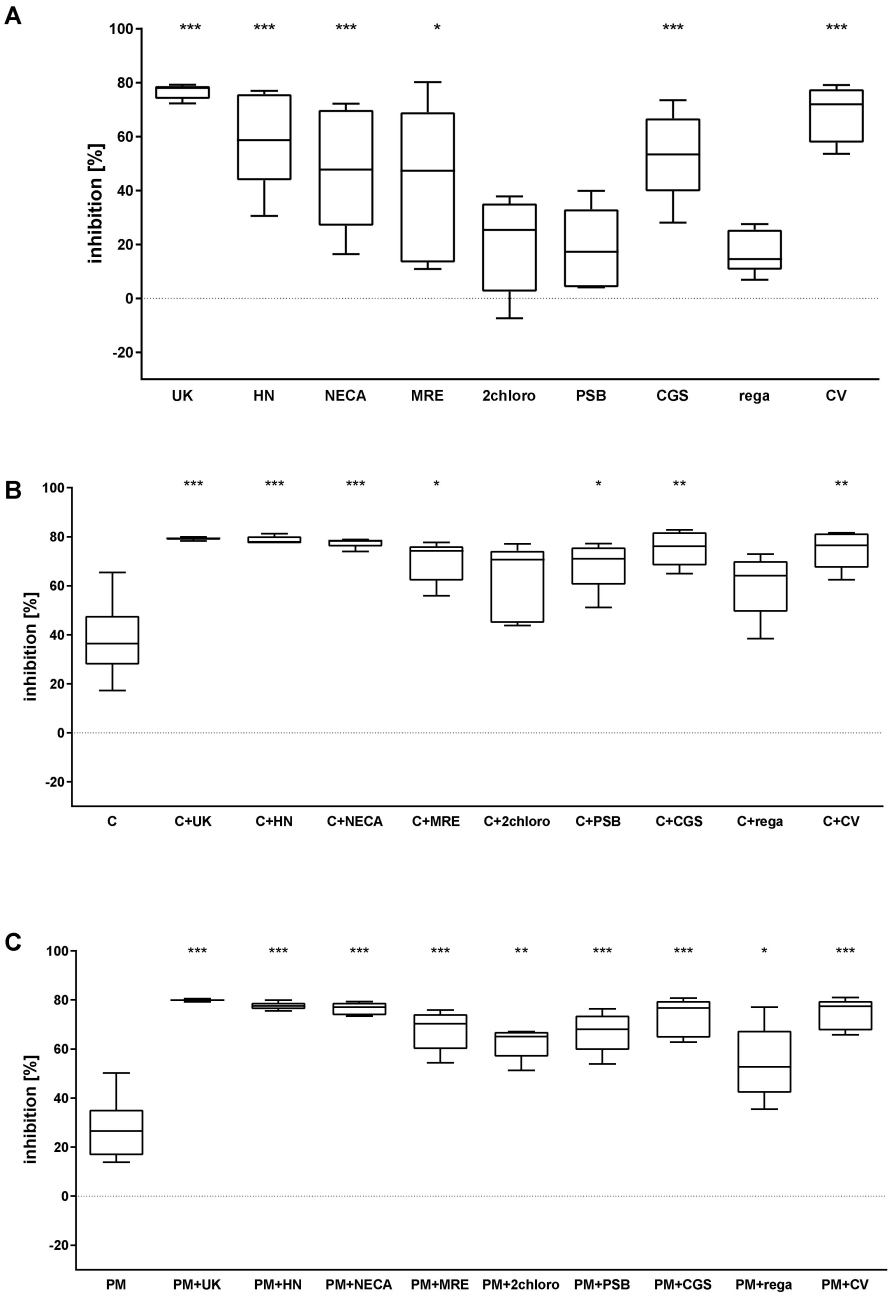

Figure 5. AR agonists strengthen the inhibitory effect on calcium ion mobilisation by of P2Y$_{12}$ antagonists (n = 5). Panels present data on the effect of AR agonist on platelets (**A**), platelets antagonised with P2Y$_{12}$ inhibitor: cangrelor (**B**) or prasugrel metabolite (PM) (**C**). Data are presented as median,

interquartile range (box), and minimum and maximum values (whiskers). Platelet reactivity was assessed after activation with 20 μM ADP, in whole blood. Samples were preincubated at 37 °C for 3 min with AR agonist and cangrelor, or 15 min with PM, all in their previously determined IC_{50}. Statistical significance estimated by one-way ANOVA for repeated measurements with the post hoc Bonferroni's multiple comparisons test or Friedman's test with Dunn's correction for multiple comparisons (groups containing AR agonist are compared to samples: untreated sample for AR agonists alone (**A**) or $P2Y_{12}$ inhibitor-treated samples for combined version (**B**,**C**)). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. UK—UK423,097, HN—HE-NECA, MRE—MRE0094, 2chloro—2-chloroadenosine, PSB—PSB0777, CGS—CGS21680, rega—regadenoson, CV—CV1808, C—cangrelor, PM—prasugrel metabolite R-138727. IC_{50} values: UK423,097 1 μM, HE-NECA 0.2 μM, NECA 0.5 μM, MRE0094 26 μM, 2-chloroadenosine 5 μM, PSB0777 23 μM, CGS21680 1 μM, regadenoson 1.2 μM, CV1808 25 μM, and cangrelor 17 nM, and PM 1.3 μM.

2.4. The Combined Action of Adenosine Receptor Agonists and P2Y$_{12}$ Antagonists on cAMP Level

The AR agonists and $P2Y_{12}$ inhibitors ability to increase VASP phosphorylation in activated platelets was measured. No significant effects were observed for AR agonist, $P2Y_{12}$ antagonist, or combinations thereof (Table S2). This could be due to the insufficient sensitivity of the applied method. The cAMP measurement was therefore performed to confirm the influence of AR agonists and $P2Y_{12}$ antagonists on this pathway.

Two AR agonists that were established to strongly inhibit platelet functions (UK423,097, and HE-NECA) and one less efficient AR agonist (PSB0777) were used in a combination with $P2Y_{12}$ receptor antagonist cangrelor. Each compound was used in its IC_{50}, with the values taken from our previous work (see previous section), or a high dosage (100 μM for AR agonists, 1 μM for cangrelor). Their impact was tested on resting and activated (20 μM ADP) isolated platelets. The results were normalized for platelet count. Positive control of platelets treated with forskolin (5 μM) yielded high results of median 4935→ (interquartile range: 2946, 15586) pmol/1 × 10^8 plt.

In resting platelets, the $P2Y_{12}$ antagonist cangrelor significantly increased cAMP formation in both tested concentrations. A significant increase was also found for the AR agonists UK423,097 (both concentrations), HE-NECA (100 μM), and PSB0777 (both concentrations). AR agonists and $P2Y_{12}$ antagonists increased cAMP formation, showing a strong antiplatelet effect, which further increased when use in combination: cangrelor + UK423,097 (both concentration pairs), cangrelor + HE-NECA (both concentration pairs), and cangrelor + PSB0777 (paring in high concentrations) (Figure 6).

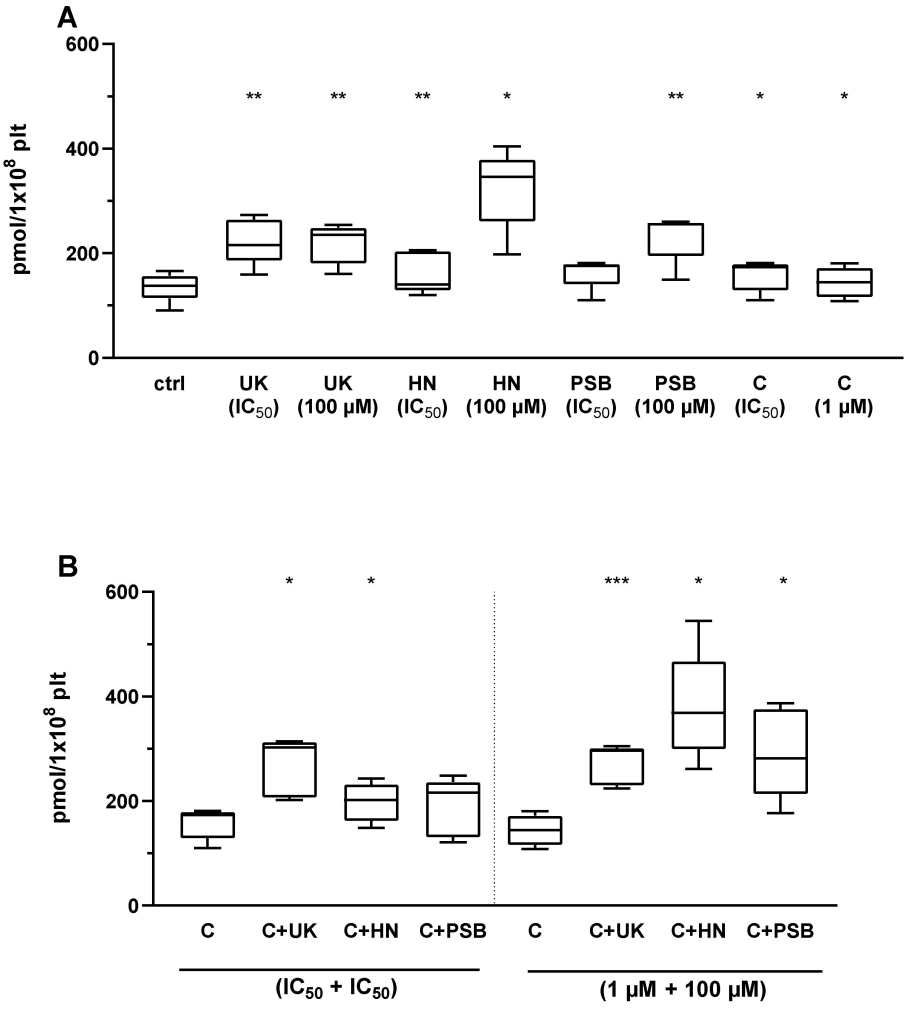

Figure 6. AR agonists strengthen the $P2Y_{12}$ inhibitory effect on platelet reactivity as measured by cAMP formation in resting platelets ($n = 5$). Panels present data on AR agonist impact on platelets in comparison to unstimulated platelets (ctrl) (**A**), platelets antagonised with $P2Y_{12}$ inhibitor: cangrelor (**B**). Data are presented as median, interquartile range (box), and minimum and maximum values (whiskers). cAMP level was measured in isolated platelets. Samples were preincubated at 37 °C for 3 min with AR agonist and cangrelor. Statistical significance estimated by one-way ANOVA for repeated measurements with the post hoc Bonferroni's multiple comparisons test or Friedman's test with Dunn's correction for multiple comparisons (samples containing AR agonist are compared to control samples with $P2Y_{12}$ inhibitor within pertinent group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. UK—UK423,097, HN—HE-NECA, PSB—PSB0777, C—cangrelor. IC_{50} values: UK423,097 1 µM, HE-NECA 0.2 µM, NECA 0.5 µM, and cangrelor 17 nM.

In ADP-activated platelets, $P2Y_{12}$ antagonist cangrelor significantly increased cAMP formation in high concentration, as well as AR agonists: UK423,097 (both concentrations), HE-NECA (100 µM), and PSB0777 (100 µM). AR agonists and $P2Y_{12}$ antagonists, when used in combination, deepened the antiplatelets effect compared to these drugs applied alone (Figure 7).

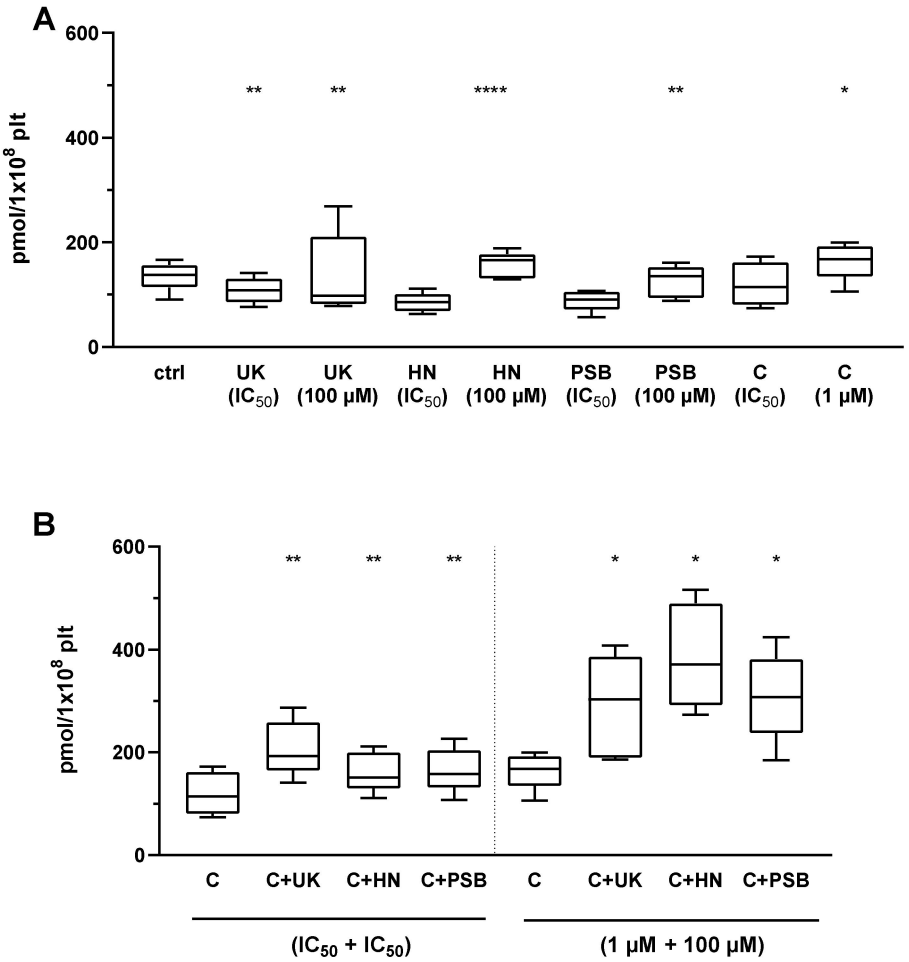

Figure 7. AR agonists strengthen the $P2Y_{12}$ inhibitory effect on platelet reactivity as measured by measured by cAMP formation in ADP- activated platelets (n = 5). Figures present data on AR agonist impact on platelets in comparison to platelets stimulated with 20 µM ADP (ctrl) (**A**), platelets antagonised with $P2Y_{12}$ inhibitor: cangrelor (**B**). Data are shown as median, interquartile range (box), and minimum and maximum values (whiskers). cAMP level was measured in isolated platelets after activation with 20 µM ADP. Samples were preincubated at 37 °C for 3 min with AR agonist and cangrelor. Statistical significance estimated by one-way ANOVA for repeated measurements with the post hoc Bonferroni's multiple comparisons test or Friedman's test with Dunn's correction for multiple comparisons (groups containing AR agonist are compared to control group with $P2Y_{12}$ inhibitor) (samples containing AR agonist are compared to control samples with $P2Y_{12}$ inhibitor within pertinent group). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$. UK—UK423,097, HN—HE-NECA, PSB—PSB0777, C—cangrelor, IC_{50} values: UK423,097 1 µM, HE-NECA 0.2 µM, NECA 0.5 µM, and cangrelor 17 nM.

3. Discussion

AR agonists are a re-emerging group of compounds with various functions which can be useful in the prevention and treatment of several human diseases [23–25]. In the literature, some encouraging results have been published for the application of AR agonists in arrhythmias, cardiac and cerebral ischaemias, neurodegenerative diseases, inflammation, sleep disorders, pain, diabetes, cancer, renal failure as well as glaucoma [26]. Interestingly, agonists of AR receptors expressed on blood platelets (A_{2A} and A_{2B}) were previously reported to have remarkable anti-platelet properties [11,13,19,21]. Hypothetically, the agonists of the A_{2A} and A_{2B} adenosine receptors could be a beneficial supplement to current antithrombotic therapy, especially in the light of frequently observed, high inter-individual variability in response to platelet inhibitors.

The antiplatelet effects of adenosine receptor agonists in combination with two $P2Y_{12}$ antagonists (administered intravenously—cangrelor, or orally—prasugrel [27]) were evaluated in this study. We provide further proof of concept and effectiveness of such parings, and investigate molecular pathways most impacted by this combination.

Our previous findings demonstrated the anti-aggregatory activity of some of the AR agonists presented here (UK 432097, MRE 0094, PSB 0777) examined in the absence or presence of the $P2Y_{12}$ antagonists (cangrelor or prasugrel metabolite) [19], and that the use of combination of a $P2Y_{12}$ antagonist and an AR agonist (regadenoson, NECA, and LUF5835) leads to increased inhibition of platelet function than the $P2Y_{12}$ antagonist alone, and that their antiplatelet effect was much more pronounced in individuals with poor response to $P2Y_{12}$ inhibitors [21]. This study supports our recent in vitro work, extending further analyses of the influence of AR agonists, on the effects of the $P2Y_{12}$ antagonists by introducing measurements of a panel of platelet activation hallmarks.

In this work, AR agonists of different selectivity to AR receptors were used—the following agonists were found to be selective for A_{2A} over A_{2B}: UK423,097, HE-NECA (also agonizes A_3 receptor, not expressed on platelets), 2-chloroadenosine (which also agonizes A_1 receptor, and weakly agonizes A_3 receptor, both not expressed on platelets), MRE0094, regadenoson, PSB0777, and CGS21680. Other AR agonists investigated in this study (CV1808, and NECA) are not selective between A_{2A} and A_{2B} receptor subtypes. Our previous papers showed that A_{2B} AR receptor selective agonist either did not show anti-platelet effect or had only minimal one, which could be explained by a slight cross-reactivity with A_{2A} AR receptor [13,19]. Even though human A_{2A} and A_{2B} ARs sequence is identical in 59%, no definite proof of a selective A_{2B} AR agonist having antiplatelet properties is available.

Two $P2Y_{12}$ antagonists: cangrelor and prasugrel were utilized to assess whether AR agonists can enhance their antiplatelet effect. Prasugrel is a pro-drug and requires metabolization to its active components, therefore its most abundant stable active metabolite R-138727 was used [28]. $P2Y_{12}$ antagonists, as well as AR agonists, were used at IC_{50} values [19,21]. The relatively high dosage (aggregation IC_{50} = 1.3 μM) required to achieve the effective platelet inhibition most likely stems from the fact that only one of the prasugrel active metabolites is used, whereas in vivo prasugrel metabolization results in a number of active metabolites, all of which may exert an antiplatelet effect of their own. We aimed to reflect suboptimal dosages of both classes of compounds in order to demonstrate the combined effect. In addition, it allowed us to work with concentrations clinically achievable in the patients' bloodstream.

A_{2A} AR is known as the important receptor on blood platelets and a mediator of the adenosine-dependent inhibition of platelet aggregation [29]. The activation of this receptor leads to the inhibition of internal calcium stores mobilization and external calcium influx, both linked with activation of adenylate cyclase and the increase of cAMP concentration in the cytosol, which leads to the inhibition of platelet activation [30]. The activation of A_{2A} AR was also reported to reduce the P-selectin expression on the platelet cell surface when platelets were stimulated with the use of thromboxane A_2 or ADP [31]. In this study, we investigated AR agonists at suboptimal concentrations to assess platelet function in vitro at pivotal points of platelet cell signalling—calcium ion mobilization and cAMP formation, also providing proof that they have an ability to further promote the ability of cangrelor and prasugrel metabolite to hinder this process. Similarly, we found that a whole set of markers of platelet activation, such as P-selectin expression, GPIIb-IIIa activation together with fibrinogen binding are reduced by AR agonists alone, and, what is noteworthy, the effects of $P2Y_{12}$ inhibitors on those markers are strengthened by the addition of AR agonists. Our results, therefore, provide further proof that such dual experimental therapy could prevent excessive blood clotting.

This publication provides for the first time the evidence of antiplatelet activity of AR agonist CV1808, demonstrating that CV1808 inhibits exogenous fibrinogen binding, strengthens the antiplatelet effect of PM across all the investigated parameters, and potentiates the ability of cangrelor to limit calcium mobilization. UK423,097 was consistently able to inhibit platelet function across tested parameters, and also HE-NECA and NECA significantly and strongly decreased platelet activation

as evidenced by most of the markers (GPIIb-IIIa activation being an exception). On the other hand, regadenoson and PSB0777 did not significantly affect any of the tested platelet activation markers, regadenoson also was unable to strengthen the antiplatelet effect of cangrelor. Overall, however, all the AR agonists studied in this work showed ability to potentiate anti-platelet effect of at least one $P2Y_{12}$ antagonist. A summary of the obtained results is presented in Figure 8.

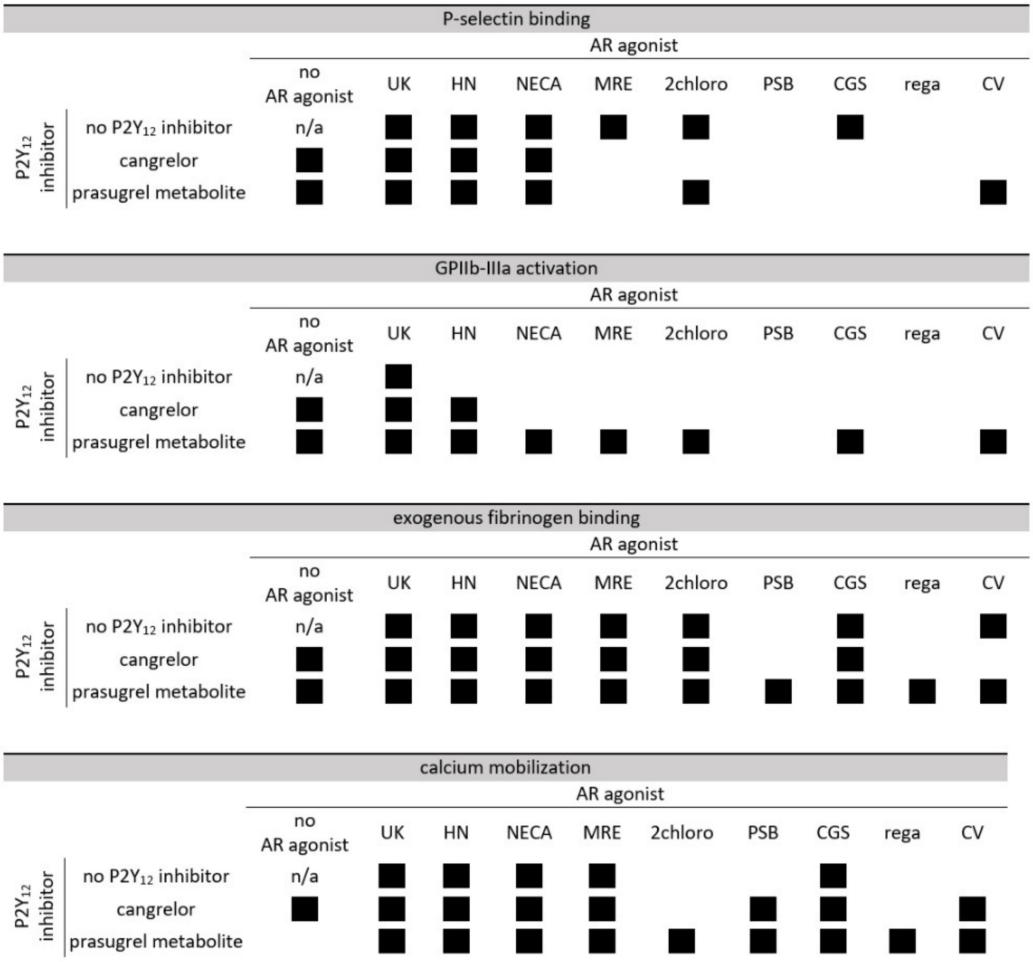

Figure 8. Comparison of AR agonist, $P2Y_{12}$ antagonist or AR agonist and $P2Y_{12}$ antagonist pairing inhibitory effectiveness of ADP-induced platelet reactivity assessed by P-selectin expression, GPIIb-IIIa activation, fibrinogen binding, and calcium ion mobilization. Black boxes indicate statistically significant inhibition for $P2Y_{12}$ antagonist alone and AR agonist alone compared to control non-treated sample, as well for AR agonist and $P2Y_{12}$ antagonist pairs compared to the sample with $P2Y_{12}$ inhibitor only. UK—UK423,097, HN—HE-NECA, MRE—MRE0094, 2chloro—2-chloroadenosine, PSB—PSB0777, CGS—CGS21680, rega—regadenoson, CV—CV1808

Interestingly, a high affinity of the A_{2A} receptor agonist does not predict a high anti-platelet effect of this compound. For example, we found a moderate antiplatelet effect for PSB0777 and CGS21680 which have been shown to have the high affinities to A_{2A} receptor (Ki = 44.4 nM [32], and Ki = 27 nM [33], respectively), while NECA was observed to have one of the most robust antiplatelet properties, despite its lower affinity to the receptor (Ki = 620 nM [34]). Similar observations were reported previously [18].

The inter-individual variation in sensitivity to AR agonists was high, especially for weaker agonists, such as PSB0777 or CV1808, the coefficient of variation frequently exceeding 100%. Such a high value and relatively small sample size may account for no significant drop being observed in

platelet function in some cases. It is noteworthy that P-selectin expression and GPIIb-IIIa activation methods were found to be less sensitive in detecting the effects of AR agonists in comparison with fibrinogen binding and calcium ion mobilization. P-selectin expression and GPIIb-IIIa activation indicate overall platelet activation and are standard markers of full platelet activation leading to thrombus formation. Additionally, GPIIb-IIIa is a key, final target for anti-platelet therapeutic intervention [35,36]. The changes in those parameters indicate an interference in platelet activation cascade and therefore the inhibition of pro-thrombotic process. Fibrinogen is the main ligand of the GPIIb-IIIa receptor, but activation of this receptor is not synonymous with having bound a molecule of fibrinogen and starting the crosslinking process [37]. In fact, the GPIIb-IIIa activation and fibrinogen binding experimental results are, as expected, highly correlated ($R^2 = 0.73$), but in our study fibrinogen binding turned out to be a more sensitive marker for detecting the inhibition caused by AR agonists. It could be speculated that binding of exogenous fibrinogen could also be mediated via other platelet-fibrinogen interactions, such as fibrinogen binding to receptors GPVI, GPIb or even non-specific binding. Hence, this parameter could also detect the impact of AR agonists and $P2Y_{12}$ inhibitors on other signalling pathways, but this issue needs further studies.

Calcium mobilization is a very dynamic process, susceptible to rapid change in response to stimulus and therefore a good marker to detect changes in platelet signalling. It governs not only platelet activation, but also secretion and aggregation [38]. It is therefore understandable that subtler changes are possible to register with appropriately sensitive measurement of calcium flux and its inhibition. Generally, as AR agonists are fast-acting compounds, the methods detecting early activation events are more suitable for assessing their antiplatelet action.

cAMP is an important mediator since its increase leads to the activation of signalling pathway (mainly protein kinase A cascade) resulting in the inhibition of platelet function [39]. To assess the effect of AR agonists and their combination with $P2Y_{12}$ antagonists, we have selected two of the most potent AR agonists (UK423,097 and HE-NECA) and one representative of the less effective AR agonists (PSB0777) in combination with cangrelor. The compounds were tested in two concentrations: one being the IC_{50} value and the second one a high concentration selected to demonstrate a maximal inhibitory effect (100 μM for AR agonists and 1 μM for cangrelor); in resting and activated (20 μM ADP) platelets. It was demonstrated that all three tested AR agonists have the ability to increase cAMP formation in human platelets ex vivo (however the weaker one—PSB0777, only in the high concentration of 100 μM). These results are in accordance with the data reported previously in in vitro cell culture studies using standard cell lines such as CHO or HEK-293 [31,40]. The obtained results are also consistent with those previously reported in the literature using human platelets and comparable methods for a different group of AR agonists [18].

Interestingly, this work provides the first report suggesting that the AR agonists alone and in the combination with a $P2Y_{12}$ antagonist increase cAMP formation in ADP-activated platelets. This suggests that such dual experimental therapy may be beneficial in pathologically upregulated platelets or in case of platelets with arrested or delayed cAMP formation rates. The observation that cAMP formation is increased by AR agonists and the fact that AR agonists enhanced the cAMP elevation caused by $P2Y_{12}$ inhibitors confirms that this dual experimental therapy is effective at the pivotal point of platelet activation control.

The phosphorylation of vasodilator-stimulated phosphoprotein (VASP) is a marker of ADP-induced platelet activation through $P2Y_{12}$ receptor [41]. We hypothesised, based on calcium mobilization and cAMP formation results which suggested the inhibition of platelet function through pathway involving VASP phosphorylation, that our proposed dual approach would suppress this process. It seems however, that the applied method (a commercial kit) does not have enough sensitivity to detect changes effected by applied low (subclinical) concentrations of AR agonists and $P2Y_{12}$ inhibitors. Additionally, the method produced inconsistent readings and very high experimental background. In our opinion, therefore, the use of VASP phosphorylation detection kits dedicated to

monitoring of $P2Y_{12}$ function in clinical setting is not suitable to research purposes aiming to detect subtle changes in platelet signalling.

The majority of the results (cAMP measurement was an exception) presented in this manuscript were obtained using methods assessing platelet function in whole blood. Such the approach was chosen deliberately since it is known that endogenous adenosine undergoes rapid and excessive uptake and metabolism by erythrocytes [42]. Synthetic AR agonists investigated in this work were reported [13,20,43] to be much more stable in blood, we decided to use the experimental conditions mimicking the physiological ones, and also our aim was to minimize a risk of the incidental platelet activation during the process of the isolation of platelet-rich plasma or platelets.

Our rationale to use methods based on flow cytometry resulted from a need to screen many experimental samples (at least 30 per experiment for each blood donor) providing an opportunity to quickly and reliably obtain a complete panel of data with minimal amount of human material (whole blood) used. Furthermore, flow cytometry analysis is especially valuable when working with blood platelets which are prone to artefactual activation. In the case of cAMP formation experiments, when platelet isolation was inevitable to perform a colorimetric assay, it requires the number of tested combinations to be limited.

4. Materials and Methods

4.1. Chemicals

Adenosine receptor agonists were purchased from Sigma (St. Louis, MO, USA)—NECA (CAS № 35920-39-9); Cayman (Ann Arbor, MI, USA)—regadenoson (CAS № 313348-27-5), 2-chloroadenosine (CAS № 146-77-0); Tocris Bioscience (Bristol, United Kingdom)—PSB0777 (CAS № 2122196-16-9), CGS21680 (CAS № 124431-80-7), CV1808 (CAS № 53296-10-9); Abcam (Cambridge, UK)—HE-NECA (CAS № 141018-30-6); Axon Medchem (Reston, VA, USA)—UK 432097 (CAS № 380221-63-6)); and MyBioSource (San Diego, CA, USA)—MRE0094 (CAS № 131865-88-8)). Cangrelor (AR-C69931MX) was from Cayman Chemicals). Prasugrel metabolite (R-138727) was obtained from BOC Sciences (Shirley, NY, USA). Calcein AM was obtained from Molecular Probes (Eugene, OR, USA). Antibodies anti-human CD61/PerCP, CD61/PE, CD62P/PE, PAC-1/FITC, mouse IgG1/PE isotype control, mouse IgG1/FITC isotype control, Cellfix, and buffered sodium citrate was purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Fibrinogen from Human Plasma, Oregon Green 488 Conjugate, and Fluo-4, AM, cell permeant were purchased form Invitrogen (Carlsbad, CA, USA). PLT VASP/$P2Y_{12}$ kit was purchased form BioCytex (Marseille, France). Cell permeant calcium indicator Fluo-4 AM acetoxymethyl ester (Fluo-4 AM), probenecid (water soluble), pluronic acid (water solution) and thapsigargin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cyclic AMP Select ELISA Kit was purchased form Cayman Chemical. Phosphate buffered saline pH 7.4 (PBS) was obtained from Corning (New York, NY, USA). Dimethyl sulfoxide (DMSO), adenosine diphosphate (ADP), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). All other chemicals, unless otherwise stated, were purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland).

4.2. Chemical Solutions Preparation

The stock and working solutions of cangrelor and prasugrel metabolite were prepared in distilled water. The 100 mM stock solutions of AR agonists were prepared in DMSO, excluding PSB0777 which was dissolved in water. Stock solutions were then diluted with DMSO and PBS to working concentrations (as observed by Boncler et al. [19], diluting AR agonist stocks may result in precipitates, which was avoided in this study), and added to the biological material. The dilution factor was chosen to maintain the maximal final concentration of DMSO not exceeding 0.1% in the biological samples in all of the assays. Each compound was used at its aggregation IC_{50} value [19,21]: PSB0777 23 μM, CGS21680

1 μM, MRE0094 26 μM, 2-chloroadenosine 5 μM, CV1808 25 μM, HE-NECA 0.2 μM, NECA 0.5 μM, regadenoson 1.2 μM, and UK423,097 1 μM, cangrelor 17 nM, and PM 1.3 μM, unless otherwise specified.

4.3. Blood Donors

Experiments were approved by the Ethics of Research in Human Experimentation Committee at the Medical University of Lodz, approval number (RNN/43/17/KE). Blood was collected from healthy donors who gave written consent (n = 34, 30% men and 70% women; mean age 27.5 ± 8.5 years) into a vacuum tube containing 0.105 mol/L buffered sodium citrate (final citrate:blood ratio of 1:9 v/v) for experiment conducted using whole blood, or into a vacuum tube containing acid citrate dextrose (ACD) (final ACD:blood ratio of 1:7 v/v) for experiments requiring isolated platelets (cAMP level measurement). All individuals stated that they had not taken medications known to influence platelet function for at least two weeks prior to the study.

4.4. Platelet Viability Assay

Platelet viability of resting platelets in the presence of AR agonist was assessed accordingly to Rywaniak et al [22]. Samples were preincubated with AR agonists for 3 min at 37 °C. Positive control (low platelet viability) was blood preincubated in the presence of 1% paraformaldehyde (PFA) for 15 min at 37 °C. Samples were then diluted 10-fold with PBS pH 7.4, labelled with anti-CD61/PE antibodies (15 min, RT) and stained with 0.1 μM calcein-AM (15 min, 37 °C). CD61/PE-positive events (5000) were gathered immediately after staining using flow cytometry (FACSCanto II, BD Bioscience, San Diego, CA, USA). The percentage of calcein-negative platelets was measured.

4.5. P-Selectin Expression and GPIIb-IIIa Activation

Whole blood was preincubated with an AR agonist and/or a $P2Y_{12}$ inhibitor for 3 (AR agonists and cangrelor) or 15 (prasugrel metabolite) minutes at 37 °C. Platelets were activated with 20 μM ADP for 5 min at RT. Samples were then diluted 10-fold with PBS, labelled with anti-CD61/PerCP, anti-CD62P/PE and PAC-1/FITC antibodies (15 min, RT), and fixed with CellFix (prepared according to manufacturer instructions) for 1h at RT. Directly before measurement, the samples were diluted 1:1 with PBS and the assay was performed, gathering 10000 CD61/PerCP-positive events, using FACSCanto II flow cytometer (BD Bioscience). The percentage of marker-positive platelets (above isotype cut-off) was measured.

4.6. Binding of Exogenous Fibrinogen

Whole blood was preincubated with an AR agonist and/or a $P2Y_{12}$ inhibitor for 3 (AR agonists and cangrelor) or 15 (prasugrel metabolite) minutes at 37 °C. Exogenous Oregon Green-labelled fibrinogen was added to the samples (3 μg/mL), which were subsequently activated with 20 μM ADP for 5 min at RT. Samples were then diluted 10-fold with PBS, labelled with anti-CD61/PE antibodies (15 min, RT), and fixed with CellFix (prepared according to manufacturer instructions) for 1 h at RT. Directly before measurement, the samples were diluted 1:1 with PBS and the assay was performed, gathering 10,000 CD61/PE-positive events, using FACSCanto II flow cytometer (BD Bioscience). The percentage of marker-positive platelets was measured.

4.7. VASP-P Measurement

A PLT VASP/$P2Y_{12}$ kit was used to monitor specific platelet ADP receptor antagonists according to manufacturer's instructions; the analysis was performed on whole blood preincubated with an AR agonist and/or a $P2Y_{12}$ inhibitor for 3 (AR agonists and cangrelor) or 15 (prasugrel metabolite) minutes at 37 °C. Afterwards, samples were supplemented with prostaglandin E1, activated with ADP, fixed, permeabilized and stained with anti VASP-P monoclonal antibodies and next with secondary polyclonal fluorescently labelled antibodies. The non-specific fluorescence was determined using

negative isotypic control. Using FACSCanto II flow cytometer (Becton-Dickinson) 10,000 events identified as platelets (CD61-positive) were acquired, and their mean fluorescence intensity was measured using FACSCanto II flow cytometer (BD Bioscience).

4.8. Calcium Mobilization

Whole blood was preincubated with an AR agonist and/or a $P2Y_{12}$ inhibitor for 3 (AR agonists and cangrelor) or 15 (prasugrel metabolite) minutes at 37 °C. Next, samples were diluted 10-fold in PBS containing 1 mM of $CaCl_2$, and incubated with Fluo-4 AM (final concentration 3 μM) for 15 min at 37 °C, with pluronic acid (final concentration of 0.02%) to facilitate the solubilisation of Fluo-4. To prevent the efflux of calcium indicator out from the cells, samples were supplemented with the inhibitor of organic-anions transporters, probenecid (at final concentration of 2.5 mM). Platelets were then labelled with anti-CD61/PerCp antibodies for 20 min, RT, and samples were diluted 10-fold in PBS containing 1 mM of $MgCl_2$. Directly before the measurement platelets were stimulated with ADP (final concentration 20 μM) and after 10 s the end-point fluorescence intensity was measured on FACSCanto II flow cytometer (BD Bioscience).

4.9. cAMP

Blood platelets were isolated by a two-step centrifugation at 37 °C: first 200× *g* for 12 min to obtain platelet rich plasma (PRP) with 50 ng/mL PGE_1 added, and subsequent centrifugation of PRP at 800× *g* for 15 min, again with 50 ng/mL PGE_1 added, and re-suspension of platelet pellet with Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, pH to 7.4) with 0.3% bovine serum album and 0.5 mM papaverine. Platelet count in the suspension was measured using Sysmex XS-800i (Sysmex, Kobe, Japan) automated morphology instrument. After at least 35 min from last PGE_1 addition, platelets were incubated with AR agonist and/or $P2Y_{12}$ antagonist for 3 min at 37 °C. If required, platelets were then incubated with 20 μM ADP for 5 min at RT. Subsequently lysis was performed using lysis buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mMEDTA, 0.1% Triton® X-100, pH 7.4) in 1:1 *v*/*v* ration to the platelet volume for 10 min at RT, and suspension was centrifuged at 10,000× g for 3 min. Cyclic AMP was measured using a Cyclic AMP Select ELISA Kit (Cayman Chemical) according to manufacturer's instructions.

4.10. Statistical Analysis

The results are expressed as median with interquartile range. The Shapiro-Wilk test and Mauchley's test were used to test the data distribution and sphericity of variances, respectively. Data with Gaussian distribution was analysed with one-way ANOVA for repeated measurements with the *post hoc* Bonferroni's multiple comparisons test. Data departing from Gaussian distribution were assessed with the Friedman's test with Dunn's correction for multiple comparisons. The statistical analysis was performed using the following software packages: Statistica v.13 (Dell Software, Round Rock, TX, USA), and GraphPad Prism (GraphPad Software, San Diego, CA, USA).

5. Conclusions

In summary, this work provides the comprehensive evidence of the antiplatelet potential of AR agonists, demonstrated on multiple levels of platelet activation process, from calcium flux inhibition, and cAMP formation increase to restriction of surface markers of platelet activation such as P-selectin expression and GPIIb-IIIa activation together with fibrinogen binding. All the AR agonists studied were able to strengthen the effect of at least one $P2Y_{12}$ receptor inhibitor; therefore, a dual experimental therapy involving combination of $P2Y_{12}$ inhibitors and AR agonists appears to be a feasible solution to overcoming problems of drug resistance leading to dosage increase and resulting in severe side effects, and a way of combating inter-individual variation [21]. The approach combining the blocking of $P2Y_{12}$ receptor and the activation of AR receptors using novel agonists of AR receptors may prove to be a

favourable strategy of preventing thrombotic events, and should therefore be further investigated (including in vivo studies in animal models).

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-8247/13/8/177/s1. Figure S1. Representative cytometric dot-plots and histograms showing dual effect of AR agonist (UK432,097) and $P2Y_{12}$ antagonists (cangrelor and prasugrel metabolite) in whole blood on the expression/binding of platelet surface activation markers and intracellular calcium mobilisation.; Table S1. Effects of AR agonists on platelet viability; Table S2. The effects of adenosine receptor agonists and $P2Y_{12}$ antagonists on VASP phosphorylation.

Author Contributions: Conceptualization, N.W., B.L., C.W. and M.R.; Data analysis, N.W., H.K., B.L. and M.R.; Formal analysis, N.W. and B.L.; Funding acquisition, C.W. and M.R.; Investigation, N.W. and H.K., Methodology, N.W., H.K., B.L. and M.R.; Project administration, M.R.; Supervision, B.L., C.W. and M.R.; Writing—original draft, N.W.; Writing—review & editing, B.L., C.W. and M.R. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by "Dual purinoreceptor-dependent approach to prevent thromboembolic events dependent on blood platelets and endothelium—approaches based on animal and cellular models" TEAM/2016-1/8 project carried out within the TEAM programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund, and "Adenosine receptor agonists and $P2Y_{12}$ receptor antagonists exhibit combined inhibitory effect on blood platelet function" 2019/32/T/NZ3/00333 ETIUDA7 doctoral scholarship founded by National Science Centre—Poland.

Conflicts of Interest: The authors declare no conflict of interest.

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STATEMENT

I declare that my contribution to the article:

Wolska, N., H. Kassassir, B. Luzak, C. Watala, and M. Rozalski. 2020. 'Adenosine Receptor Agonists Increase the Inhibition of Platelet Function by P2Y12 Antagonists in a cAMP- and Calcium-Dependent Manner', Pharmaceuticals, 13. DOI: 10.3390/ph13080177

consisted of participation in a study design, execution of the experiments, data analysis, the manuscript review and editing, and preparing the response to reviewers.

I agree to use of the above publication by Nina Wolska, MSc. in her doctoral dissertation.

OŚWIADCZENIE

Oświadczam, że mój wkład w artykuł:

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Wyrażam zgodę na wykorzystanie powyższej publikacji przez mgr inż. Ninę Wolską w jej pracy doktorskiej.

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