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$P2Y_{12}$ antibody inhibits platelet activity and protects against thrombogenesis

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ABSTRACT

Given that platelet hyperactivity is known to give rise to thrombotic disorders, new and/or novel antiplatelet therapies are constantly being developed to add to, or to complement the current arsenal of agents. To this end, adenosine diphosphate (ADP) is an important platelet activator that acts by binding to the G-protein coupled $P2Y_1$ and $P2Y_{12}$ receptors. Although the contribution of the $P2Y_{12}$ receptor to the genesis of thrombosis is well established, the parenteral arsenal of drugs targeting this receptor in clinical use is limited to cangrelor. In this study, we investigated the potential antiplatelet activity of an antibody targeting the ligand-binding domain of the $P2Y_{12}$ receptor (abbreviated $P2Y_{12}$Ab). Our *in vitro* studies revealed that the $P2Y_{12}$Ab could effectively inhibit aggregation induced by ADP, as well as that triggered by the thromboxane receptor agonist U46619. Additionally, using FACS analysis, we observed reduced P-selectin, phosphatidylserine exposure and integrin activation in the presence of $P2Y_{12}$Ab. As for its *in vivo* effects, the $P2Y_{12}$Ab also demonstrated protection against thrombus formation; albeit this was accompanied with a bleeding diathesis (longer bleeding time). Notably, this inhibitory profile is consistent with that observed with oral anti-$P2Y_{12}$ agents. Collectively, our findings demonstrate that the $P2Y_{12}$Ab functionally blocks platelet activity *in vitro* and *in vivo*, and support the notion that it can be purposed as a parenteral antiplatelet agent, to be used in conjunction with and/or as a complement to current antiplatelet therapies.

1. Introduction

ADP is the endogenous ligand for two platelet G-protein coupled receptors, namely $P2Y_1$ and $P2Y_{12}$. Although both of these receptors are activated upon ADP binding, they utilize separate mechanisms for initiation of platelet activation, i.e., the Gq and the Gi signaling pathways [1]. Co-activation of both P2Y receptors is required for a robust activation of platelets; however, it has been noted that inhibition of only one of them is sufficient for reducing ADP-induced platelet activity [2]. To this end, the antiplatelet agent clopidogrel (plavix®) directly and irreversibly targets the $P2Y_{12}$ receptor [3]. Currently, there remains drug-development interest in the $P2Y_{12}$ receptor, amongst academic and industry research laboratories. This derives, in part, from multiple clinical studies that found that co-treatment with both clopidogrel and aspirin improves efficacy and reduces the number of major thrombotic events, when compared to aspirin alone [4].

In terms of FDA-approved antibody-based antiplatelet therapeutic agents, currently there is only one, namely abciximab (ReoPro®), which is a noncompetitive/irreversible inhibitor of active GPIIb/IIIa. By functionally blocking platelet surface integrins, abciximab effectively reduces the fibrinogen network that allows platelets to bridge together forming an aggregate. One pharmacokinetic advantage of abciximab is that it does not have to be adjusted, dosage wise, for patients with renal failure, as it is metabolized by proteolytic cleavage [5]. However, given its "broad spectrum" inhibition of platelets, bleeding is one of its major limitations [6]. Based on these considerations, there is a need to develop other parenteral antiplatelet agents, perhaps that specifically target receptors that are known to play a central role in thrombosis in order to create a more tailored or personalized

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antiplatelet therapy [7]. In this connection, we have previously shown that antibodies targeting the (ligand-binding domain) second extracellular loop of the $P2Y_1$ receptor and the thromboxane receptor (TPR) exerted function-blocking activity with regard to aggregation, and thrombosis, underscoring their potential as parenteral antiplatelet therapy [8,9]. The results of these studies suggest that the use of novel antibody antiplatelet therapies could be an effective complement or alternative for the prevention of thromboembolic disorders such as heart attack or stroke.

It is noteworthy that the availability of parenteral/intravenous (IV) and oral agents that target the same receptor would allow for a therapeutic regimen where the patient is started on that IV agent, and then prescribed the oral one for maintenance purposes, rather than having to use agents (IV and oral) that have different targets (e.g., platelets and the clotting system). Thus, should a parenterally (IV) effective $P2Y_{12}$ targeting agent become available, one may initiate a therapeutic regimen were its use is followed by an orally active agent (against the $P2Y_{12}$ receptor) such as clopidogrel. This is not possible with our aforementioned $P2Y_1$ and TPR antibodies, since there are no oral (or parenteral) FDA-approved drugs for these receptors. Moreover, agents (e.g., an antibody) directly targeting the $P2Y_{12}$ receptor could have an "added" benefit in terms of their efficacy because it plays a more central role in platelet function/thrombosis than the $P2Y_1$ receptor [10]. Of note and thus far, clinical practice is faced with the challenge that cangrelor [11] is the only available IV $P2Y_{12}$ inhibitor, and its use is associated with a number of side effects [12] including bleeding (greater in incidence compared to clopidogrel in some studies), decreased alertness and/or labored breathing. Hence, there is clear need for additional/alternative IV $P2Y_{12}$ blockers (with a better safety profile).

In the present studies, we characterize an antibody targeting the platelet $P2Y_{12}$ receptor (abbreviated $P2Y_{12}$Ab) in terms of its function-blocking capacity, under *in vitro* and *in vivo* experimental conditions.

2. Materials and methods

2.1. Reagents and materials

ADP was from Chronolog Corporation (Havertown, PA), whereas U46619 and PGI_2 were from Cayman Chemical (Ann Arbor, MI). Fibrinogen and apyrase were from Sigma Aldrich (St. Louis, MO). Microscope slides were from Fisher Scientific (Hanover Park, IL). Coverslips were from Neuvitro Corporation (Vancouver, WA). CD62P, Annexin V, and PAC-1 FITC antibodies were from BD Biosciences (San Jose, CA). The rabbit antipeptide $P2Y_{12}$-specific polyclonal antibody (corresponding to amino acids 270–282 of the human $P2Y_{12}$ receptor) was from Alomone Labs (Jerusalem, Israel). All other reagents were of analytical grade.

2.2. Animals

C57BL/6 J mice were from Jackson Laboratories (Bar Harbor, ME). All mice used for experiments were 8–10 weeks of age, and housed in groups of 1–4 at 24 °C, under 12/12 light/dark cycles, with access to water and food *ad libitum*. All experiments involving animals were performed in compliance with the relevant laws and institutional guidelines, and approved by the Institutional Animal Care and Use Committee.

2.3. Human subjects

Human blood studies were approved by the Institutional Review Board, and donors were asked to sign a written consent, and a subjects' bill of rights.

2.4. Human and mouse platelet preparation

Platelets were prepared as previously described [13]. Blood was drawn from healthy volunteers, followed by centrifugation at 237xg for 10 min at room temperature (RT). Platelet-rich plasma (PRP) was recovered and platelets were pelleted at 483xg for 10 min at RT. The pellets were resuspended in buffer, then washed and resuspended again. Platelets were counted with an automated hematology analyzer (Drew Scientific Dallas, TX) and the counts adjusted to the indicated concentrations.

2.5. In vitro platelet aggregation

The PRP from our human blood samples was incubated with vehicle or 2.4–4.8 μg $P2Y_{12}$Ab for 3 min. The samples were then stimulated with 10 μM ADP or 1 μM U46619. Platelet aggregation was measured using the turbidometric method, with models 490 or 700 aggregometry systems (Chronolog Corporation, Havertown, PA).

2.6. Flow cytometric analysis

Flow cytometric analysis for P-selectin, Annexin-V or PAC1 was carried out as we described before [14]. Briefly, human platelets (2×10^8) were incubated in the presence or absence of $P2Y_{12}$Ab (2.4 μg) for 5 min and then stimulated with ADP (10 μM) or U46619 (1 μM) for 3 min. Platelets were then incubated with FITC-conjugated anti–P-selectin to measure α-granule secretion, FITC-conjugated anti-Annexin V to measure phosphatidylserine exposure, or PAC-1 antibodies to measure GPIIb-IIIa activation. Finally, fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using compatible software CFlow Plus (BD Biosciences, Franklin Lakes, NJ).

2.7. Immunofluorescence microscopy

Immunofluorescence microscopy was used to assess platelet spreading, as previously described [15]. In brief, washed platelets were allowed to incubate in the presence or absence of $P2Y_{12}$Ab (2.4 μg) for 5 min. Then, they were left as is (resting) or treated with 10 μM ADP for 5 min at 37 °C and placed onto fibrinogen-coated coverslips for 30 min. Platelets were fixed, rinsed/washed and incubated with TRITC-conjugated phalloidin. Coverslips were mounted and examined with an Eclipse Ti-5 epifluorescence microscope (Nikon, Melville, NY).

2.8. Tail bleeding time essay

Mice were IV injected with $P2Y_{12}$Ab (4.8 μg) or saline and the tail bleeding assay was performed after 1 h, as described before [14]. Briefly, mice were anesthetized before the tail was transected at a distance of 5 mm from the tip. The tail was then immediately immersed in saline maintained at 37 °C at constant temperature, and the time to bleeding cessation was measured (observed visually).

2.9. $FeCl_3$ carotid artery injury thrombosis assay

Mice were IV injected with $P2Y_{12}$Ab (4.8 μg) or saline and the $FeCl_3$-induced thrombosis model was performed after 1 h, as described before [14]. An hour post injection, animals were anesthetized, and a midline incision of the skin was made and a segment of the left common carotid artery was exposed and cleaned. Baseline carotid blood flow was measured and recorded before thrombosis was caused by placing a filter paper