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Differential anti-thrombotic benefit and bleeding risk profiles of antagonists of protease-activated receptor 1 and 4 in Cynomolgus Macaques

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ABSTRACT

Platelet activation plays a crucial role in hemostasis and thrombosis. Thrombin, the most potent stimulus of platelet activation, mediates platelet activation via the protease activated receptors (PARs). The platelet PAR repertoire in mediating thrombin's action differs across species. Only nonhuman primate (NHP) platelet activation is known to be similar to humans, mediated by PAR1 and PAR4, hence limiting translational in vivo studies of PAR's role in thrombosis and hemostasis to NHPs. Earlier studies have demonstrated a range of distinct in vitro activities of PAR1 and 4 in platelet activation yet the implications of these events in vivo is unclear. The objective of this study is to investigate and compare the roles of PAR1 and PAR4 in hemostasis and thrombosis in a relevant animal species. NHP models for pharmacokinetic, ex vivo platelet aggregation responses, FeCl3 injury-mediated arterial thrombosis and template bleeding were developed in Cynomolgus Macaques. Potent and selective small molecule antagonists of PAR1 and PAR4 were characterized in an array of in vitro assays, and subsequently examined head-to-head in the NHP models. Treatment of NHPs with antagonists of PAR1 or PAR4 both resulted in strong inhibition of ex vivo platelet aggregation. At doses that led to similar inhibition of platelet aggregation, animals treated with the PAR4 antagonist showed similar levels of anti-thrombotic efficacy, but longer bleeding times, compared to animals treated with the PAR1 antagonist. These findings suggest that PAR1 antagonism will likely produce a larger therapeutic index (ie. a larger anti-thrombotic efficacy over bleeding risk margin) than PAR4 antagonism.

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1. Introduction

Platelet activation plays a crucial role in normal hemostasis preventing blood loss after injury, but also in the development of thrombosis that leads to clinical manifestations of atherothrombotic diseases. Platelets can be activated by a number of different mechanisms, including stimulation by adenosine diphosphate (ADP), thromboxane A_2, collagen and thrombin [10]. Clinical benefits of inhibiting platelet activation are well documented with aspirin treatment inhibiting the synthesis of thromboxane A_2, and clopidogrel treatment which blocks the activation of major platelet ADP receptor $P2Y_{12}$. Aspirin and clopidogrel represent the standard-of-care for the treatment of recurrent thrombotic complications in patients with manifestation of atherosclerotic disease [1,13].

Thrombin is the most potent platelet agonist and can stimulate platelet activation at subnanomolar concentrations [2]. Thrombin-induced platelet activation is mediated by a group of G protein coupled receptors (GPCR), named protease-activated receptors (PARs). Of the four known subtypes of PARs, PAR1, PAR3 and PAR4 are activated by thrombin, while PAR2 is activated by trypsin and trypsin-like protease but not by thrombin [2,8]. PAR activation by thrombin is mediated by a unique tethered ligand mechanism [8,29]. These receptors carry their own

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ligand, but they remain silent until activation by receptor cleavage. Upon binding to the receptor, thrombin recognizes the N-terminal exodomain of the receptor, and cleaves the peptide bond, leading to unmasking of a new amino terminus. This newly exposed NH2 terminus sequence functions as a tethered ligand, binds intramolecularly to the body of the receptor which then elicits transmembrane signaling. Synthetic peptides that mimic the sequence of the tethered ligand will thus function as agonists for the PAR receptor independent of receptor cleavage [14,15].

Thrombin binds to PAR1 and PAR4 with differential affinity. EC50s of thrombin binding were estimated to be 50 pM for PAR1 and 5 nM for PAR4 [2]. Differences in the requirement of thrombin levels to activate these two receptors appear to be at least in part due to the presence of a hirudin-like sequence in the N-terminal exodomain of PAR1 but not PAR4 [19]. Instead of binding to a hirudin-like sequence, PAR4 utilizes a negatively charged cluster of amino sequence, thus dissociates with slow kinetics from the positively charged thrombin [18]. In addition to differences in binding affinity, a growing body of evidence demonstrates many differences in PAR1- and PAR4-mediated platelet activation, such as differences in kinetics and the magnitude of response [9,25], and downstream signaling pathways [5,12,17,21,28].

Thrombin-mediated platelet activation also differs across animal species. For example, platelet activation by thrombin is mediated by PAR1 and PAR4 in humans, in contrast to PAR3 and PAR4 in mice, and PAR1 and PAR3 in guinea pig [2]. The nonhuman primate (NHP) is the only known species wherein its platelet activation by thrombin is also mediated by PAR1 and PAR4, similar to humans [2,8]. Given their important roles in platelet activation, PAR1 and PAR4 are attractive targets for novel antiplatelet therapies. Vorapaxar, a novel first-in-class PAR1 antagonist, was recently approved by the FDA for reduction of thrombotic cardiovascular events in patients with a history of myocardial infarction or with peripheral arterial disease [3]. The objective of this study was to compare the effects of PAR1 vs. PAR4 antagonism on hemostasis and thrombosis. Potent and selective small molecule antagonists of PAR1 and PAR4 were synthesized and profiled in vitro. NHP were chosen as study subjects due to their similarity in platelet expression of the PAR receptor repertoire to humans [2,8,11]. NHP models of pharmacokinetics (PK), pharmacodynamics (PD), arterial thrombosis, as well as template bleeding were developed. Animals treated with PAR1 and PAR4 antagonists were then evaluated in head-to-head comparisons.

2. Materials and methods

2.1. Reagents

Antagonists of PAR1 (MRK-PAR01) and PAR4 (MRK-PAR04) were prepared by Merck & Co., Inc. (Kenilworth, NJ, see Table 1 for their respective potency and selectivity). Other reagents were purchased from the following companies: clopidogrel from Beta Pharma Scientific (Branford, CT), adenosine diphosphate (ADP) from Chrono-Log (Havertown, PA), haTRAP [H-Ala-Phe(4-fluoro)-Arg-Cha-homoArg-Tyr-NH2] from Elim Biopharmaceuticals (Hayward, CA), TRAP (H-Ala-Phe(para-Fluoro)-Arg-Cha-Cit-Tyr-NH2) and AYPGKF from Peptides International (Louisville, KY), human alpha-thrombin and human gamma-thrombin enzymes from Haematologic Technologies Inc. (Essex Junction, Vermont), ketamine from Wyeth (Fort Dodge, IA), and $FeCl_3$ (Iron(iii)chloride Anhydrous) from Acros Organics (Geel, Belgium).

2.2. In vitro experiments

2.2.1. FLIPR assays with PAR1 or PAR4 expression cell lines

HEK293 cells (endogenously expressing human PAR1) were grown in DMEM plus 10% heat inactivated FBS (Gibco, Grand Island, NY). U2OS cells stably expressing human PAR4 were grown in McCoy's 5A medium modified (Biowhittaker, Walkersville, MD) plus 10% heat inactivated FBS. 12,000 cells per well were plated into a 384 well plate (Greiner, Monroe, North Carolina) in 20 μL of cell growth media. After 24 h, the cell growth media was removed and 25 μL FLIPR assay buffer was added to each well. Compounds were added to the plate at a final DMSO concentration of 0.2%. One vial of Calcium 5 dye (Molecular Devices, Sunnyvale, CA) was reconstituted with 10 mL of FLIPR assay buffer (HBSS without phenol red with $Mg2+/Ca2+$, 20 mM Hepes, 2.5 mM Probenecid). 20 μL of this dyer solution was added to each well. Plate was centrifuged for 1 min at 600 rpm and then incubated for 60 min at room temperature in the dark. The Tetra FLIPR (Molecular Devices) added agonist and read the plate at Excitation 470–495, Emission 515–575. Cell activation was induced by treatment with respective agonists, including 3 μM of TRAP (PAR1), 35 μM of AYPGKF (PAR4), 3 nM of human alpha thrombin, or 400 nM human gamma thrombin. The concentrations of these agonists were selected based on average of pilot studies where approximately 80% of maximum cell activations (EC80) were observed with the respective agonist.

2.2.2. Platelet FLIPR experiments

Human blood was collected from healthy donors into 3.2% citrated tubes, and all steps were performed at room temperature. The blood was centrifuged at 200 $\times g$ for 20 min. Platelet rich plasma (PRP) from the top layer was transferred to a 15 mL polypropylene tube and treated with 500 nM Prostaglandin E1 (Sigma-Aldrich, Oakville, Ontario) for 5 min. The tube was centrifuged for 5 min at 2500 rpm to pellet the platelets. The supernatant was poured off and the platelets were gently washed with 10 mL platelet wash buffer (10 mM Hepes, 130 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM monopotassium phosphate, 4 mM sodium bicarbonate, 11.5 mM glucose, 0.2 mM ethylene glycol tetraacetic acid, and 0.2% BSA, at pH 6.5). The tube was centrifuged again at 2500 rpm, the supernatant removed, and the platelets were resuspended in 5 mL of platelet suspension buffer (10 mM Hepes, 130 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM monopotassium phosphate, 4 mM sodium bicarbonate, 11.5 mM glucose, 1.2 mM magnesium chloride, 1.8 mM calcium chloride, pH 7.4). One vial of calcium 4 dye (Molecular Devices, Sunnyvale, CA) was reconstituted with FLIPR buffer (HBSS without phenol red without Ca/Mg, 20 mM Hepes, 0.04% Chaps, 2.5 mM Probenecid). 1.5 million platelets (in 2460 μL) were plated into each well with 11,070 μL FLIPR buffer and 820 μL Calcium 4 dye. Cells were treated with increasing concentrations of compounds at 0.2% DMSO and incubated for 1 h in the dark. The

Table 1
In vitro activities of MRK-PAR01 and MRK-PAR04.

Cell	Agonist	MRK-PAR01 (IC50: nM)	MRK-PAR04 (IC50: nM)
HEK	PAR1-AP	21	6181
U2OS	PAR4-AP	3965	13
Platelet	PAR1-AP	54	5300
Platelet	PAR4-AP	9100	1
Platelet	Alpha-Thrombin	29	4200
Platelet	Gamma-Thrombin	>7390	32

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