Inhibitory Effects of MNS and BDA-410 on Human Platelet Aggregation

G.J. Czaplewski

Department of Bioengineering, University of Illinois, Chicago, IL 60607

A.J. Wieschhaus and A.H. Chishti

Department of Pharmacology, University of Illinois, Chicago, IL 60612

Cysteine proteases and tyrosine kinases are two classes of enzymes that have been implicated in the coagulation cascade. This cascade is responsible for the aggregation of human platelets and their role in thrombosis. Understanding the interactions between these two systems is essential in developing treatments to reduce the risk of acute myocardial infarction and ischemic stroke. A modified model for gel-filtered platelet isolation was developed and used in testing a known cysteine protease inhibitor, BDA-410, and a known tyrosine kinase inhibitor, MNS. MNS decreased platelet aggregation as previously reported. BDA-410 also decreased platelet aggregation but required relatively higher concentrations. Importantly, MNS and BDA-410 in combination exerted a near additive effect. Further testing would be required in order to explain the relationship between the two inhibitors on platelet aggregation. Together, these results suggest a role of combination therapy as a potential modality to reduce platelet aggregation and thrombosis in vivo.

Keywords : tyrosine kinase; cysteine protease; inhibitor;

Abbreviations: DMSO, dimethyl sulfoxide; MNS, 3,4-methylenedioxy-β-nitrostyrene; BDA-410, (2S)-N-{(1)-l-[(S)-hydroxy(3-oxo-2-phenyl-1-cyclopropen-1-yl)methyl]-2-methylpropyl}-2-benzenesulfony-lamino-4-methylpentanamide; ACD, acid citrate dextrose; PRP, platelet rich plasma; TRAP, thrombin receptor-activating peptide;

Introduction

Acute myocardial infarction and ischemic stroke are the first and third leading causes of death, respectively, in the United States.[1] These events are associated with the role of platelets in thrombosis and thromboembolism. Preventing an excessive accumulation of platelets on the endothelial cell lining is paramount to reducing these risks. Because platelet adhesion and the coagulation cascade are essential to proper wound healing, reducing the extent of thrombosis can be alternately achieved by inhibiting platelet aggregation. By using a cysteine protease inhibitor and a tyrosine kinase inhibitor and measuring platelet aggregation, a relationship can be established between the two systems with respect to aggregation. The relationship between the two systems should provide insight into their step-wise interactions within the coagulation cascade. The advantage of combining two low doses of combinatorial therapy instead of a high dose of a single drug is that side effects stemming from high doses can be reduced. Targeting the cysteine protease and tyrosine kinase systems using a combinatorial therapy should further reduce platelet aggregation and reduce the risk of acute myocardial infarction and ischemic stroke if the combination has an additive inhibitory effect.

Platelet aggregation describes platelet-platelet interactions resulting in the formation of aggregates. Before aggregation, each platelet is activated by an outside-in signaling pattern. When agonists such as thrombin, collagen, ADP, and fibrinogen are present in high enough concentrations outside the platelet, they bind to G-protein coupled receptors (GPCR) or diffuse directly into the platelet. GPCRs are present in abundance on the outer platelet membranes with the driving force for initial aggregation, integrin GPIIb-IIIa, expressing about 120,000 copies per platelet. [2-3] Binding to GPCRs causes the release of G-proteins inside the cell while diffusion can bypass this step and directly activate the platelet. G-proteins are a class of secondary messengers involved in the secondary cascade and ultimately regulate downstream intracellular signals and processes. G-proteins regulate the activation of cysteine proteases, tyrosine kinases, and other enzymes, which cause the signal to initiate a rise in intracellular Ca^{2+} levels and increased tyrosine phosphorylation. Phosphorylation increases cause formational and operational changes in the platelet. [4] The formational changes include a shape change in which pseudopodia extend from the previously smooth spherical exterior. These projections allow platelets to interrelate with each other and form aggregates. [5] In the presence of fibrin, the aggregates form a thrombus and prevent blood loss from the blood vessel. This process becomes harmful when a large enough percentage of the vessel becomes blocked, which causes a decrease in oxygen levels and ultimately infarction.

Combination effects of MNS and BDA-410

The exact path of intracellular signaling in platelet aggregation is an active area of research. Two systems that have been implicated in the process are the cysteine proteases and the Syk and Src family tyrosine kinases. Whether these two systems act in series, parallel, or independently in the signaling cascade is unknown. The calcium-dependent cysteine protease targeted here is the calpain protease system consisting of calpain-1, calpain-2, and calpastatin. [6] BDA-410 ((2S)-N-{(1)-l-[(S)-hydroxy(3-oxo-2-phenyl-1-cyclopropen-1-yl)methyl]-2-methylpropyl}-2-benzenesulfony-lamino-4-methylpentanamide) is a known inhibitor of calpain protease activity and has a molecular weight of 484.6 Da. [7-8] In this experiment, BDA-410 and MNS(3,4-

methylenedioxy-β-nitrostyrene) are tested in a combinatorial therapy in an attempt to inhibit platelet aggregation. To validate the platelet isolation procedure prior to testing, an established Syk and Src tyrosine kinase inhibitor known as MNS is used. MNS has a molecular weight of 193.16 Da, and has been shown to inhibit platelet aggregation particularly by preventing GPIIb/IIIa activation through the inhibition of tyrosine kinases. [9] To test the effectiveness of these two inhibitors, platelet aggregation was initialized through doses (≤0.15μM) of thrombin and (≤200μM) TRAP-4 (thrombin receptor-activating peptide).

Materials and Methods

Preparation of gel-filtered platelets: PRP (platelet rich plasma) anticoagulated with ACD (acid citrate dextrose) was obtained from volunteer donors who had not taken aspirin in the last 48 hours. A 10 ml column was built using Sepharose 2B gel layered over filter paper. The column was equilibriated with one washing of deionized water and three washings of Tyrode's modified buffer (10.0 mM HEPES, 12 mM $NaHCO_3$, pH 7.5, 137 mM NaCl, 2.5 mM KCl, 5 mM glucose, 0.35% bovine serum albumin and 1.0 mM $MgCl_2$). PRP was loaded at the top of the column and platelet elutant was collected. Less than 2 ml of platelets were collected to avoid plasma content. Platelet count was adjusted to $3x10^8$ cells/ml (in Tyrode's modified buffer) using a spectrophotometer. Platelets were allowed to rest for 30 minutes at room temperature prior to stimulation.

Inhibitor and platelet preparation: 100 μM BDA-410 and 10 μM MNS, using DMSO (dimethyl sulfoxide) as the vehicle (≤1% DMSO by volume), were added separately and jointly into platelet mixtures and incubated at room temperature for 30 minutes with slow rotation (5-10 rpm) prior to stimulation.

Platelet aggregation: Gel-filtered human platelet aggregation was measured using a CHRONO-LOG Whole Blood Aggregometer with 1,200 rpm stirring. [10] After 30 minutes of rest, platelets were placed into cuvettes with stir bars and incubated at 37°C for 3 minutes. After 3 minutes, platelets were supplemented with 1.0 mM $CaCl_2$ and placed inside the aggregometer for 2 minutes. Platelets were then treated with an agonist and aggregation was measured for 5 minutes.

Results

Platelets were isolated by two methods from human PRP anticoagulated with ACD. First, platelets were extracted from two cycles of centrifugal spinning of PRP at 2,400 rpm for 10 minutes with citrate-glucose-saline washing and re-suspension in between cycles. This isolation caused premature activation, resulting in poor response [Fig. 1] to high doses of thrombin. Platelets extracted from the gel-filtering process were responsive [Fig. 2] to low doses of thrombin. Similar curves were obtained for both methods but concentrations of thrombin varied greatly (0.05-1.0U). Subsequent experiments [Fig. 3] show a refinement in the gel-filtration process and a reduced dose of thrombin. Therefore, the gel-filtered procedure was adopted for the inhibitor experiments.

The gel-filtered procedure was then used to obtain results from the cysteine protease and tyrosine kinase inhibitors. MNS demonstrated an ability to inhibit aggregation [Fig. 4] as was shown recently. [9] At a concentration of 100 μM [Fig. 5], BDA-410 demonstrated the ability to inhibit platelet aggregation. Because 100 μM is the established IC_{50} for BDA-410 using thrombin as an agonist in neuroblastoma cultures, [11] these results in platelets seem plausible. Further studies should be conducted to determine the long term effects of 100 μM BDA-410 on platelet and cell cultures.

Using a dose of 0.15U/ml thrombin and a one-way analysis of variance with GraphPad Prism software, the means ($n = 3$) were not significantly different ($p = 0.0791$) at the 5-minute mark during aggregation tests. The p-value is almost significant and performing more trials could result in a significant difference. Furthermore, using a post-hoc Dunnett's test (column to control column comparison), the MNS and BDA-410 combination means were significantly different ($p < 0.05$) from the vehicle means. Each bar graph [Fig. 6] shows mean platelet aggregation ± SEM from 1 to 5 minutes after thrombin injection.

Using doses of 100-200 μM TRAP-4 and a one-way analysis of variance, the means ($n = 4$) were not significantly different ($p = 0.145$) at the 5-minute mark during aggregation tests. Running more trials using newly acquired TRAP-4 stored at -70°C with aliquots for each trial might produce more significant results and require lower doses for aggregation. Each bar graph [Fig. 7] shows mean platelet aggregation ± SEM from 1 to 5 minutes after TRAP-4 injection.

Discussion

This report details evidence for the effect of two inhibitors on human platelet aggregation. BDA-410 was more effective at inhibiting thrombin induced aggregation than at inhibiting TRAP-4 induced aggregation. MNS was also effective at inhibiting thrombin induced aggregation but not as effective using TRAP-4. In combination, MNS and BDA-410 had an additive effect at inhibiting thrombin induced aggregation. Because thrombin is one of the most potent platelet agonists, it is not surprising that the inhibitory effects were more easily seen in thrombin than in TRAP-4. ADP, collagen, U46619, TRAP-4, and other agonists require more platelet sensitivity usually found in fresh platelets while thrombin is effective even with less responsive or outdated platelets.

The requirement for higher doses of BDA-410 to reduce aggregation is unknown and could be the result of many factors. It is possible that BDA-410 is not very competitive with thrombin receptors or GPCRs, allowing platelet aggregation to overwhelm low levels of cysteine protease inhibition. Also, the role of cysteine proteases in aggregation could be less pronounced in comparison to tyrosine kinases, especially when using powerful agonists like thrombin. Performing more experiments using less potent agonists could answer this question.

Devising a consistent and effective method of platelet isolation was a preliminary problem that required an alteration of previously established methods. [12] Most of the procedure was obtained from Kuchay *et al.* [13] However, two anticoagulants, apyrase and PGE_1, were not added during platelet isolation because the desired platelet reactivity was achievable without further need for anticoagulants.

Collecting more than 3-4 ml of platelets at the onset of PRP addition to the column caused an unwanted amount of fibrinogen and other factors to remain unfiltered. Because these factors play major roles in the secondary coagulation cascade, they induced undesirable aggregation curves with 50-90% increases in aggregation in less than 30 seconds (data not shown). These rapid and dramatic increases in aggregation made it impossible to measure and observe the amount of inhibition using any agonist. Because fibrinogen plays a crucial role in the formation of fibrin, unwanted fibrinogen left in the platelet solution caused aggregated platelets to form accretions, which caused substantial amounts of noise on the aggregometer read-out. The presence of fibrinogen after 3 ml of elutant suggests that it might descend through the column more rapidly than its antagonist, the enzymatic precursor plasminogen.

Another problem that was encountered was the inconsistent response of the expired platelets to thrombin doses. Because there are many independent variables that affect the response of platelets including the donor, collecting agency, expiration date, and amount of anticoagulants present, it becomes difficult to maintain experimental consistency. A refined procedure such as the one used in this experiment is crucial when many uncontrollable variables are present. This is another reason why relatively large ranges of thrombin (0.003-1.5U) were used in preliminary experiments when testing isolation methods on expired platelets, prior to the final data being collected with fresh human platelets.

Conclusion

This project develops a consistent and detailed procedure for filtering platelets and reports results on the effectiveness of inhibitors on the cysteine protease and tyrosine kinase systems. BDA-410 exhibited inhibition of platelet aggregation as did MNS. A combination of the two treatments resulted in even further inhibition when using thrombin as the agonist. The requirement of high doses of BDA-410 might be due to a number of factors including the possibility of low or noncompetition with thrombin binding sites or GPCRs. Understanding the relationship between the two systems is crucial in producing combinatorial therapies that effectively and safely reduce the risk of acute myocardial infarction and ischemic stroke. Further experiments should be conducted using other agonists in order to comprehend these interactions and to determine the effects of long term exposure to 100 μM BDA-410.

Acknowledgements

This project was funded by the National Science Foundation (NSF-REU) and the Department of Defense (DoD ASSURE).

References

[1] Heron MP, Hoyert DL, Murphy SL, Xu J, Kochanek KD, Tejada-Vera B. Deaths: Final Data for 2006. National vital statistics reports; 2009 Apr; Vol 57 No 14. Hyattsville, MD: National Center for Health Statistics.

[2] Wagner CL, Mascelli, MA, Neblock, DS, Weisman, HF, Coller BS, Jordan RE. Analysis of GP IIb/IIIa receptor number by quantification of 7E3 binding to human platelets. Blood 1996; 88: 907– 914.

[3] Youssefian T, Masse JM, Rendu F, Guichard J, Cramer EM. Platelet and megakaryocyte dense granules contain glycoproteins Ib and IIb– IIIa. Blood 1997; 89: 4047– 4057.
[4] Shattil SJ, Kashiwagi H, Pampori N. Integrin signalling. The platelet paradigm. Blood 1998; 91: 2645– 2657.
[5] Gresele, P (Ed.) Page, CP (Ed.) & Fuster, V (Ed.). Platelets in Thrombotic and Non-thrombotic Disorders: Pathophysiology, Pharmacology and Therapeutics. Port Chester, NY, USA: Cambridge University Press, 2002.
[6] Goll DE, Thompson VF, Li H, et al. The calpain system. Physiol Rev 2003; 83:731–801.
[7] Li X, Chen H, Jeong JJ, Chishti AH. BDA-410: A novel synthetic calpain inhibitor active against blood stage malaria. Mol Biochem Parasitol. 2007 Sep; 155(1):26-32.
[8] Battaglia F, Trinchese F, Liu S, et al. Calpain inhibitors, a treatment for Alzheimer's disease: position paper. J Mol Neurosci 2003; 20(3):357–62.
[9] Wang WY, Wu YC, Wu CC. Prevention of platelet glycoprotein IIb/IIIa activation by 3,4-methylenedioxy-β-nitrostyrene; a novel tyrosine kinase inhibitor. Mol Pharmacol. 2006 Oct; 70(4):1380-9.
[10] Born, G. V. 2005. Light on platelets. J. Physiol. 568:713–714.
[11] Trinchese F, Fa M, Liu S, Zhang H, Hidalgo A, Schmidt S, Yamaguchi H, Yoshii N, Mathews P, Nixon R, Arancio O. Inhibition of calpains improves memory and synaptic transmission in a mouse model of Alzheimer disease. J Clin Invest. 2008 Aug; 118(8):2796-807.
[12] Vollmar B, Slotta JE, Nickels RM, Wenzel E, Menger MD. Comparative analysis of platelet isolation techniques for the in vivo study of the microcirculation. Microcirculation. 2003 Apr; 10(2):143-52.
[13] Kuchay SM, Kim N, Grunz EA, Fay WP, Chishti AH. Double knockouts reveal that protein tyrosine phosphatase 1B is a physiological target of calpain-1 in platelets. Mol Cell Biol. 2007 Sep; 27(17):6038-52.

Figures

Figure 1: Percent aggregation of platelets obtained by centrifugation using Thrombin as the agonist. Thrombin concentrations 0.1 U to 1.0 U.

Figure 2: Percent aggregation of platelets obtained by gel-filtration using Thrombin as the agonist. Thrombin concentrations 0.1 U to 0.5 U. Excessive noise is probably due to unfiltered fibrinogen causing activated platelets to bind together forming a pellet.

Figure 3: Percent aggregation of platelets obtained by gel filtration using Thrombin as the agonist. Thrombin concentrations 0.003 U to 0.05 U. Noise is no longer present, indicating the successful removal of fibrinogen during filtration.

Figure 4: Percent aggregation of platelets obtained by gel filtration using Thrombin as the agonist and MNS as the inhibitor. This data confirms a previous publication of the effectiveness of MNS as a platelet aggregation inhibitor.

Figure 5: Percent aggregation of platelets obtained by gel filtration using Thrombin as the agonist and BDA-410 as the inhibitor.

Figure 6: Bars show mean platelet aggregation ± SEM at 1 minute intervals after thrombin addition. (n = 3)

Figure 7: Bars show mean platelet aggregation ± SEM at 1 minute intervals after TRAP-4 addition. (n = 4)