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Interaction between thrombin mutant W215A/E217A and direct thrombin inhibitor

Kenichi A. Tanaka^a, Andras Gruber^b, Fania Szlam^a, Leslie A. Bush^c, Stephen R. Hanson^b, and Enrico Di Cera^c

^aDepartment of Anesthesiology, Emory University School of Medicine, Atlanta, Georgia

^bDepartment of Bioengineering, Oregon Health Science University, Beaverton, Oregon

^cDepartment of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St Louis, Missouri, USA

Thrombin mutant W215A/E217A acts as an anticoagulant when administered intravenously by activating plasma protein C in concert with endothelial thrombomodulin [1,2]. The double mutation in the catalytic domain of the W215A/E217A molecule leads to compromised interactions with fibrinogen and chromogenic thrombin substrates [1,3]. Among direct thrombin inhibitors (DTIs), argatroban exerts antithrombotic activity by a rapid binding to the catalytic domain of thrombin, while lepirudin and bivalirudin occupy both exosite I and catalytic domains [4]. We hereby report on the changes in DTI-interaction with W215A/E217A, using thrombin substrate hydrolysis [1,3], activated partial thromboplastin time (aPTT) and the Thrombinoscope methods (Thrombinoscope BV, Maastricht, Netherlands) [5].

Based on the inhibition of H-D-Phe-Pro-Arg-*p*-nitroanilide hydrolysis, argatroban and lepirudin interact with the catalytic domain of W215A/E217A with a 7000-fold reduced affinity relative to wild-type thrombin (Table 1) [6]. This reduction coincides with the 19 000-fold decrease in affinity for fibrinogen [1]. On the contrary, lack of inhibition of the substrate hydrolysis with bivalirudin suggests that W215A/E217A binds to bivalirudin utilizing exosite I without blocking the active site. This finding was confirmed in studies looking at the competitive inhibitory effect of bivalirudin on the activation of protein C by W215A/E217A in the presence of thrombomodulin (Table 1).

Although the direct interaction between W215A/E217A and DTIs seems to be weak, addition of W215A/E217A in DTI-treated plasma corrected prolongation of aPTT toward normal (Fig. 1). Prolonged aPTT with DTIs was not reversed with active site mutant S195A [7] or active-site blocked thrombin (wild-type thrombin inactivated with H-D-Phe-Pro-Arg-CH₂Cl; FPR-WT). Inactive thrombin species, S195A and FPR-wild-type, occupy thrombin binding sites on fibrin(ogen) and cause dose-dependent inhibition of clotting [8]. It is thus speculated that the catalytic domain of W215A/E217A exerts some procoagulant function through activation of factor V [9]. Anticoagulant effects of DTIs have been shown to be mitigated in the presence of factor Va [10,11].

Using the Thrombinoscope system that continuously monitors thrombin generation based on the hydrolysis of fluorogenic substrate, Z-Gly-Gly-AMC (Bachem Bioscience, King of Prussia, Pennsylvania, USA), we showed that in plasma the combination of W215A/E217A

Correspondence to Kenichi A. Tanaka, MD, MSc, Associate Professor of Anesthesiology, Division of Cardiothoracic Anesthesiology, Department of Anesthesiology, Emory University School of Medicine, 3B South EUH, 1364 Clifton Road, NE Atlanta, Georgia 30322, USA Tel: +1 404 778 3900; fax +1 404 778 5194; e-mail: E-mail: ktanaka@emory.edu.

(5µg/ml~135 nmol/l) and thrombomodulin (0.75µg/ml; Asahi Kasei Pharma, Oh-hito, Japan) [12], but not W215A/E217A alone exerted anticoagulant effects based on the reduced peak levels of thrombin generation (Fig. 2a). The addition of W215A/E217A was found to reverse prolonged thrombin generation lag time for all DTIs (Fig. 2). Reduced peak thrombin levels were not significantly recovered with W215A/E217A at therapeutic levels (0.5–1µg/ml) of argatroban (Fig. 2b) [13]. Our present data are in agreement with the reversal of melagatran-prolonged lag time in thrombin generation with factor Va [10]. For bivalent inhibitors, it is plausible that bivalirudin and lepirudin reduce the interaction of factor V with W215A/E217A by binding to exosite I [9]. FPR-wild-type (30µg/ml) reversed delayed onset of thrombin generation with bivalirudin, but clotting was extensively inhibited due to occupation of thrombin binding sites of fibrin with FPR-wild-type (Fig 1 and Fig 2c) [8]. Lepirudin obliterated thrombin generation, but this was restored toward normal after the addition of W215A/E217A, especially at therapeutic level (1µg/ml) of lepirudin (Fig. 2d).

In summary, the catalytic-site double mutant, W215A/E217A, shortens DTI-prolonged aPTT and the onset of endogenous thrombin generation *in vitro*. Further modifications of the catalytic domain or exosites of thrombin molecule or both may lead to a development of antidotes for DTI-related bleeding complications [10].

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Fig. 1.

Activated partial thromboplastin time, measured in normal volunteer plasma, spiked with a direct thrombin inhibitor ($n = 8$). Mean values ($\pm$ SD) of activated partial thromboplastin time (aPTT) (in seconds). Note aPTT values were shortened by W215A/E217A at 5 µg/ml at respective anticoagulant level. Panel a: argatroban 0.5 and 1 µg/ml; Panel b: bivalirudin at 5, 10, and 15 µg/ml; Panel c: lepirudin at 0.1, 1, and 5 µg/ml. † $P < 0.01$ for control vs. W215A/E217A at respective anticoagulant levels. Note inactive thrombins, S195A or active-site occupied thrombin (inactivated with H-D-Phe-Pro-Arg-CH₂Cl; FPR-WT), do not shorten aPTTs (* $P < 0.05$). WE, W215A/E217A.

Fig. 2.

Effects of W215A/E217A on thrombin generation. Panel a: a representative series of thrombin generation curves. The peak thrombin generation is slightly decreased by the addition of W215A/E217A, 5 μ g/ml, and modestly decreased by recombinant human soluble thrombomodulin (rhsTM, 0.75 μ g/ml). Addition of both W215A/E217A and thrombomodulin further blunts thrombin generation. Panel b: a representative series of thrombin generation curves exemplifies that the argatroban at 0.5 and 1 μ g/ml prolongs the onset and reduces the peak thrombin generation. Addition of W215A/E217A, 5 μ g/ml, shortens the onset, but does not reverse the reduced peak level of thrombin formation. Panel c: a representative series of thrombin generation curves exemplifies that the bivalirudin (Bival, 10 μ g/ml)-induced prolongation of the lag time can be reversed with W215A/E217A, 5 μ g/ml. Panel d: a representative series of thrombin generation curves exemplifies that the lepirudin at 1 and 5 μ g/ml blunts thrombin formation (flat lines). Addition of W215A/E217A, 5 μ g/ml, shortens the onset and restores the peak thrombin generation. FPR, thrombin inactivated with H-D-Phe-Pro-Arg-CH₂Cl; TM, thrombomodulin; WE, W215A/E217A.

Table 1

Binding of wild-type and mutant thrombin to thrombin inhibitors and substrates

	WT	WE
K_i (nmol/l)		
Argatroban	5.6	42×10^3
Bivalirudin	9.2	no inhibition (145) *
Lepirudin	0.000045	0.31
k_{cat}/K_m ($M^{-1}s^{-1}$)		
Fibrinogen	17×10^6	0.89×10^3
Platelet PAR1	26×10^6	26×10^3
Antithrombin	13×10^6	4×10^3
Thrombomodulin	220×10^3	33×10^3

The inhibitory constant (K_i) of direct thrombin inhibitors (DTI) toward wild-type and mutant thrombin (W215A/E217A). K_i values (nmol/l) were calculated from thrombin-mediated hydrolysis of H-D-Phe-Pro-Arg-*p*-nitroanilide in the presence of respective DTI. Experimental conditions are 5 mmol/l Tris, 0.1% polyethylene glycol (PEG), 145 mmol/l NaCl, pH 8.0, 25°C. The mean values of triplicate experiments are shown.

* Determined from competitive inhibition of protein C activation in the presence of thrombomodulin. k_{on} ($M^{-1}s^{-1}$) values for DTIs are adapted from [6]. k_{cat}/K_m values for thrombin and mutants are adapted from [1]. For respective DTI, k_{cat}/K_m values were calculated from thrombin-mediated hydrolysis of H-D-Phe-Pro-Arg-*p*-nitroanilide in the presence of DTI. Experimental conditions are 5 mmol/l Tris, 0.1% PEG, 145 mmol/l NaCl, pH 8.0, 25°C. The mean values of triplicate experiments are shown. WE, W215A/E217A; WT, wild-type; PAR1, protease activated receptor 1.