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Enzyme Assays

The rate of increase in absorbance at 405 nM due to hydrolysis of synthetic chromogenic peptide substrates (from American Diagnostica; 5-100 μM H-D-HHT-L-Ala-L-Arg-pNA for thrombin, 0.1-3 mM Cbo-Gly-D-Ala-Arg-pNA for trypsin, 20-500 μM H-D-Nle-HHT-Lys-pNA for plasmin, 30-700 μM H-D-Lys-(ε-Cbo)-Pro-Arg-pNA for protein Ca, 30-1000 μM CH_3SO_2-D-CHT-Gly-Arg-pNA for tPA, 70-3000 μM H-D-Nle-HHT-Lys-pNA for streptokinase) was measured in the presence and absence of inhibitors (I) with a microplate reader at 37 °C. The enzyme reaction is started by the addition of enzyme (0.9 nM human α-thrombin, 3.2 U/mL bovine trypsin, 1.4 μg/mL (0.049 CU) human plasmin, 3.63 mM activated human protein C, 724 IU/mL Bowes melanoma 2-chain tPA activity standard, 15.1 IU/mL streptokinase in the presence of 6.5 μg/mL Lys-type human plasminogen). Data were collected over a period of 30 min and the initial rate of substrate hydrolysis [V_o (mOD/min)] was calculated. Following Michaelis-Menten kinetics, the affinity of the enzyme for the substrate, in the absence of the inhibitor (K_m) and in the presence of an inhibitor (K_p), is determined to be the negative inverse x-intercept of Lineweaver-Burk plots. The K_i values were calculated by using the equation: $K_i = (K_m[I])/(K_p - K_m)$.

Trypsin-catalyzed hydrolysis rates were measured using the same type of procedure. Bovine type-1 trypsin (Sigma) and Spectrozyme® TRY (Cbo-Gly-D-Ala-Arg-pNA•AcOH; American Diagnostics) replaced their thrombin equivalents in a concentration range of 3.2 U/mL trypsin and 0.1-0.3 mM Spectrozyme®.

Enzyme Inhibition: K_i Values[a]

cmpd	trypsin	plasmin	tPA	prot. Ca	SK
2	3.1 ± 1.7 (3)	2,200 ± 600 (5)	620 ± 32 (6)	3,600 ± 1,500 (5)	1,200 ± 600 (6)
efegatran	3.9 ± 1.3 (6)	330 ± 80 (5)	1,300 ± 700 (8)	720 ± 200 (5)	1,700 ± 200 (6)
argatroban	2,900 ± 1200 (6)	400 ± 58 μM (3)	370 ±140 μM (3)	no inhibition	no inhibition

a. K_i values, mean, and standard error are given in nM, except were noted otherwise; the number of experiments (N) is given in parentheses. Abbreviations: tPA = tissue-type plasminogen activator, Prot. Ca = activated protein C, and SK = streptokinase.

Human Platelet Aggregation Methods

Platelet concentrate (Biological Specialties, Inc) was centrifuged at 1000 rpm (Sorvall RT 6000B) at 25 °C for 10 min. The resultant platelet-rich plasma (PRP) was gel filtered (Sepharose 2B, Pharmacia) in Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.76 mM Na_2HPO_4, 5.5 mM glucose, 5.0mM Hepes, and 2 mg/mL BSA; pH = 7.4). The gel-filtered platelets were diluted with Tyrode's buffer, compound solution in buffer, and 2 mM $CaCl_2$ to achieve 100,000 pl/μL final assay concentration on the 96-well assay plates. Platelet aggregation was initiated by the addition of a concentration of human α-thrombin (American Diagnostica) shown in preliminary experiments (on the day of the test) to achieve 80% aggregation (0.005-0.25 NIH U/mL). The assay plate was stirred and intermittently placed in a microplate reader (Molecular Devices) to read optical density (650 nM; ΔSOFT) at 0 and 7 min after the addition of thrombin. Aggregation was calculated to be the decrease in optical density between the time-zero and 7-min measurements. The assay was determined to be valid when the percent aggregation of the control was >75%. Each concentration of inhibitor, vehicles, and buffer controls were run in duplicate.