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IN VITRO EFFECTS OF INOGATRAN, A SELECTIVE LOW MOLECULAR WEIGHT THROMBIN INHIBITOR

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Abstract The thrombin inhibitor inogatran is a synthetic peptidomimetic with a molecular weight of 439 dalton. *In vitro* studies have shown that inogatran is a classical competitive inhibitor of the active site of thrombin with a K_i of 15 x 10^{-9} mol/l. Inogatran doubles the thrombin clotting time in human plasma at 20 x 10^{-9} mol/l, APTT at 1.2 x 10^{-6} mol/l, and prothrombin time at 4 x10^{-6} mol/l. The effects on rat and dog plasma are similar although slightly weaker. IC_{50} for inhibition of thrombin-induced aggregation of human platelets is 17 x 10^{-9} mol/l. Inogatran has no effect on platelet aggregation induced by ADP or collagen. Up to a concentration of 10 x 10^{-6} mol/l inogatran does not inhibit t-PA-induced fibrinolysis as seen in an ECLT system. Inogatran has good selectivity for thrombin as compared to several other serine proteases occurring in the blood. It is concluded that the properties of inogatran *in vitro* make the compound suitable for further studies in animals and man.

For the last decade much interest has been focused on developing selective thrombin inhibitors for treatment of arterial and venous thrombosis. Various lines of development have been followed.

In 1957 Markwardt isolated hirudin, a selective thrombin inhibitor from the medicinal leech (1). It was later shown that hirudin was a polypeptide of about 60 amino acids (2). Hirudin is now produced by gene technology and used as an anticoagulant, currently in phase III of clinical testing (cf. 3).

Key words: thrombin, thrombin inhibitor, dipeptide, inogatran, anticoagulant, selectivity
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Parallel with hirudin several LMW thrombin inhibitors have been developed. Argatroban, first described by Okamoto et al. (4), is a low molecular weight compound with a unique structure, designed to selectively inhibit thrombin. Argatroban was registered in Japan as early as 1990 and is in phase II/III studies in Europe and North America (cf. 5). Other lines for development of LMW thrombin inhibitors are based on benzamidines or amidinopiperidines (6,7).

In the sixties, Blombäck and co-workers showed that fibrinopeptide A and some smaller peptides derived from fibrinopeptide A inhibited thrombin-induced clotting, probably by means of competitive inhibition (8). This observation has been the basis for the development of a series of thrombin inhibitors. Good anticoagulant and antithrombotic effects have been obtained with inhibitors derived from the tripeptide D-Phe-Pro-Arg, exemplified by PPACK (9), the tripeptide aldehyde, described by Bajusz (10), the boroarginines (11), and the transition state analogues described by Jones et al. (12).

Hirulog is an elegant composition of D-Phe-Pro-Arg tripeptide and the C-terminal of hirudin (13). Like hirudin, hirulog is in clinical development.

The structure of inogatran, the thrombin inhibitor presented here, is based on the tripeptide D-Phe-Pro-Arg, although profoundly changed in order to improve pharmacodynamic and pharmacokinetic properties. The inhibiting activity on thrombin in purified systems, inhibition of plasma coagulation and platelet aggregation, as well as selectivity against several other serine proteases and fibrinolysis, are described.

MATERIALS

Inogatran

The thrombin inhibitor inogatran is a peptidomimetic with the structure HOOC-CH_2-(R)Cha-Pic-Nag, where Cha=cyclohexylalanine, Pic=pipecholic acid and Nag=noragmatine (*Glycine, N-[2-[2-[[[3-[(aminoiminomethyl)amino]propyl]amino]-carbonyl]-1-piperidinyl]-1-(cyclohexylmethyl)-2-oxoethyl]-, [2R-[2S]]-*) (14). The compound, which has a molecular weight of 439 dalton, is a crystalline, stable, water-soluble, and stereochemically pure substance. It was produced by Astra Hässle AB, Mölndal, Sweden and Astra Production Chemicals, Södertälje, Sweden. In the present study, it was used as a dihydrochloride, a dihydrobromide or as the pure base.

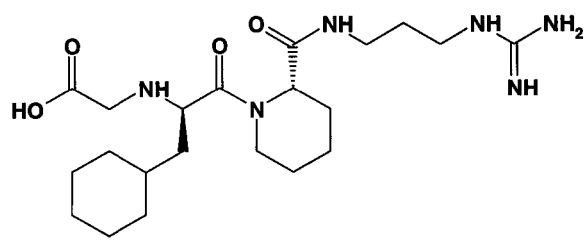

FIG. 1

Structure of inogatran

Pooled platelet-poor plasma from man, the rat and the dog.
Human blood was drawn from 20-30 healthy volunteers into one part of 0.13 mol/l trisodium citrate to nine parts of blood. The blood was centrifuged and the plasma separated, snap-frozen and stored at -80°C until use. Rat blood was collected from 20 anaesthetised Sprague-Dawley rats by puncture of the aorta, and dog blood from the cephalic vein of three male beagle dogs. The animal blood was anticoagulated with 0.11 mol/l trisodium citrate and otherwise treated as described for human blood.

Platelet-rich plasma and washed platelets from humans.
Citrated blood for preparation of platelet-rich and platelet poor plasma, and blood anticoagulated with EDTA-K_3 for isolation of platelets, was collected from healthy volunteers. The platelets were washed and isolated by differential centrifugation at 20°C. After the last centrifugation the platelets were resuspended in PBS buffer according to Dulbecco (Biochrom KG, Germany, cat no 04-001815) to a count of $200\text{-}230 \cdot 10^9$/l.

Enzymes
Human *thrombin* (E.C.3.4.21.5), cat no T 6759, and *trypsin,* type I, from bovine pancreas (E.C.3.4.21.4), cat no T 8003, were from Sigma Chem. Co., USA. Human *factor* X_a (E.C.3.4.21.6) cat no 00910, was purchased from Diagnostica Stago (Asnières, France). Human *factor* XI_a (E.C.3.4.21.27) and human *activated protein C* (E.C.3.4.21.69), were obtained from Enzyme Research Laboratories Inc. (South Bend, IN, USA). Human *factor* β-XII_a (E.C.3.4.21.38), cat no 233 496, *kallikrein* from human urine (E.C.3.4.21.35), cat no 420303, and human *neutrophil elastase* (E.C.3.4.21.37.), cat no 324681, were products of Calbiochem (San Diego, USA). Human *plasmin* (E.C.3.4.21.7) and human *plasma kallikrein* (E.C.3.4.21.34) were obtained from Chromogenix AB (Mölndal, Sweden). Human purified *complement component* $C\bar{1}\,s$ (E.C.3.4.21.42) (a generous gift from Dr Mats Rånby), human *single-chain t-PA* from melanoma cells (E.C.3.4.21.68), cat no 102115, and human *two-chain t-PA* from melanoma cells, cat no 102215, were products of Biopool AB (Umeå, Sweden). *Urokinase* (E.C.3.4.21.73) (*Abbokinase®*), was from Abbott Laboratories (North Chicago, USA).

Chromogenic substrates.
Flavigen® C1s, from Biopool AB (Umeå, Sweden), cat no 101356, was a generous gift from Dr Mats Rånby. The substrates *S-2238, S-2251, S-2288, S-2222, S-2366, S-2302, S-2266, S-2484* and *S-2444* were obtained from Chromogenix AB.

Other reagents
APTT reagent kit PTT-Automate-5 (cat no 0480) and *SPA reagent SPA 50* (cat no 100121) were both from Diagnostica Stago. *ADP reagent* (cat no. 885-3) and *Collagen reagent* (cat no. 885-1) were obtained from Sigma Chemical Co. *F.T.H.50-Reptilase® Reagent* was obtained from Diagnostica Stago, (cat no 00481). Each vial was reconstituted to 10 BU/ml. *Bovine serum albumin (BSA)* fraction V was a product of ICN Biomedicals Ltd., High Wycombe, Bucks, GB (cat no 810033). *PEG 6000* ("for Biochemistry") was obtained from BDH Ltd, Poole, England. (cat no 44271). *Aprotinin* (Trasylol®) was from Bayer, Leverkusen, Germany, *Tween 80* (cat no P1754) from Sigma Chemical Co, and *Triton X-100* (cat no 12298), *Glycerol* (cat no 4094) and *DMSO* (cat no 2950) from Merck, Darmstadt, Germany.

Buffers and solvents

Tris-HCl buffer, 0.05 mol/l, pH 7.4, ionic strength 0.15 adjusted with sodium chloride, with or without 10 g/l BSA, was used in all experiments unless otherwise stated. For most K_i-determinations the same Tris buffer, usually supplemented with BSA, Triton X-100, Tween 80 or PEG 6000 and aprotinin, was used. *Tris buffer* with $CaCl_2$ 2 mmol/l, was used for the determination of K_i for active protein C. K_i for trypsin was measured in *Tris buffer*, 0.05 mol/l, pH 8.2, containing $CaCl_2$, 20 mmol/l.

Equipment

Cobas Bio Centrifugal Analyser (Roche, Basel, Switzerland) was used for chromogenic assays, *Coagulometer KC10* (Amelung GmbH, Lemgo, Germany) for the clotting assays, *Platelet Aggregation Profiler, Model PAP-4* (Biodata Corporation, Harboro, USA) for aggregation studies, and *THR 147 Compact Thrombocyte Analyser* (Analysinstrument AB, Stockholm Sweden) for platelet counting. *Thermomax Microplate Reader* (Molecular Devices Corp., Menlo Park, USA) was used for fibrinolysis studies.

METHODS

Determination of K_i

The inhibition of each enzyme was studied at three substrate concentrations and usually seven inhibitor concentrations, at 37°C and in a final volume of 400 µl. The inhibitor was first mixed with enzyme and the reaction started by addition of substrate solution. The absorbance was monitored at 405 nm in the Cobas Bio analyser. Reagents and final concentrations are listed in Table I. The steady-state reaction rates were used for construction of dixon plots, and when possible the K_i value was calculated. The error of the method for determination of K_i for thrombin was 9.6 %, as estimated earlier by 10 parallel determinations of K_i (data not shown).

Thrombin time

Inogatran in saline (0-5 µmol/l), 100 µl, was mixed with 100 µl of pooled rat, dog or human plasma. Thrombin solution, 100 µl, giving a final concentration 1.4 NIH units/ml, was added and the clotting time measured.

APTT

Inogatran in saline, 0-2000 µmol/l, 10 µl, was added to 90 µl of rat, dog or human plasma. APTT was measured according to the instructions for the reagent.

Prothrombin time

Inogatran was dissolved and diluted to 0-2000 µmol/l in reagent buffer supplied with the kit. Plasma from rats, dogs or man, 100 µl, was added to 100 µl of inhibitor solution and 500 µl of reagent buffer. Assay reagent, 200 µl, was added to 100 µl of this mixture and the clotting time measured.

Platelet aggregation with ADP or collagen

Human platelet-rich plasma, 400 µl, and inogatran solution or buffer, 50 µl, were mixed and the aggregation started by addition of 50 µl of either ADP reagent to a final concentration of 2 µmol/l, or collagen reagent to a final concentration of 0.2 mg/ml. The final concentrations of inogatran were 1.0, 3.0 and 10.0 µmol/l.

TABLE I

Reagents and Final Concentrations in the K_i Determinations

Enzyme	Enzyme conc./act.	Enzyme nmol/l	Substrate μmol/l	Inogatran μmol/l
Thrombin	0.125 NIHU/ml	0.85	S-2238 (16,24,50)	0-0.10
Plasmin	0.035 CU/ml	25	S-2251 (200,350,800)	0-200
Tct-PA	781 IU/ml	13	S-2288 (200,300,500)	0-20
Factor Xa	0.04 U/ml	6.2	S-2222 (750,1050,1500)	0-200
Factor XIa	2.27 μg/ml	14	S-2366 (100,150,200)	0-200
Factor βXIIa	0.0080 U/ml	670	S-2302 (100,200,400)	0-10
Plasma kallikrein	0.25 nkat/ml	2.5	S-2302 (125,200,400)	0-20
Active protein C	0.25 μg/ml	4.1	S-2366 (200,400,600)	0-40
Compl.Comp $C\bar{1}$ *s*	5 μg/ml	57	Flavigen C1s (250,286,500)	0-50
Tissue kallikrein	0.01 IU/ml	40	S-2266 (40,60,150)	0-500
Leucocyte elastase	1.05 μg/ml	36	S-2484 (188,263,375)	0-1000
Urokinase	150 IU/ml	≤93	S-2444 (100,200,300)	0-1000
Trypsin	0.15 μg/ml	6.3	S-2222 (15,25,50)	0-0.50

Platelet aggregation with thrombin

A suspension of washed human platelets, 400 μl, inogatran solution or buffer, 50 μl, and thrombin solution, 50 μl, was mixed and the aggregation monitored. The final concentration of inogatran was 0-100 μmol/l and of thrombin 0.1 NIH units/ml.

Euglobulin clot lysis time

A euglobulin fraction was precipitated at pH 5.9. The precipitate was dissolved in buffer and mixed with sct-PA solution to give a final assay concentration of 1.2 IU/ml. Inogatran and Reptilase®, 10 μl of each, were mixed with 200 μl of the euglobulin-t-PA solution in microtiter wells and the absorbance (450 nm) monitored for three hours. The lysis times were plotted against the inhibitor concentrations, and two concentrations, IC_{50LT} and IC_{LT}, were determined by interpolation. IC_{50LT} was defined as the inhibitor concentration which doubled the lysis time and IC_{LT} as the lowest inhibitor concentration which significantly inhibited the fibrinolysis, *i.e.* caused a 25 % increase in the lysis time, as judged from the precision of the method.

RESULTS

Enzymatic properties

The inhibition constant K_i, determined from steady-state measurements of the effect of inogatran on thrombin, was 15±1.5 nmol/l (mean and SD, n=4). According to the Lineweaver Burk and Dixon plots (example shown in Figures 2A and 2B) inogatran could be classified as a purely competitive inhibitor.

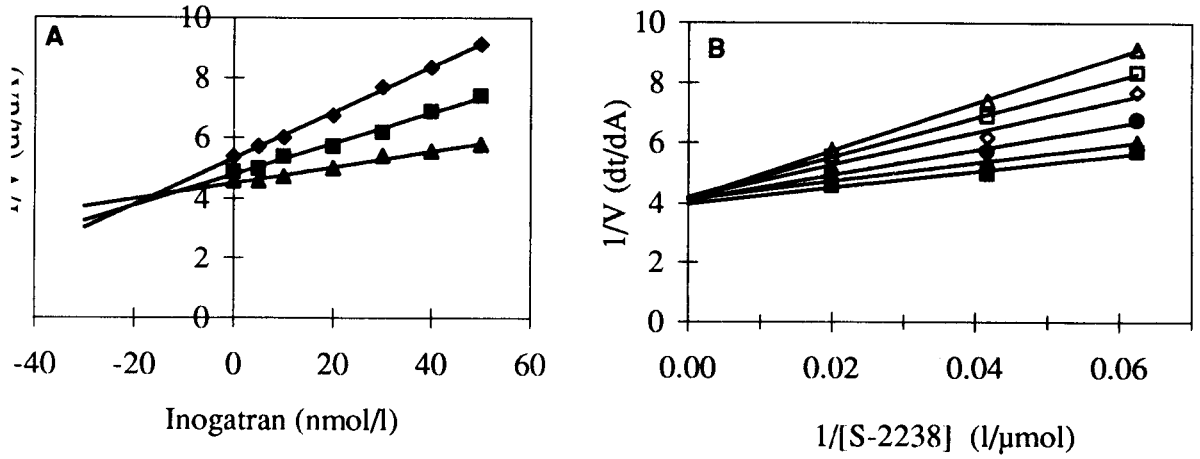

FIG. 2

Panel A: Dixon plot for the inhibition of thrombin (0.125 NIH U/ml) by inogatran measured at 16 ◆, 24 ■ and 50 ▲ µmol/l of S-2238. Panel B: Lineweaver-Burk plot of the same data as in panel A. Inogatran 5 ■, 10 ▲, 20 ●, 30 ◊, 40 ❑ and 50 Δ nmol/l.

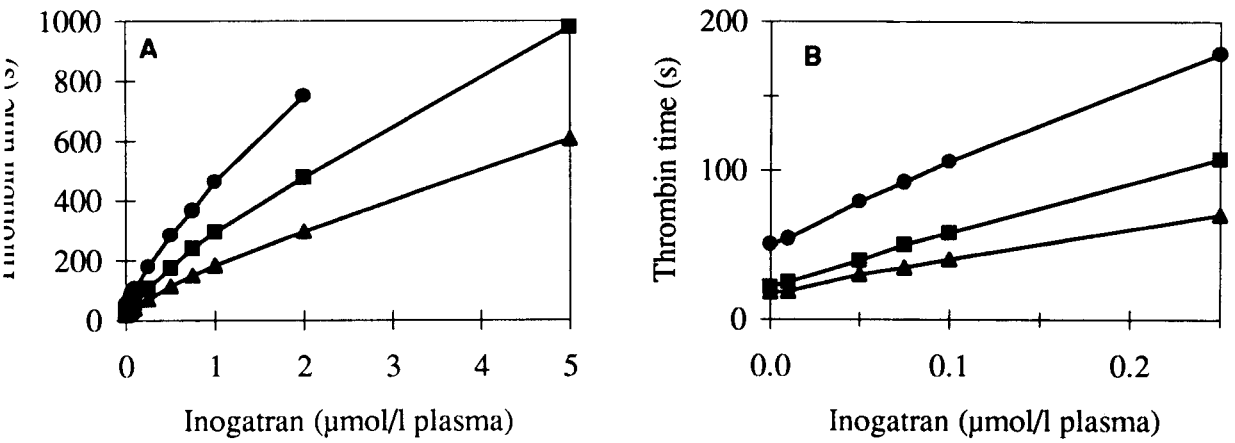

FIG. 3

Thrombin time in plasma from the rat ●, the dog ▲, and man ■ as a function of inogatran concentration. A. High concentrations. B. Low concentrations.

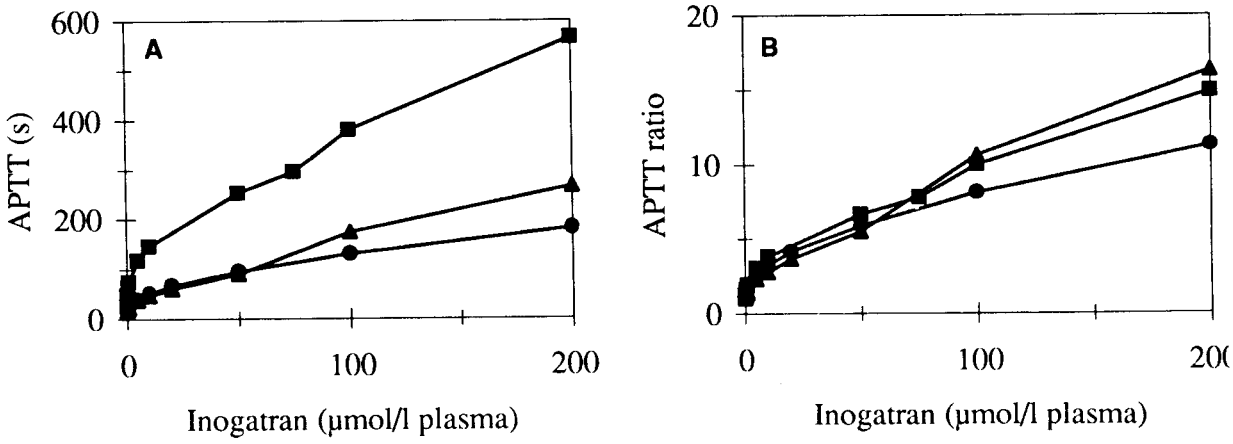

FIG. 4

Activated partial thromboplastin time (APTT) in plasma from the rat ●, the dog ▲, and man ■ as a function of inogatran concentration. A. Expressed in seconds. B. Expressed as multiples of the baseline value.

Effect on plasma coagulation

The thrombin time for plasma from the rat, the dog and man with different concentrations of inogatran, is shown in Figures 3 A and B. Although at different levels, the curves were similar in shape. The concentration of inogatran in the reaction mixture that doubled the clotting time, IC_{50TT}, was 31 nmol/l for rat, 28 nmol/l for dog, and 20 nmol/l for human plasma.

The results of APTT determinations in different species are shown in Figures 4A and 4B. There was a concentration-dependent prolongation of the APTT for all three species. The baseline values for APTT differed, with around 16 s clotting time for rat and dog plasma and 38 s for human plasma. Furthermore, the method also seemed to be more sensitive to inhibition of coagulation in human plasma than in the animal plasmas. However, when expressed as multiples of the baseline APTT, there was better agreement between the concentration-effect curves for the three species. Used in this way, APTT values will give useful information about the level of anticoagulation, which in turn can be related to the antithrombotic effect of inogatran. In this experiment, the plasma concentration that doubled the APTT value, IC_{50APTT}, was 2.5 µmol/l for rat plasma, 3.2 µmol/l for dog plasma, and 1.2 µmol/l for human plasma.

The results of the measurements of the prothrombin time are shown in Figures 5A and B. Like in the other assays, there was a concentration-dependent increase in the clotting time, with somewhat different baseline values for the three species. Expressed as multiples of the baseline values, the results with rat plasma differed from those with dog and human plasma. The IC_{50}-values were 6.3 µmol/l for rat, 3.2 µmol/l for dog and 4.0 µmol/l for human plasma.

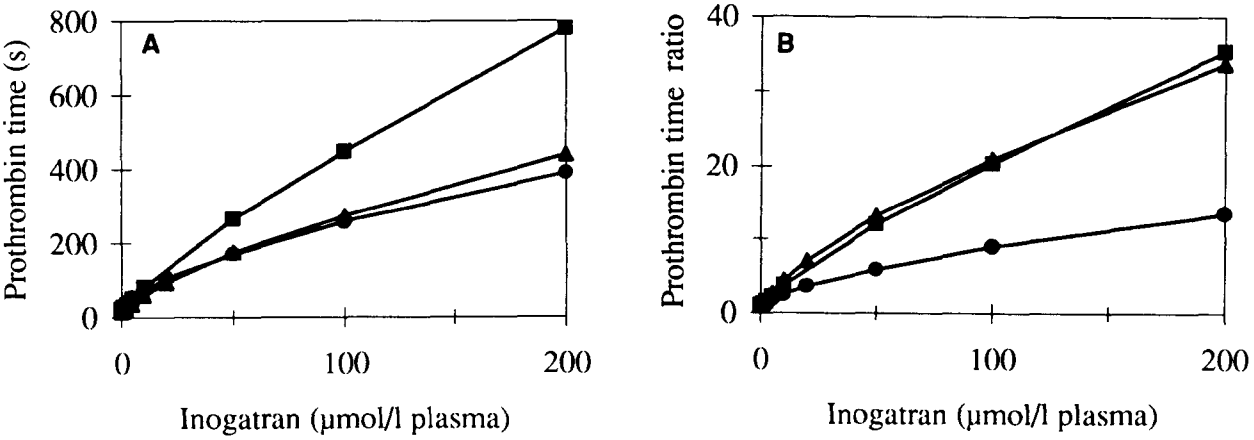

FIG. 5

Prothrombin time in plasma from the rat ●, the dog ▲, and man ■
A. Expressed in seconds. B. Expressed as multiples of the baseline value.

Effect on human platelets

Before the experiments with the inhibitor, optimal concentrations of ADP and collagen were established for the individual plasmas (n=5) and were found to be 2.0 μmol/l and 0.2 mg/ml, respectively. Inogatran in concentrations of 1, 3 or 10 μmol/l inhibited neither ADP- nor collagen-induced platelet aggregation as judged by the lag phase, slope and maximum aggregation. Furthermore, inogatran alone in concentrations of 1, 3, or 10 μmol/l did not influence platelet aggregation at all, as judged by the slope and maximum aggregation.

Three platelet suspensions from three different donors were used with 2-6 aggregation experiments for each inogatran concentration. Inogatran inhibited the thrombin-induced aggregation in a concentration-dependent way (Fig. 6). Under the experimental conditions, the IC_{50}-value for thrombin-induced platelet aggregation amounted to 17 nmol/l.

Effect on various serine proteases

Inogatran inhibited most serine proteases to some extent but at concentrations that were far above whatever could be reached in plasma in a therapeutic situation. Trypsin, having a K_i of 0.67 μmol/l for inogatran, was the most sensitive enzyme. For most enzymes, the inhibition was classified as competitive as judged by a Dixon plot. K_i values for inhibition of the separate enzymes with inogatran are shown in Table II, where also the K_i-ratios between the separate enzymes and thrombin are given.

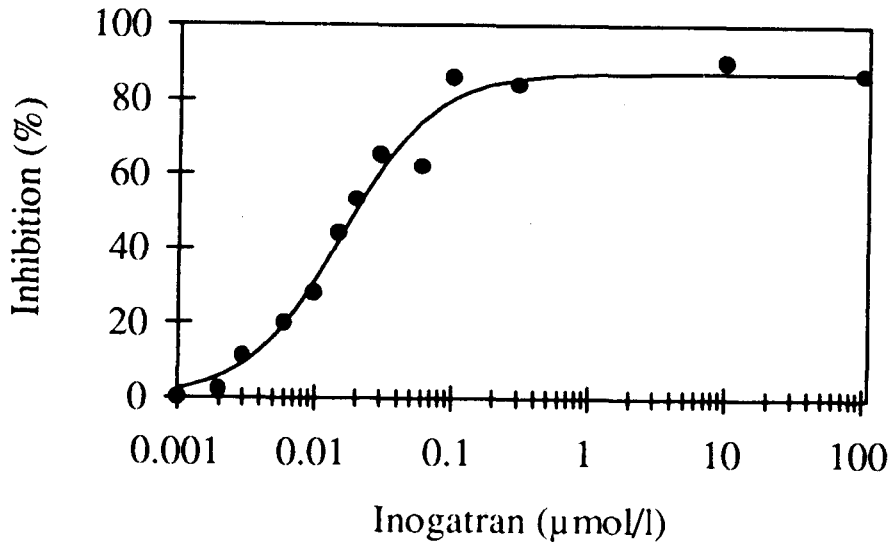

FIG. 6

Effect of inogatran on thrombin-induced platelet aggregation.

The data for % inhibition 100-((agg_{sample}/agg_{blank})x100) were fitted to a sigmoidal concentration-effect function for determination of inhibitory potency according to the formula Y = min + (max-min)/(1+(X/I), where Y = % inhibition, X = inogatran concentration and I = the concentration at half-maximum effect (calculated). The minimum value (min) was set to 0 %, and the maxium value (max) was a calculated parameter. The calculations were performed according to the method of least squares, using software RS/1, BBN Software Product Co, Cambridge, MA, USA.

Effect on global fibrinolysis

The formation and lysis of reptilase-induced fibrin clots, measured as absorbance at 450 nm with various concentrations of inogatran, are shown in Figure 7. There was no prolongation of ECLT up to 10 µmol/l of inogatran. However, prolongation was noticed at 30 µmol/l and higher. When the interpolated lysis time values were plotted versus the concentration of inogatran, IC_{LT} was calculated to be 34 µmol/l and IC_{50LT} to 175 µmol/l.

DISCUSSION

Inogatran is a peptidomimetic based on the tripeptide R-Phe-Pro-Arg, which in turn is based on the structure of fibrinopetide A close to the thrombin splitting site in the Aα chain of fibrinogen. As judged from Lineweaver-Burk and Dixon plots after steady state measurements, inogatran is a classical, reversible, purely competitive inhibitor of thrombin. In a more extensive study of the enzymatic properties of inogatran Nilsson et al. (to be published) have shown that inogatran binds to thrombin with an association rate constant, k_{on}, of 24 +/– 2 $\cdot 10^6$ $(mol/l)^{-1} \cdot s^{-1}$ and a dissociation rate constant, k_{off} of 0.44 +/- 0.04 s^{-1}, i.e. the rate of binding is rapid and of the same

TABLE II

Selectivity of the Thrombin Inhibitor Inogatran against some other Serine Proteases

Enzyme	K_i µmol/l	K_i-ratio Enzyme/Thrombin
Thrombin	0.015*	1
Plasmin	115	7700
Two-chain t-PA	12	790
Factor Xa	78	5200
Factor XIa	190	12900
Factor XIIa	6.6	440
Plasma Kallikrein	11	730
Active Protein C	72	4800
Compl. Comp. $C\bar{1}s$	78	5200
Tissue Kallikrein	630	41900
Leucocyte Elastase	>1000	>66700
Urokinase	770	51400
Trypsin	0.67	45

* Mean value (n=4)

order of magnitude as found for many natural inhibitors. From the K_{off} the half-life of the inogatran-thrombin complex can be calculated to approximately 1.6 s.

The enzyme kinetics of inogatran has similarities to that of another LMW thrombin inhibitor, argatroban, which also is a rapidly reacting competitive inhibitor (Nilsson et al and ref 5). Hirudin likewise binds rapidly to thrombin but the half-life of the complex is five to eight hours.

The binding of hirudin is very tight with an exceptionally low K_i value of 20 fmol/l (15). Other inhibitors like efegatran and DUP 714 are also potent but bind relatively slowly to thrombin (Nilsson et al and ref 11). The clinical value of different binding properties of thrombin inhibitors remains to be established.

Theoretically inogatran should prolong the clotting time in any assay based on thrombin addition or generation. As expected, inogatran prolonged the thrombin time, APTT and prothrombin time in a concentration dependent manner. The results obtained in this *in vitro* study are very close to the results obtained in phase I studies, where blood samples were drawn after inogatran treatment and plasma coagulation assays correlated to the concentration of inogatran (16).

The thrombin time was by far the most sensitive assay and measurements of the thrombin time may be an excellent bioassay for determination of low concentrations of inogatran in plasma. The inogatran concentration that doubled the APTT was about 50 fold of that necessary for doubling of the thrombin time. APTT is used in the clinic to control treatment with heparin and thrombin inhibitors, and a doubling of the APTT is a common goal for therapy. Assuming this is valid also for inogatran the results obtained here indicate that a clinically relevant concentration may be

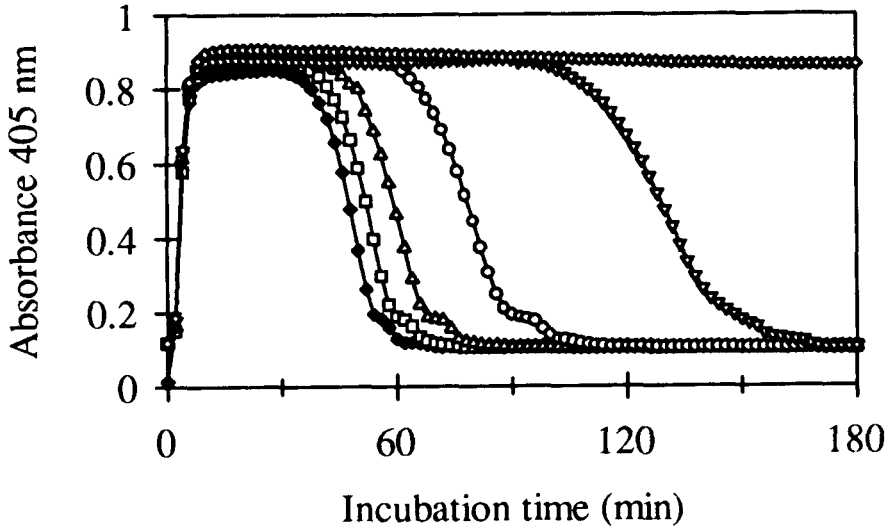

FIG. 7

The effect of various concentrations of inogatran on the lysis of fibrin clots, measured as change in absorbance. Inogatran 0 ◆, 10 ❑, 30 Δ, 100 O, 300 ∇ and 1000 ◊ μmol/l.

about 1 μmol/l, i.e. about 50 times the K_i-value. The discrepancy between the Ki-value and an assumed therapeutic concentration is consistent with the power and complexity of thrombin formation *in vivo* (17).

As thrombin is a potent activator of platelets the effect of inogatran on thrombin-induced platelet aggregation was studied. By use of washed human platelets an IC_{50} value in the same order of magnitude as the IC_{50} for thrombin time and the K_i value was found. The effect on platelet aggregation might be of value particularly in arterial thrombosis, where platelets are believed to play a pivotal role. However, the antiaggregating effect of inogatran is expected to be less strong than that of GPIIb/IIIa blockers, which in contrast to thrombin inhibitors, block aggregation induced by all types of inducers.

To assess the selectivity of the thrombin inhibitor inogatran, the inhibition constant K_i was determined for several other serine proteases, some of them essential for the normal coagulation or/and fibrinolysis, and some with other effects. In high concentrations inogatran acted as a reversible competitive inhibitor against most of the enzymes, but the selectivity of inogatran for thrombin was high. Trypsin was the most sensitive enzyme, with a selectivity ratio of 45. However, trypsin does not normally occur in the blood and is not involved in haemostasis.

Other enzymes in the coagulation cascade had K_i-values that were between 440 and 19000 times higher than that of thrombin. It has long been debated whether thrombin inhibitors, factor Xa inhibitors or more unselective inhibitors are the best antithrombotic drugs (19). LMW heparin was developed with the assumption that inhibition of factor Xa as well as thrombin is of importance. Synthetic, unselective inhibitors have so far not been used as antithrombotics, but

recent studies indicate that selective thrombin inhibitors as well as selective Xa inhibitors, both are effective antithrombotic agents. The most efficient antithrombotic mechanism is still an open question and might be different for different thrombotic diseases.

The enzymes from the fibrinolytic system had K_i values that were at least 790 times higher than that for thrombin. The low influence of inogatran on the fibrinolytic system was also confirmed with a more physiologic assay, the euglobulin clot lysis time, and later in animal experiments (18).

In conclusion, inogatran is a rapidly binding, reversible, competitive inhibitor of thrombin. It prolongs plasma coagulation and inhibits thrombin-induced platelet aggregation and has negligible effects on fibrinolysis. It is sufficiently selective against relevant plasma serine proteases. Results from animal experiments (20, 21, 22, 23, 24) and phase I studies (16) are consistent with these *in vitro* results.

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