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Inhibition of trypsin by heparin and dalteparin, a low molecular weight heparin

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Abstract: The interaction between trypsin, a prototype S1 serine protease, with heparin and its low molecular weight derivative dalteparin were investigated. Direct inhibition of the proteolytic activity of trypsin by heparin and dalteparin, used in concentrations typical for their clinical application, was detected. The half-maximum inhibition of the trypsin activity was achieved at 15.25±1.22 μg/mL for heparin and was estimated to be at 58.47±15.20 μg/mL for dalteparin. Kinetic analyses showed that heparin and its low molecular weight derivative dalteparin inhibited trypsin by occupation of an exosite, producing noncompetitive and mixed inhibition, respectively. Heparin as a noncompetitive inhibitor with constant of inhibition $K_{i1,2}$ = 0.151±0.019 μM and dalteparin with K_{i1} = 0.202±0.030 μM and K_{i2} = 0.463±0.069 μM in mixed inhibition both represent moderate inhibitors of serine protease trypsin. The obtained constants of inhibition indicate that under the clinically applied concentrations of heparin and dalteparin, trypsins and their homolog S1 serine proteases could be directly inhibited, influencing the delicate control of proteolytic reactions in homeostasis.

Keywords: serine proteases; heparin; dalteparin; inhibition.

INTRODUCTION

Trypsin (EC 3.4.21.4) is a member of the serine protease S1 family. The catalytic activity of the S1 trypsin family is provided by a charge relay system involving serine, histidine and aspartic acid. The sequences in the vicinity of the serine and histidine residues in the active site are well conserved in this family.[1] Proteases known to belong to the serine protease S1, often called trypsin family, are: blood coagulation factors VIIa, IXa, Xa, XIa and XIIa, thrombin (IIa), plas-

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min, activated protein C, trypsins I, II, III, and IV, chymotrypsins, *etc*.[2] Several distinct mechanisms exist for the control of peptidase activity, but inhibition is of particular importance. Peptidases from the family S1 are inhibited by diverse group of inhibitors, including low molecular weight natural and synthetic inhibitors for research or therapeutic purposes, and also natural proteinaceous inhibitors. Natural inhibitors of proteases represent a structurally heterogeneous group of substances from low-molecular weight to macromolecular, originating from animals, plants and microorganisms.[2] Their great importance and particular reason for intensive and constant investigation are related to their potential application in therapy of different diseases, including such serious ones as Alzheimer or AIDS.[3,4]

Heparin is naturally occurring polyanionic glycosaminoglycan with a molecular mass of about 15–20 kDa, and extreme acidic properties.[5] Heparin causes significant structural and functional alterations of trypsin, as the prototype S1 serine protease, in a paradoxical manner which strongly depends on heparin dose, trypsin–heparin ratio and the experimental conditions (pH, presence of salts, time),[5] including inhibition at concentrations of up to 60 mg/L and almost complete losts of activity at concentrations from 120–400 mg/L.[6] Specific, but less intense alterations of the structure and function of trypsin caused by heparin in concentrations from 6–60 mg/L have been recognized as an oxidative mechanism of radical generating binding property of heparin.[6] Actually, it was demonstrated that heparin is capable of generating radicals after its binding to trypsin, which is of particular physiological importance. Additionally, even at the lowest concentrations which trigger radical production, heparin specifically binds to trypsin.[6] Acting as a very efficient anticoagulant, heparin binds to serpin (serin protease inhibitor) peptidase inhibitor antithrombin III (AT-III), which inactivates thrombin and other proteases involved in blood clotting.[7] It should be mentioned that from an administrated dose of heparin of about 32000 U per 24 h for 70 kg patients, by continuous infusion of 40 U/mL, only one third binds to antithrombin.[8] It was found that the inhibitory effect of heparin is mostly indirect, even though it may affect proteolytic enzymes through direct inhibition.[9]

Low-molecular-weight-heparin (LMWH), dalteparin, has a molecular mass of 4–6 kDa and a chain length of 13–22 sugars. Results obtained in clinical trials of heparin and dalteparin as anticoagulants confirmed the significance of both used glycosaminoglycans in anticoagulation by an indirect inhibition of blood clotting serine proteases.[10]

The specific interactions between heparin and LMWH with S1 serine proteases from blood clotting have been investigated and described.[11,12] However, to date, no kinetic data of the direct inhibition of S1 serine proteases by heparin and low molecular weight heparin have been reported. The present investigation was designed as a model system to evaluate the interaction between the prototype S1 serine protease trypsin with heparin and its derivative dalteparin, as inhibitory

substances. Kinetic data and an inhibition model of heparin and dalteparin on the proteolytic activity of serine protease trypsin were determined.

EXPERIMENTAL

Chemicals

Bovine trypsin (T-4665, Sigma Chemicals Co., St. Louis, MO) was used without further purification. Heparin (Galenika, Belgrade, Serbia) and LMWH – dalteparin (Hemopharm, Vršac, Serbia, in cooperation with Sanofipharm, France) were used in appropriate dilutions in the corresponding buffers. BAPNA (N^{α}-benzoyl-DL-arginine-*p*-nitroanilide) and casein Hammerstein were purchased from Sigma Chemicals Co., St. Louis, USA. All chemicals were of analytical grade.

Standard enzyme assays

The proteolytic activity of trypsin on N^{α}-benzoyl-DL-arginine-*p*-nitroanilide as the substrate was determined according to a modified method of Erlanger.[13] BAPNA, in a final concentration of 10 mM (stock solution), was prepared by dissolving in 0.050 M Tris buffer pH 8.2 containing 0.020 M $CaCl_2$ and 2 % (v/v) dimethylformamide. Into 0.50 mL of the BAPNA solution (4.5 mM), 0.10 mL of enzyme solution, containing 0.10 mg of trypsin (9.0 μg/mL) (specific activity on N^{α}-benzoyl-L-arginine-ethyl ester hydrochloride (BAEE): 8750 U/mg) was added, the volume adjusted to the final 1.1 mL with Tris buffer pH 8.2 and the reaction mixture was incubated 15 min at 37 °C. The absorbance of the clear supernatant was measured at 410 nm using a spectrophotometer, Ultrospec K, Sweden. The concentration of *p*-nitroaniline was calculated using a standard curve. One unit of enzyme activity was defined as the amount of enzyme that liberated 1.0 μmol of *p*-nitroaniline per minute under the test conditions.

The proteolytic activity on casein as the substrate was determined according to a modified method of Kunitz[14] and Van der Walt.[15] Casein was dissolved in Tris buffer pH 8.5 to a final concentration of 1.0 % (m/v). Into 0.50 mL of casein solution, 0.10 mL of enzyme solution containing 0.10 mg of lyophilized trypsin powder (9.0 μg/mL, specific activity on BAEE 8750 U/mg) was added and reaction mixture was incubated 15 min at 37 °C. The reaction was stopped with 1.0 mL of trichloroacetic acid (TCA) solution (30 %) and centrifuged at 3000 rpm for 10 min. The absorbance of the clear supernatant was measured at 280 nm. One unit of enzyme activity was defined as the amount of enzyme that decreased the absorbance by 0.0010 after 15 min under the test conditions.

The effect of heparin and dalteparin on the trypsin activity

The effect of different concentrations of heparin: 500 IU (0.21 μM), 1000 IU (0.42 μM), 1500 IU (0.63 μM), 2000 IU (0.84 μM), 2500 IU (1.05 μM); and dalteparin: 1025 IU (1.31 μM), 2050 IU (2.62 μM), 3075 IU (3.93 μM), 4100 IU (5.24 μM), 5125 IU (6.55 μM) on the trypsin activity (the same concentration as in the standard assay) against BAPNA and casein was performed. A mixture of total volume of 1.1 mL containing: 0.10 mL of enzyme solution and 1.0 mL of inhibitor solution was pre-incubated for 5 min at 37 °C. After the addition of 0.50 mL of BAPNA or casein solution, activity was monitored as described in the standard assay methods. The control was the enzyme solution without inhibitors, supplemented with buffer solution to a final volume of 1.1 mL. The activity of the control was defined as 100 %. The half-maximum inhibitory concentrations (IC_{50} values) of heparin and dalteparin for trypsin were determined mathematically by derivation of the best-fit line using an online IC_{50} calculator from BioFitData software package (ChangBioscience).[16]

Kinetic parameters

Enzyme assays on trypsin were performed with BAPNA as the substrate, in concentrations of: 10.0 mM, 5.00 mM, 2.50 mM, 1.25 mM, 0.623 mM and 0.313 mM and different concentrations of inhibitors, *i.e.*, heparin: 0.84 μM, 0.42 μM, 0.21 μM; and dalteparin: 3.93 μM, 2.62 μM, 1.31 μM. The final volume of 1.1 mL of the reaction mixture contained: 0.10 mL of enzyme solution and 1.0 mL of inhibitor. After pre-incubation for 5 min at 37 °C, 0.50 mL of BAPNA solution was added and absorbance at 410 nm was monitored after the 1st and 2nd minute of reaction. The control was the enzyme solution without inhibitor.

To determine kinetic parameters and mode of action of the tested substances on the trypsin activity, the curve fitting software package Ez-fit was used.[17] All enzyme assays were performed in duplicate with the control (test without inhibitor) and the data were fitted to the equations:

$$V_0 = \frac{V_{max}[S]}{\alpha K_m[S]} \text{ for competitive,}$$

$$V_0 = \frac{V_{max}[S]}{\alpha K_m[S] + \alpha[S]} \text{ for noncompetitive,}$$

$$V_0 = \frac{V_{max}[S]}{\alpha' K_m[S] + [S]} \text{ for uncompetitive and}$$

$$V_0 = \frac{V_{max}[S]}{\alpha' K_m[S] + \alpha[S]} \text{ for mixed type of inhibition.}$$

The kinetic constants (V_{max}, K_m and K_i values) and statistical parameters, including the Akaike information criterion (*AIC*), were calculated by the Ez-fit software. By comparing the values of Akaike's information criterion (*AIC*) for the tested inhibition models, the preferred fit was chosen as the one at least 2 units smaller than the rival model.[18] Lineweaver–Burk graphs were plotted using the Microcal Origin program (version 6.1).

Statistical analysis

Graphs were plotted using the Microcal Origin program (version 6.1). The kinetic constants and their standard errors are presented as means ± *SEM* (obtained by linear regression analysis). The statistical comparisons were performed by the Student's *t*-test for paired observations. The means of at least five observations is quoted in the text and $p < 0.01$ was considered statistically significant.

ESI-MS of trypsin

Mass measurements of proteins were performed on a MS system consisting of a 6210 Time-of-Flight LC/ESI-MS (G1969A, Agilent Technologies). A sample of trypsin was dissolved in a mobile phase consisting of a 50:50 mixture of solvent A (0.20 % formic acid in water) and solvent B (acetonitrile). The mass spectrometer was run in the positive electron spray ionization (ESI) mode. A personal computer system running Agilent MassHunter Workstation Software was used for data acquisition and Agilent MassHunter Workstation Software and Analyst QS were used for data processing.

RESULTS AND DISCUSSION

Enzyme and substrate selection

Of many animal trypsins, bovine trypsin has been studied and used for many years as the prototype of serine endopeptidases from the S1 family. The forms of

trypsins present in higher animals share not only high structural but also sequence identity.[2] From the list of trypsins available at the MEROPS database, as one of the protease information systems, only bovine and human trypsins were selected for comparison. Sequences of bovine and human trypsins were aligned using the SIB BLAST network service and trypsins with high identities are given in Table I.[19]

TABLE I. Similarity search of bovine and human trypsins using SIB BLAST Network Service of ExPASy Proteomics Server

Entry name in UniProtKB/-/Swiss-Prot	Accession number	Protein name	Synonyms	No. of amino acids[a]	Identity	Score
TRY1_BOVIN	P00760	Cationic trypsin (precursor)	EC 3.4.21.4 Beta-trypsin	243	243/243 (100 %)	462 bits (1188)
TRY2_BOVIN	Q29463	Anionic trypsin (precursor)	EC 3.4.21.4	247	165/223 (73 %)	362 bits (928)
TRY1_HUMAN	P07477	Cationic trypsin-1 (precursor)	EC 3.4.21.4 Trypsin I Cationic trypsinogen Serine protease 1 Beta-trypsin	247	168/223 (75 %)	364 bits (935)
TRY2_HUMAN	P07478	Anionic trypsin-2 (precursor)	EC 3.4.21.4 Trypsin II Anionic trypsinogen Serine protease 2	247	166/223 (74 %)	355 bits (911)
TRY3_HUMAN	P35030	Trypsin-3 (precursor)	EC 3.4.21.4 Trypsin III Brain trypsinogen Mesotrypsinogen Trypsin IV Serine protease 3 Serine protease 4	303	162/223 (72 %)	351 bits (901)

[a]The length of the sequence of the unprocessed precursor

The high similarity between bovine and human cationic trypsin is obvious. The native form of bovine trypsin, referred to as cationic trypsin or β-trypsin, consists of a single chain polypeptide of 223 amino acid residues. A molecular mass of 23305 Da was computed using Expasy ProtParam Tool. The bovine trypsin preparation (*ex* Sigma) used in this research was analyzed by ESI-MS to confirm the presence of the dominant trypsin form. Intensive signals at 23294 and 23312 Da corresponding to β- and α-trypsin, were detected, similar to a reported

ESI-MS spectrum of bovine trypsin.[20] The cationic form of bovine trypsin, widely used in a variety of medical and scientific applications, is well characterized in terms of kinetic parameters, particularly for low molecular weight synthetic substrates with ester and amide bonds.[21,22] To compare the potential inhibition of trypsin activity by heparin, a proteinaceous substrate casein and a synthetic substrate BAPNA (Fig. 1) were preliminarily investigated. As Fig. 1 shows, the inhibition patterns obtained by both substrates were similar, although the chromogenic substrate BAPNA seemed slightly more suitable for investigations of inhibittion. For the further kinetic study, bovine trypsin and the BAPNA chromogenic substrate were selected.

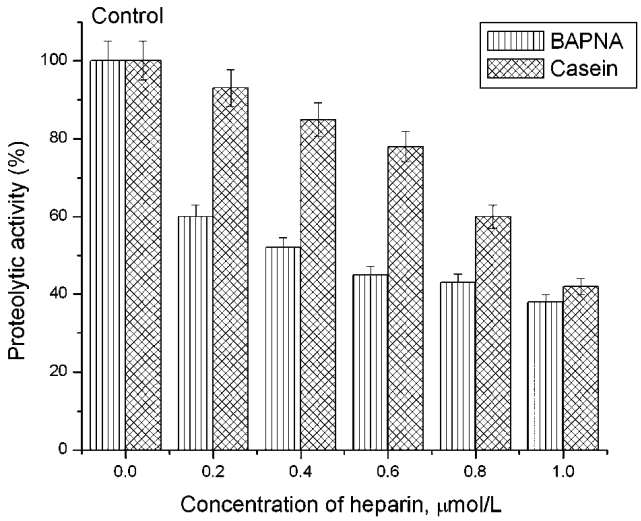

Fig.1. Activity of trypsin in the presence of different concentrations of heparin on BAPNA and casein. The activity of trypsin without inhibitors was 100 %. The results are expressed as the mean percentage of enzyme activity, from at least three independent experiments, performed in triplicate.

The effect of heparin and dalteparin on the activity of trypsin

The results of a preliminary experiment designed to evaluate the effects of different concentrations of heparin and dalteparin on the activity of trypsin are shown in Fig. 2.

Heparin at a concentration of 1.05 μM reduced the trypsin activity to 37 % ($p < 0.05$), while LMWH – dalteparin at a concentration of 7.55 μM inhibited the trypsin activity by 40 % ($p < 0.05$). It is clear from Fig. 2 that the inhibitory effects are dose-dependent, *i.e.*, increasing the concentration of the test substances decreased the proteolytic activity of trypsin on BAPNA as the substrate. In micro-molar concentrations, heparin and dalteparin act as inhibitors of the S1 serine protease trypsin.

The half-maximum inhibition concentration (IC_{50}) of heparin on trypsin was found to be 15.25±1.22 μg/mL. By derivation of the best-fit line, the IC_{50} value of dalteparin was mathematically estimated to be 58.47±15.20 μg/mL. The obtained IC_{50} values for heparin and LMWH were slightly higher than those referenced.[23]

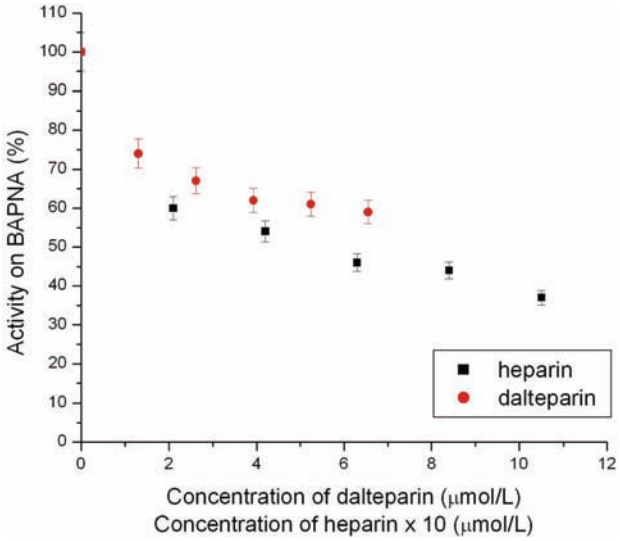

Fig. 2. The effect of different concentrations of heparin and dalteparin on the activity of trypsin on BAPNA. The activity of trypsin without inhibitors was 100 %. The results are expressed as a mean percentage of enzyme activity, from at least three independent experiments, performed in triplicate.

Kinetic study

To investigate the type of inhibition of the trypsin activity by heparin and dalteparin, kinetic analysis of the inhibition pattern using the Ez-fit software package was undertaken. Initial velocity data obtained from the inhibition of trypsin activity were fitted to four inhibition models. The Akaike information criterion (*AIC*) for competitive, uncompetitive, noncompetitive and mixed model of trypsin inhibition by heparin and dalteparin is shown in Table II. Based on a comparison of the values of the Akaike information criterion, the inhibition model was selected that was at least 2 units smaller than the rival model.

It is obvious from the *AIC* values that neither of the tested substances showed competitive inhibition. In addition, uncompetitive inhibition, in which the inhibitor binds to the enzyme–substrate complex, is unlikely to occur. Finally, inhibition in which the inhibitor binds to a site different from the active site, with possibility to bind to either the free enzyme or the enzyme–substrate complex with the same (non-competitive) or different (mixed) constants of inhibition, are the most likely scenarios. Both substances, with the lowest *AIC* for heparin of 177.44

TABLE II. The Akaike information criterion (*AIC*) for competitive, uncompetitive, noncompetitive and mixed model of trypsin inhibition by heparin and LMWH – dalteparin. The kinetic constants and *AIC* were calculated by Ez-fit software. By comparing the *AIC* values for the tested inhibition models, the preferred fit was chosen as the one at least 2 units smaller than the rival model

Tested substance	*AIC* (competitive inhibition)	*AIC* (mixed inhibition)	*AIC* (noncompetitive inhibition)	*AIC* (uncompetitive inhibition)
Heparin	196	179.14	177.44	180.38
Dalteparin	237.17	113.14	115.02	134.55

for noncompetitive and for dalteparin of 113.14 for mixed inhibition (Table II), actually inhibit trypsin by binding to an exosite. A recently identified activity modulation of serine protease fIXa as a homolog of trypsin by occupation of the heparin-binding exosite[11,12] supports the data obtained in this kinetic study. The obtained kinetic data clearly show that there is a specific interaction between trypsin and the tested substances based on the binding of heparin and dalteparin to an exosite of the enzyme, demonstrating a mixed or noncompetitive inhibition pattern. Lineweaver–Burk graphs of the most probable inhibition models of trypsin by heparin and dalteparin are shown in Fig. 3.

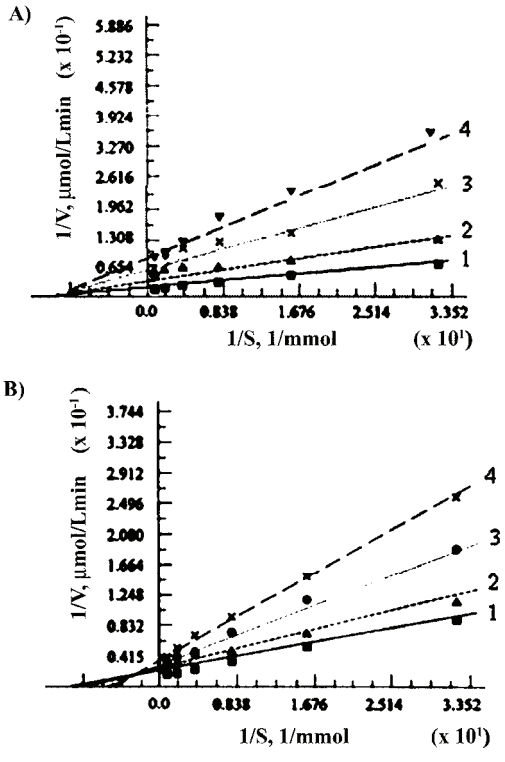

Fig. 3. Lineweaver–Burk plot of a series of kinetic measurements of trypsin activity on BAPNA in the presence of different concentrations of A) heparin and B) dalteparin. Enzyme assays on trypsin were performed with BAPNA as the substrate, in the concentration range 0.313–10.0 mM and different concentrations of inhibitors, *i.e.*, heparin: 0.42–0.84 µM, and dalteparin: 2.9–8.79 µM. The kinetic parameters and mode of action of the tested substances on the trypsin activity were determined using the curve fitting software package Ez-fit. All enzyme assays were performed in duplicate with a control. A) The concentrations of heparin: 1, 0 µM; 2, 0.21 µM; 3, 0.42 µM and 4, 0.84 µM. B) The concentrations of dalteparin: 1, 0 µM; 2, 1.31 µM; 3, 2.62 µM and 4, 3.93 µM.

Heparin, with constant of inhibition $K_{i1,2}$ = 0.151±0.019 μM for noncompetitive inhibition, and dalteparin, with K_{i1} = 0.202±0.030 μM and K_{i2} = 0.463± ±0.069 μM for mixed inhibition, represent moderate inhibitors of the serine protease trypsin. In addition to the constants of inhibition, it was found that the K_m value for BAPNA of 0.99 mM (the K_m for BAPNA is referenced to be 0.94 mM)[22] was not changed in noncompetitive inhibition by heparin, while it was found to be 1.35 mM in mixed inhibition. Data for comparison of S1 serine protease inhibition by heparin and LMWH were not found. However, some inhibitions of fIXa, one of the serine proteases in the human blood-coagulation cascade, were found to be: K_i = 3.2 μM for the 8-hydroxyquinoline family of inhibitors[24] and K_i = 1.73 nM for KFA-1411, a synthetic low molecular weight inhibitor, the inhibition constant of which on trypsin was K_i = 6.1 μM.[17]

CONCLUSIONS

Being involved in complex biological processes, the activity of serine proteases is regulated by sophisticated mechanisms, including the delicate balance between proteolytic and inhibitory reactions in homeostasis. The present model study clearly shows the potential of well known substances, such as heparin and LMWH, to act as inhibitors of trypsin, the reference serine protease.

Research of peptidase inhibitors is an active and rapidly growing field focused mainly on two objectives: construction and screening of new chemical entities with inhibitory activity, and the search for natural proteinaceous inhibitors. A high throughput screening of non-proteinaceous chemical entities for identification of serine protease inhibitors, potentially applicable as drugs has been employed and robust data collections have been generated to date. However, the activity of some well-known (“old”) molecules, such as heparin and LMWH, as potential inhibitors has not been evaluated yet. The present study shows that heparin and dalteparin can specifically inhibit trypsin, producing noncompetitive and mixed kinetic inhibition pattern.

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ИЗВОД

ИНХИБИЦИЈА ТРИПСИНА ХЕПАРИНОМ И ДАЛТЕПАРИНОМ

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У раду је испитивана интеракција трипсина, као прототипа S1 серин-протеазе, са хепарином и његовим нискомолекулским дериватом далтепарином фрагмином. Утврђена је ди-

ректна инхибиција трипсина како хепарином тако и далтепарином, у концентрацијама типичним за њихову клиничку употребу. Одређена је IC_{50} трипсина хепарином: 15,25±1,22 μg/mL и далтепарином: 58,47±15,20 μg/mL. Кинетичка анализа је показала да хепарин и његов нискомолекулски дериват далтепарин инхибирају трипсин по моделу некомпетитивне и мешовите инхибиције, редом. Хепарин са константом инхибиције $K_{i1,2}$ =0,151±0,019 μM (некомпетитивна) и далтепарин са K_{i1} = 0,202±0,030 μM и K_{i2} = 0,463±0,069 μM (мешовита), представљају умерене инхибиторе трипсина, као референтне серин-протеазе. Добијене константе инхибиције указују да при клинички апликованим концентрацијама хепарина и далтепарина, трипсини и хомологе S1 серин-протеазе могу бити директно инхибиране и тиме утицати на деликатну контролу активности серин-протеаза у хомеостази.

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REFERENCES

1. A. J. Barret, N. D. Rawlings, *Handbook of proteolytic enzymes*, 1st Ed. on CD, Academic Press, London, 1998
2. A. J. Barrett, N. D. Rawlings, E. A. O' Brien, *J. Struct. Biol.* **134** (2001) 95
3. G. Hook, V. Y. Hook, M. Kindy, *Biol. Chem.* **388** (2007) 979
4. B. Luna, M. U. Townsend, *Clin. Ther.* **29** (2007) 2309
5. P. Finotti, S. Manente, *Biochim. Biophys. Acta* **1207** (1994) 80
6. P. Finotti, C. Corvaja, A. Pagetta, *Free Radic. Biol. Med.* **27** (1999) 1378
7. T. S. Olson, B. E. Pock, J. Kvassman, U. J. D. Shore, D. A. Lawrence, D. Ginsburg, I. Bjork, *J. Biol. Chem.* **270** (1995) 30007
8. J. Hirsh, T. Warkentin, S. Shaughnessy, S. Anand, J. Halperin, R. Raschke, C. Granger, E. Ohman, J. Dalen, *Chest* **119** (2001) 64S
9. P. A. Patson, M. Schapira, *Biochem. Biophys. Res. Comm.* **230** (1997) 597
10. I. Ahrens, K. Peter, C. Bode, *Haemostaseologie* 27 (2007) 328
11. P. F. Neuenschwander, *Biochemistry* **43** (2004) 2978
12. P. F. Neuenschwander, S. R. Williamson, A. Nalian, K. J. Baker-Deadmond, *J. Biol. Chem.* **281** (2006) 23066
13. B. F. Erlanger, N. Kokowsky, W. Cohen, *Arch. Biochem. Biophys.* **95** (1961) 271
14. M. Kunitz, *J. Gen. Physiol.* **30** (1947) 291
15. S. J. Van der Walt, F. J. Joubert, *Toxicon* **9** (1971) 153
16. BioDataFit 1.02, *Data Fit for Biologists* (http//www.changbioscience.com/stat/ec50.html), Chang Bioscience Inc., 2008
17. W. F. Perella, *Anal. Biochem.* **174** (1988) 437
18. R. H. Jones, E. B. Reeve, G. D. Swanson, *Comp. Biomed. Res.* **17** (1984) 277
19. S. F. Altschul, T. L. Madden., A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res.* **25** (1997) 3389
20. D. S. Ashton, A. E. Ashcroft, C. R. Beddlerl, D. J. Cooper, B. N. Green, R. W. A. Oliver, *Biochem. Biophys. Res. Commun.* **199** (1994) 694
21. K. A. Walsh, *Methods Enzymol.* **19** (1970) 41
22. K. D. Johnson, A. Clark, S. Marshal, *Comp. Biochem. Physiol. B* **131** (2002) 423
23. E. Glusa, C. Adam, *Br. J. Pharmacol.* **133** (2001) 422
24. N. H. Mueller, N. Pattabiraman, C. Ansarah-Sobrinho, P. Viswanathan, T. C. Pierson, R. Padmanabhan, *Antimicrob. Agents Chemother.* **52** (2008) 3385.