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Letter to the Editors-in-Chief

Multiple inhibitory kinetics reveal an allosteric interplay among thrombin functional sites

Dear Editors,

Thrombin (EC 3.4.21.5, 36 kDa) is a key enzyme of the blood coagulation system, accomplishing limited proteolysis of fibrinogen and, thus, producing a network from fibrin fibers. This process, along with platelet aggregation, provides effective blood clotting [1]. Besides, thrombin is involved in a variety of processes including ones linked with thrombin production and inhibition, platelet activation, and regulation of inflammation. To perform these diverse functions thrombin employs several spatially separated sites; in particular, the main ones are exosite I, exosite II, and the active site [2]. The exosites are enriched with lysine/arginine residues and have affinity to negative charged ligands. The active site is represented by His_{57}, Asp_{102}, and Ser_{195} triade is exposed at the bottom of the substrate binding groove in thrombin [1]. The efficient hydrolysis is possible through forming either simultaneous specific interaction with thrombin exosites and active site (macromolecular substrates) or additional interactions with active site cleft (peptidomimetics). Thrombin substrate selectivity can be clearly seen on fibrinogen which has only 4 thrombin-sensitive peptide bonds out of 376 trypsin-sensitive bonds [3].

A variety of factors that determine thrombin activity poses a question on an ideal thrombin inhibitor. A set of proteins, peptides, peptidomimetics, DNA, RNA, and carbohydrates was reported to inhibit thrombin activities to various extents. Known inhibitors can be readily classified by their target site: exosite I-targeted (hirugen [4], G-quadruplex-based DNA aptamers [5]), exosite II-targeted (heparin [6], RNA aptamer Toggle-25 t [7]), active site-targeted (PPACK [8], argatroban [9]), and also mixed types – 'exosite I plus active site'-targeted (hirudin [10], bivalirudin [4]) along with 'exosite II plus active site'-targeted inhibitors (antithrombin III with heparin [11]). Generally, active site-targeted inhibitors directly affect thrombin peptidase activity. Exosite I-targeted inhibitors disturb fibrinogen hydrolysis. Whereas exosite II-targeted inhibitors interfere with heparin binding.

An attempt to compare already existing data for inhibition capacity of different substances faces the problem of diversity of applied techniques mainly because of fundamentally different inhibition mechanisms of these substances. Clearly, conventional amidolytic assay provides little or no information about exosite-targeted inhibitors [12]. In principle, techniques based on natural thrombin substrate hydrolysis have obvious advantages; but until recently there have been no assays suited for inhibitor screening.

In this paper the effect of thrombin inhibitors on fibrinogen hydrolysis has been investigated using turbidimetric assay reported recently [13]. Also GPRP-peptide [14], as an inhibitor of the next step, fibrin association, was characterized kinetically for the first time. A collected set of data makes possible to compare directly different thrombin inhibitors, to understand the enzyme activity regulation in order to gain knowledge for rational development of new antithrombotic substances.

Materials and Methods

Inorganic salts and Tris were purchased from MP Biomedicals (France). Recombinant human thrombin with a specific activity of 3.6 kIU/mg (HTI, USA) and fibrinogen from human plasma (Calbiochem, Germany) were used. Recombinant hirudin with specific antithrombin activity 16 kIU/mg (GenWay, USA); antithrombin III from human plasma with 0.71 mole of active substance per mole of protein (HTI, USA); unfractionated sodium heparin (Synthes, Russian Federation); bivalirudin trifluoroacetate (Selleck Chemicals, USA); hirugen (AnaSpec, USA); PPACK·2HCl (HTI, USA); argatroban (Sigma-Aldrich, USA); RNA aptamer Toggle-25 t, 5'-GGGAACAAAG-CUGAAGUACUUACCC-3' (Synthol, Russian Federation); and GPRP-peptide (Sigma-Aldrich, USA) were used.

Turbidimetric Assay

The thrombin inhibition experiments were carried out at 37 °C in the buffer with the salt composition close to blood plasma (20 mM Tris-acetate, pH 7.4, 0.14 M NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). Fibrinogen concentration was 0.5, 1.0 or 2.0 μM; and the thrombin was added to final concentration 1-5 nM. The sample turbidity was detected as described earlier [15] with the spectrophotometer WPA Biowave II + (Biochrom, UK).

In the inhibition experiments an appropriate amount of inhibitors in 0.1 nM – 300 μM range was added in the assay straight before thrombin. The inhibition coefficient was defined according to the Eq. (1):

$$IC = \sqrt{\frac{W^S_{\max}}{W_{\max}} \frac{t_{\max}}{t^S_{\max}}}, \quad (1)$$

where $W^S_{\max}$ and $t^S_{\max}$ are the parameters of the turbidimetric curve for the standard sample, and *IC* is the inhibition coefficient which reflects decreasing of the active thrombin portion by the inhibitor. The inhibition types as well as the constants were determined according to Zavyalova et al. [13]. The following equations were applied for description of different inhibition types:

1) complete competitive inhibition type

$$IC = 1 + \frac{K_I}{1 + K_0 C^0_F} C^0_I; \quad (2)$$

2) complete non-competitive inhibition type

$$IC = 1 + K_I C^0_I; \quad (3)$$

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3) partial non-competitive inhibition type

$$IC = \frac{1 + K_I C_I^0}{1 + \beta K_I C_I^0}, \quad 4$$

where C_I^0 and C_F^0 are the concentrations of inhibitor and substrate, correspondingly; K_I and K_0 are apparent association constants of thrombin-inhibitor and thrombin-fibrinogen complexes.

Hirudin curves were treated with tight-binding kinetic equations for amidolytic assay published earlier [16,17]. Heparin apparent inhibition constant was recalculated per one monosaccharide residue since heparin binds through nonspecific electrostatic interactions [18]. Antithrombin III and GPRP-peptide curves were treated in the following way: concentration of inhibitors that doubled *IC* at different fibrinogen concentrations were interpolated to zero fibrinogen concentration (the dependence was linear, data are not shown). For antithrombin III the simplified estimate of the second-order rate constant was performed.

Data Treatment

The data were treated with Origin 8.1 (OriginLab, USA). Non-linear curve fitting using least squares and linear regression were used to calculate the apparent inhibition constants. Images of X-ray structures of thrombin complexes were extracted from PDB using PyMOL 0.99 software (DeLano Scientific LLC, USA).

Results and Discussion

A wide range of thrombin inhibitors along with a fibrin association inhibitor has been studied with the turbidimetric assay. Experimental curves are depicted in Figs. 1-4 as well as X-ray structures of thrombin-inhibitor complexes reported earlier. Constants and inhibition types were determined as it was stated in 'Materials and methods'. Novel data are summarized in Table 1 along with constants determined earlier. Obtained information on inhibition constants is mainly consisting with previous data indicating a high relevancy of the turbidimetric assay. Because of the persistent lack of the data on kinetics of inhibition of fibrinogen hydrolysis *per se*, the most valuable profit of turbidimetric assay is the ability to determine the inhibition types. Obtained data on inhibition types are mostly unique and reveal interesting regularities in thrombin function.

Another type of inhibitors, inhibitors of fibrin association, also can be characterized with turbidimetric assay. GPRP-peptide impairs fibrin association through its binding to fibrinogen D-domains, thus, replacing N-terminal residues generated by thrombin in E-domain [32]. Inhibitors of fibrin association can be distinguished by the curve non-linearity at inhibitor concentrations much higher than the thrombin one. In this concentration range the true competitive thrombin inhibitor has linear relations [13].

Thrombin inhibitors were characterized to have competitive and non-competitive inhibition types. Because of the large area of thrombin-fibrinogen interacting surface it is hard for an inhibitor to compete with fibrinogen binding directly. Therefore it is understandable that two completely competitive inhibitors are the macromolecular substances: hirudin (Fig. 2B) and antithrombin III (Fig. 4B); their interacting surfaces are comparable with the fibrinogen one. Low-molecular weight substances interacting with both active site and exosite I (Figs. 1A, B, 2A, and 4A) are unable to hinder fibrinogen binding to thrombin, but they do able to form nonproductive complexes yielding non-competitive inhibition.

Interacting surface implication can be clearly seen from the examples of DNA aptamers with different sizes targeted to the thrombin exosite I [13]. 15-mer oligonucleotide, 15-TBA, is a complete non-competitive inhibitor whereas its double-size derivative - 31-mer, 31-TBA, is the complete competitive inhibitor.

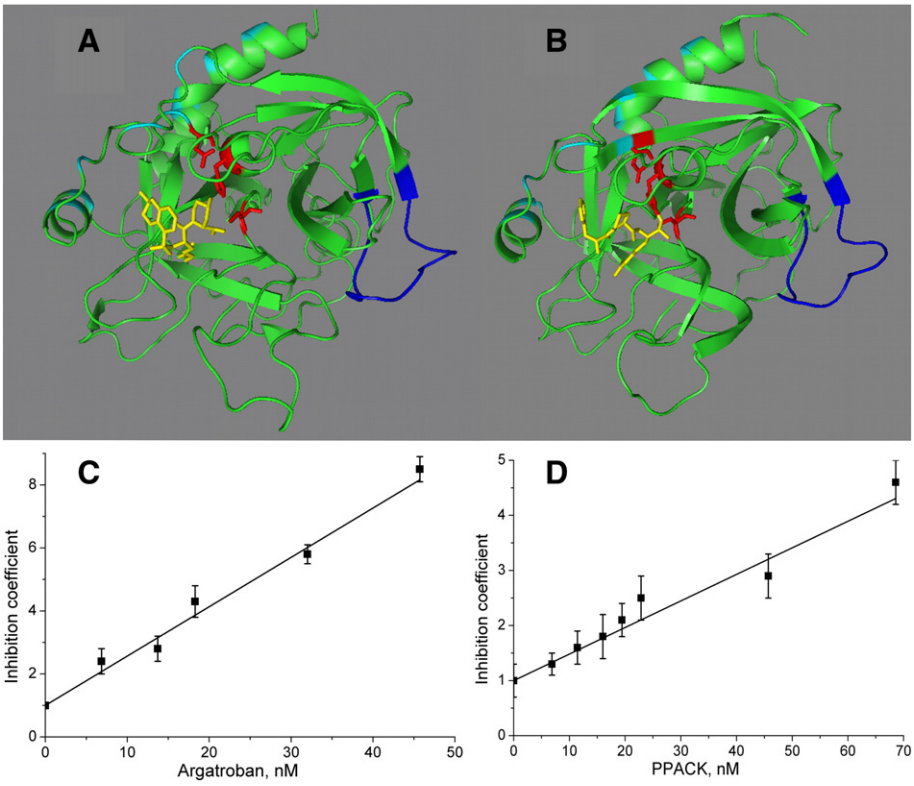

Fig. 1. Active site-targeted inhibitors. The X-ray structures of the human thrombin-argatroban complex, PDB entry 1DWC (a), and the covalent human thrombin-PPACK complex, PDB entry 1PPB (b). Thrombin active site is shown in red, exosite I is shown in dark blue, exosite II is shown in light blue, inhibitors are shown in yellow. The inhibition curves of argatroban (c) and PPACK (d).

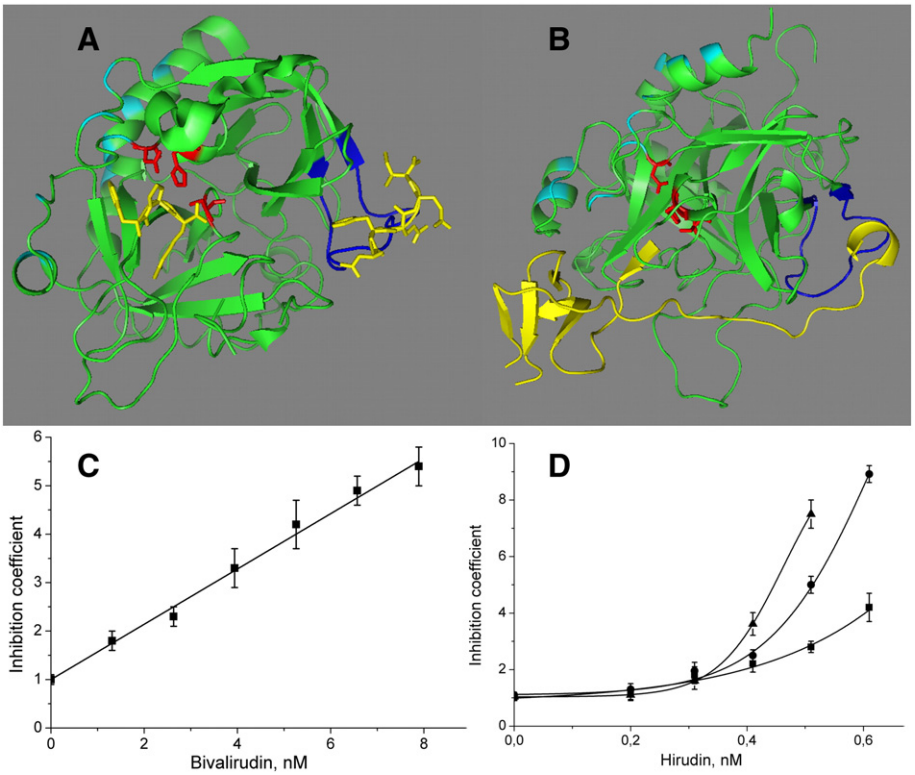

Fig. 2. Exosite I and Active site-targeted inhibitors. The X-ray structures of the thrombin-bivalirudin complex, PDB entry 2HGT (a), and the thrombin-hirudin complex, PDB entry 4HTC (b). Thrombin active site is shown in red, exosite I is shown in dark blue, exosite II is shown in light blue, inhibitors are shown in yellow. Polyglycine linker between active site and exosite I-targeted moieties of bivalirudin is fully disordered in crystal structure. The inhibition curves of bivalirudin (c), and hirudin (d): ■ – 2.0 μM of fibrinogen; ● – 1.0 μM of fibrinogen; ▲ – 0.5 μM of fibrinogen.

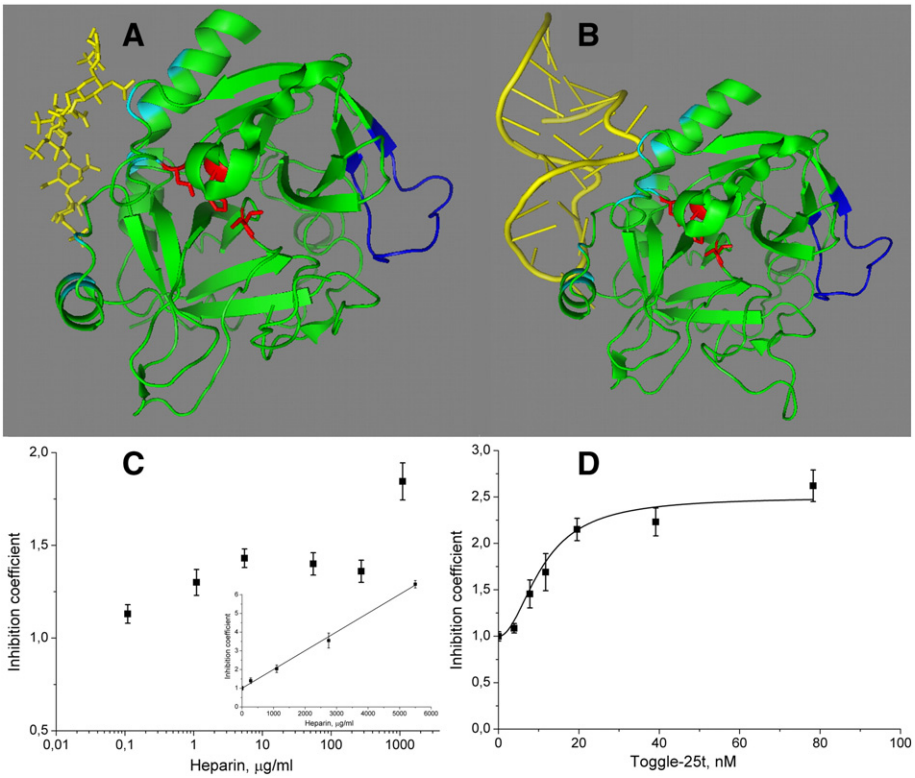

Fig. 3. Exosite II-targeted inhibitors. The X-ray structures of the thrombin-heparin complex, PDB entry 1XMN (a), and the thrombin-Toggle-25 t, RNA aptamer, PDB entry 3DD2 (b). Thrombin active site is shown in red, exosite I is shown in dark blue, exosite II is shown in light blue, inhibitors are shown in yellow. The inhibition curves of unfractionated heparin at the heparin concentration ranges below 1 mg/ml and over 1 mg/ml (inset) (c), and Toggle-25 t (d).

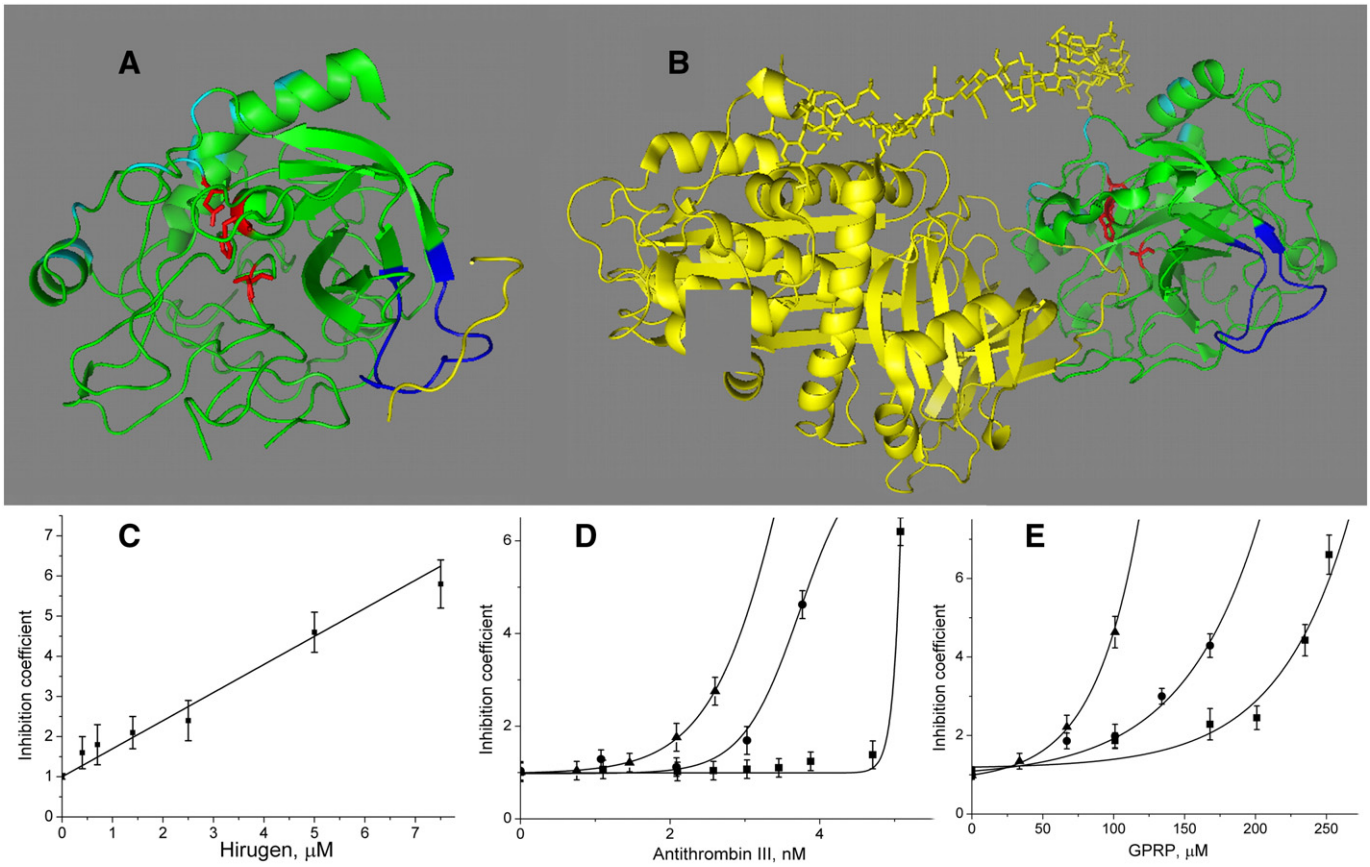

Fig. 4. Hirugen, antithrombin III, and GPRP-peptide. The X-ray structures of the thrombin-hirugen complex, PDB entry 1HGT (a), and the ternary thrombin-heparin-antithrombin III complex, PDB entry 1 TB6 (b). Thrombin active site is shown in red, exosite I is shown in dark blue, exosite II is shown in light blue, inhibitor is shown in yellow. The inhibition curves of hirugen (c), of antithrombin III in presence of 20 μg/ml of heparin (d), and GPRP-peptide, inhibitor of fibrin association: ■ – 2.0 μM of fibrinogen; ●– 1.0 μM of fibrinogen; ▲– 0.5 μM of fibrinogen.

Thrombin exosite II ligands are generally considered not to be inhibitors of fibrinogen hydrolysis. Though heparin and RNA aptamer Toggle-25 t (Fig. 3A,B) exhibited some slight inhibition of the reaction, but the kinetic details have not been determined [29,30]. Due to the sensitivity of the turbidimetric assay it was possible to obtain kinetic data for these inhibitors. Both ligands exhibit partial non-competitive

Table 1
Comparison of inhibition constants and types from turbidimetric assay with previous data. 'Complete competitive' inhibition type means pseudo-competitive type: 1) GPRP-peptide inhibits fibrin association but not thrombin; 2) thrombin inhibition with antithrombin III is interfered with fibrin leading to complicated inhibition kinetics [19].

Target	Inhibitor	Inhibition constants from turbidimetric assay	Inhibition type	Constants from refs
Thrombin active site	Argatroban	$K_I^{app} = 6.4 \pm 0.5$ *nM*	complete non-competitive	$K_d = 5.0 \pm 0.3$ *nM* [20] (intrinsic fluorescence of thrombin) $K_I = 39 \pm 2$ *nM* [21] (chromogenic assay)
	PPACK	$K_I^{eff} = 20.8 \pm 1.4$ *nM*	complete non-competitive	$K_I = 24-45$ *nM* [22,23] (chromogenic assay)
Thrombin exosite I	Hirugen	$K_I^{app} = 1.50 \pm 0.06$ *μM*	complete non-competitive	$K_I = 1.3 \pm 0.2$ *μM* [24] (intrinsic fluorescence of thrombin) $K_A = 3.2 \pm 0.2$ *μM* [12] (chromogenic assay)
Thrombin exosite I and active site	Bivalirudin	$K_I^{app} = 1.75 \pm 0.04$ *nM*	complete non-competitive	$K_I = 1.9-2.6$ *nM* [25–27] (chromogenic assay)
	Hirudin recombinant	$K_I^{app} = 3 \pm 2$ *pM*	complete *competitive*	$K_I^{app} = 19 \pm 2$ *pM* [28] (chromogenic assay, in 0.14 NaCl)
Thrombin exosite II	Toggle-25 t	$K_I^{app} = 10 \pm 3$ *nM* $\beta = 0.33 \pm 0.08$	partial non-competitive	$K_d = 0.54 \pm 0.10$ *nM* [29] (affinity)
	Heparin	$K_I^{app} = 2.65 \pm 0.14$ *μM* $\beta = 0.7 \pm 0.2$	partial non-competitive	Partial inhibition of fibrinogen hydrolysis (1.2-1.6-fold), constants weren't determined [30]; $K_d = 6-10$ *μM* [18] (affinity)
		$K_I^{app} = 4.80 \pm 0.10$ *mM*	complete non-competitive	Not determined
Thrombin exosite II and active site	Antithrombin III and heparin	$IC_{50} = 1.4 \pm 0.1$ *nM* $k_{cat}^{app} = 1.7 \pm 0.5 \cdot 10^9$ M^{-1}, min^{-1}	pseudo-complete *competitive*	$k_{cat}^{app} = 1.33 \pm 0.15 \cdot 10^9$ M^{-1}, min^{-1} [31] (chromogenic assay)
Inhibitor of fibrin association	GPRP-peptide	$IC_{50} = 27 \pm 2$ *μM*	pseudo-complete *competitive*	$K_d = 20 \pm 2$ *μM* [32] (affinity)

inhibition type with the degrees of partiality of 0.70 for heparin and 0.33 for Toggle-25 t. This inhibition mode indicates clearly the close allosteric linkage between two thrombin exosites. Allosteric regulation of thrombin activity toward low-molecular substrates was previously demonstrated for exosite I ligands and Na-binding site [1,12,33]. Described here exosite II ligands are another interesting example of allosteric interplay in thrombin molecule.

This kinetic study has explored a repertoire of different inhibition types for the set of inhibitors, and also has revealed a close allosteric interplay within the thrombin macromolecule. The main conclusion is a close relation between binding and inhibitory modes of the thrombin ligands. Active site- and exosite I-targeted ligands are able to inhibit fibrinogen hydrolysis completely. Macromolecular ligands affect fibrinogen hydrolysis in a competitive manner due to creation of the interacting area comparable to the thrombin-fibrinogen one. Low-molecular ligands probably form nonproductive complex interfering the appropriate orientation of substrate on the thrombin. Exosite II-targeted ligands are capable for only partial inhibition, indicating the fine tuning of the thrombin enzymatic activity via allosteric effects. These results are undoubtedly important for understanding of the thrombin activity regulation and the rational development of new antithrombotic substances.

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Elena Zavyalova*
Alexey Kopylov
Chemistry Department M.V. Lomonosov Moscow State University, Moscow, Russian Federation
APTO-PHARM, LLC, Moscow, Russian Federation
*Corresponding author at: Chemistry Department M.V. Lomonosov Moscow State University, Leninskie gory 1-3, Moscow, 119991 Russian Federation. Tel.: +7 495 939 31 49; fax: +7 495 939 31 81.
E-mail address: zlenka2006@gmail.com (E. Zavyalova).

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