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Molecular Modeling as a New Approach to the Development of Urokinase Inhibitors

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Proteolytic activity of urokinase plays an important role in negative remodeling of blood vessels, restenosis, tumor angiogenesis, and metastasizing, which necessitates the development of selective urokinase inhibitors. Using methods of computer modeling (docking, post processing, and direct docking) and quantum chemistry, we selected substances from the large compound database, analyzed their structures, and experimentally verified their inhibitor activity. New urokinase inhibitor candidates were proposed based on the theoretical predictions and experimental verification of compound activities. The process of modifying urokinase inhibitors based on (benzothiazol-3-yl)guanidine was developed. A new urokinase inhibitor (5-brom-benzothiazol-3-yl)guanidine, that can be effective for regulation of vascular remodeling and tumor angiogenesis, was created.

Key Words: *urokinase; proteolysis; inhibitors; molecular modeling*

Urokinase (uPA) is a multidomain multifunctional protein converting plasminogen into plasmin that apart from fibrinolysis regulation contributes to the expression of proteins and activation of proteolytic cascades. uPA and plasmin generated in this reaction take part in degradation of extracellular matrix and basement membrane proteins, activation of matrix metalloproteinases and growth factors (HGF, VEGF-189, and TGF-β) [1]. These mechanisms induced by uPA stimulate migration and proliferation of cells, which are pivotal processes of angiogenesis, vascular remodeling, tumor growth and spreading [4].

Experimental models show that uPA acts as a key regulator of negative arterial remodeling [13].

Antibodies neutralizing uPA prevent arterial stenosis induced by ballooning [8]. Results of our experiments show that uPA mediates the signals for induction of angiogenesis [13]. Proteolytically active uPA stimulates the development of capillaries and arterioles, reduces the size of myocardial infarction, and prevents the development of ischemic limb necrosis [14]. In addition, the role of uPA in the processes of growing and spreading of tumors is well known nowadays [6]. Taking to account these facts, the development and usage of selective inhibitors of uPA seem to be promising for possible suppression of negative vascular remodeling and tumor angiogenesis.

Specific binding of the majority of inhibitors to the proteolytic center of uPA is primarily determined by its amino acid residue Asp189 located in the base of primary anchor site for substrates termed specificity pocket S1 [11]. The crystal structure of uPA catalytic domain displays a trypsin-like topology in which the

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Asp189 is retained, conferring to the S1 site an affinity for positively charged Arg and Lys residues [11]. Thus, the majority of synthetic uPA inhibitors share a common structural feature consisting of a mono- or biaromatic moiety performing the function of amidine or guanidine and acting as arginine mimetic.

Recently, new low-molecular inhibitors of uPA were developed [15]. However, only WX-UK1 inhibitor (WILEX) was included to clinical trials in combination with cytostatic agents. At the present time, there is no uPA inhibitor widely used in clinical practice and development of these inhibitors is an urgent problem.

Here we developed new effective low-molecular synthetic inhibitor of proteolytic center of uPA using computer molecular modeling [2].

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Molecular Model of uPA. Sixty structures were found in the Protein Data Bank (PDB) database for the development of the model of uPA active center. Forty-five of these uPA structures were bound with direct reversible non-covalent inhibitors of its proteolytic active site [2,3]. Analysis of active center and localization of inhibitors revealed binding pockets and main interactions. According to crystalline structures, a molecular model of the protein was constructed: hydrogen atoms were added to the protein crystal structure using APLITE software, and interaction types in MMFF94 force field were analyzed [7]. A docking area with a form a cube with the edge of 22 Å corresponding to the proteolytic active site was chosen. The potential grid in this area was made using SOLGRID software. This procedure was performed on 45 complexes uPA–ligand. A protein of ISQO complex was chosen from PDB for virtual screening, post processing, and quantum chemistry.

Docking: SOL program. We used SOL program, which finds global minimum of protein-ligand potential energy by genetic algorithm in MMFF94 force field [7] for fixed protein and flexible ligand. Validation of SOL program showed high docking quality: good repeatability of ligand native positions in 80 proteins and good detection of inhibitors among large amount of inactive agents by sorting of ligands by scoring function. SOL program also showed good results during testing of CSAR2011–CSAR2012 and developing of new thrombin inhibitors [10].

Observed after docking (using SOL software) poses of 45 various native ligands from uPA PDB-complexes differed from native by no more than 3 Å in 80% of cases, which reflects the high quality of ligand positioning. Forty-five native ligands were divided into inhibitors and inactive agents (threshold of scoring function was -5.5 kkal/mol). Two false positive and 7 false negative results (correlation coefficient 0.35) were found after comparison with experimental data.

Post processing: DISCORE program. DISCORE program for post processing was used after docking for estimation of energy of protein–ligand binding. Local optimization by L-BFGS in MMFF94 force field was performed by L-BFGS method in a solvent. Free energy of protein-ligand binding is a linear combination of various compounds of energy calculated for system configuration observed during local optimization [5]. We used the following compounds in DISCORE program: coulomb and van der Waals' interactions between ligand and protein, polar and non-polar part of desolvation energy, energy of internal stress of ligand and entropy contribution. Formula of free energy includes 7 compounds and 7 adjustment coefficients, which are chosen to make binding energies of some sets of protein–ligand similar to experimentally determined energies. We used the coefficients adjusted for experiments using the set of 50 complexes of uPA with various inhibitors and inactive substances. DISCORE post processing improves the reliability of predictions and increases correlation coefficient for SOL from 0.35 to 0.52 and decreases the amount of false predictions from 38% (for SOL) to 34%.

Quantum chemistry. Post processing was also performed by quantum chemical semi-empirical method PM7 (MOPAC package) [9]. High accuracy of calculations by PM7 is similar to it by density functional method. Local optimization was performed for all ligand atoms with fixed proteins from ligand pose in a protein obtained with SOL docking taking into account solvent at the last step of optimization. Ranging of ligands and selection of candidates for uPA inhibitors were conducted in accordance to the calculated enthalpy of protein–ligand binding. The threshold (50 kkal/mol), which separates inhibitors from inactive substances, was chosen in accordance to the comparison of calculations and experimental data for known uPA inhibitors. Correlation coefficient between binding energies calculated using MOPAC and experimental values was 0.68, other adjusting coefficients were not used.

Generalized direct docking: FLM program. FLM program (Find Local Minima) was developed for increasing of accuracy for estimation of free protein–ligand binding energy. In this program docking is gridless, direct, and generalized, because as distinct from SOL program the grid of preliminary calculated potentials of interactions between trial atoms and protein is not used, and calculation of interaction between a ligand in each pose with protein are performed directly in the MMFF94 force field without adjusting parameters, which allows to conduct local optimiza-

tion of energy using protein atoms and ligands [12]. Coefficient of correlation between calculated using FLM program binding energies with experimental values for various sets of complexes (*e.g.* with uPA) was 0.50-0.65 with the threshold separating inhibitors and inactive substances of about -80 kkal/mol.

In vitro **testing of urokinase inhibitors**. Catalytic activity of uPA was estimated by the breakage of specific chromogenic substrate S-2444 (Pyro-Glu-Gly-Arg-pNA). Measuring of uPA activity in the presence of inhibitors was performed in 96-wells flat-bottomed plates form non-binding plastic using a plate spectrophotometer. uPA (Sigma) in a dose of 100 nm was preliminary incubated with plasminogen (molar ratio 1:50) in PBS containing 0.1% BSA and 0.05% Twin-20 for 10 min at 37ºC. Final volume of the sample was 100 μl. Inhibitors in a dose of 500 μM (per 200 μl) were added to the samples and incubated for 10 min at 37ºC. Ecotin (specific protein, uPA inhibitor) was added in the molar ratio of 1:4. Substrate S-2444 in a dose of 206 μM in 100 μl PBS containing 0.1% BSA and 0.05% Twin-20 was added to the samples. The analyzed sample (200 μl) contains 50 nM uPA, 500 μM inhibitors, and 103 μM substrate. Catalytic activity was estimated by the release of para-nitroanilide at each minute at $\lambda=405$ nm at 37ºC. Given by the Laboratory of Genetic Engineering of the Russian Cardiology Research-and-Production Complex, Institute of Experimental Cardiology, the Ministry of Health of the Russian Federation, recombinant protein ecotin, which specifically and irreversibly inhibits pro-

teolytic activity of uPA, was used as a positive control. This method of the inhibitory activity measurement is suitable for calculation of inhibition percentage, EC_{50}, IC_{50}, and K_i.

RESULTS

Docking and virtual screening of database with known substances. Search of uPA inhibitors in databases of known medicinal substances was performed for the development of new inhibitors. We used international ZINC database, which is a catalogue of substances prepared to docking. Docking of about 800 thousands substances from the section with lead-compound agents (small molecules, on the base of which bigger inhibiting molecules can be constructed) was conducted. Calculations were conducted using SOL docking program in parallel with calculations on a supercomputer "Lomonosov" of the M. V. Lomonosov Moscow State University. Then the ligands with scoring function <-5.5 kkal/mol were analyzed and 43 of them were experimentally tested. Fourteen of these substances had slight inhibiting activity (IC_{50} ~200 μM). Both positive and negative results were used for the following stages for the development of new uPA inhibitors.

Docking program SOL can find new inhibitors of uPA showing activity in experiment using a large database of real and virtual substances. Other programs used in our investigation (DISCORE, FLM, and MOPAC with PM7) are useful for the following
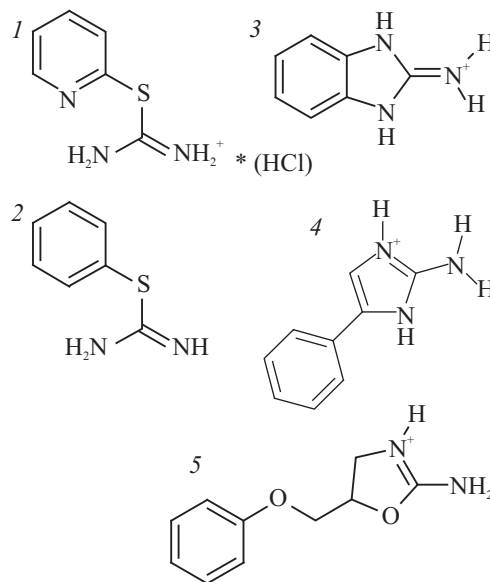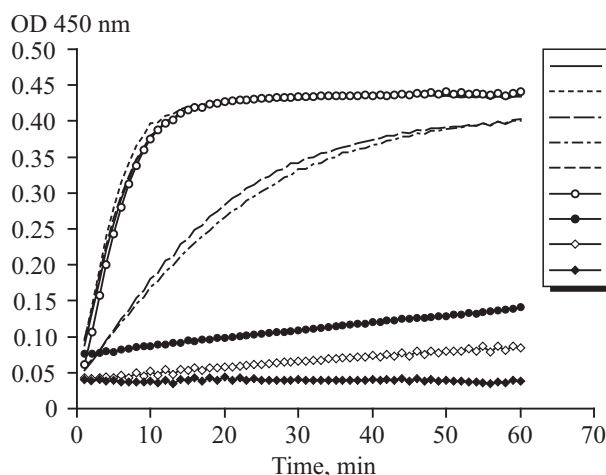
Fig. 1. Inhibition of proteolytic activity of uPA (50 nm) by inhibitors (500 μm) estimated by degradation of the specific chromogenic substrate of uPA S-2444. Amilorid (uPA inhibitor with half-inhibitory concentration 7 μM) was used as positive control. Slope of curve characterizes reaction rate: the lower is the slope in comparison with the control (uPA without inhibitors), the better the inhibitor works. Samples *1-5*, examples of synthesized and tested substances. Here and in Fig. 3: OD, optical density. Ecotin and amilorid, uPA inhibitors. Background, sample without uPA.
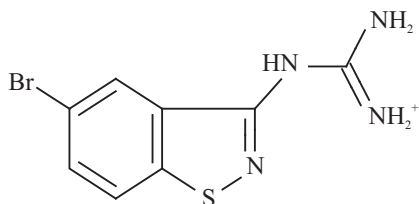
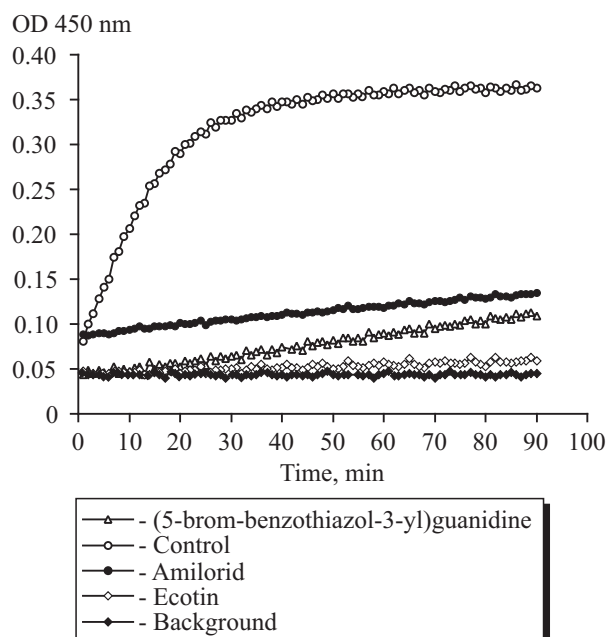**Fig. 2**. Structure of (5-brom-benzothiazol-3-yl)guanidine.

- △ - (5-brom-benzothiazol-3-yl)guanidine
- ○ - Control
- ● - Amilorid
- ◇ - Ecotin
- ◆ - Background

Fig. 3. Inhibition of proteolytic activity of uPA (50 nm) by (5-brom-benzothiazol-3-yl)guanidine (50 μm) estimated by degradation of the specific chromogenic substrate of uPA S-2444.

selection of best candidates from a variety of substances selected by SOL program, and for design of new substances for the following synthesis.

Estimation of activity of candidates into uPA inhibitors. More than 30 substances, which were chosen in accordance to the analysis of chemical structures of existing agents from the databases showing good results during docking, were experimentally analyzed. These list included (2-pyridyl)-isothiouronium chloride, S-phenylisourea, 3-amino-isochinoline C9H8N2, 2-aminobenzimidazole, (dl)-5-phenoxymethyl-4,5-dihydro-1,3-oxazole-2-amin, (5-bromopyridil-2)-isothiouronium chloride, 2-amino-S-nitrobenzimidazolum, aminoimidazole, *etc*. The majority of compounds did not inhibit uPA. Agents with positively charged group (amidine, guanidine, amino-, or aminoimidazole groups), which approaches S1 pocket of proteolytic center of uPA and interacts with Asp189, revealed inhibiting activity (IC_{50} ~200 μM).

Modifications of (benzothiazol-3-yl)guanidine, which has maximal inhibiting activity (half-inhibitory concentration 33 μM) were analyzed for the develop-

ment of uPA inhibitor with high efficiency. Docking of derivatives of (benzothiazol-3-yl)guanidine was performed using SOL program; score function, clusterization, and possibilities of synthesis were analyzed. Thirty substances with score functions <-5.5 kcal/mol were selected. Some of them was excluded due to difficulties with synthesis. The majority of 20 of synthesized substances did not inhibit uPA (Fig. 1). All tested substances were small molecules and their enlargement and addition of new substituting groups can positively or negatively affect their activity. Nine substances revealed inhibiting activity ($IC_{50} \leq 200$ μM or lower); (5-brom-benzothiazol-3-yl)guanidine had score function -5.6 kcal/mol (Fig. 2) and revealed maximal ability to inhibit uPA.

The substance in the doses of 500, 250, 100, 0, 10, and 1 μM was used for experiments. (5-brom-benzothiazol-3-yl)guanidine was dissolved in warm water. Its half-inhibitory concentration was ~5 μM (Fig. 3), and its half-inhibitory concentration for plasmin was 85 μM.

Results of our study are an attempt to use computer modeling for the development of new uPA inhibitors. We developed low-molecular inhibitor of proteolytic center of uPA, as low molecules are perspective for production of new medicinal substances. We showed an opportunity to produce new inhibitors of uPA. New basic structure of uPA inhibitor can be used for the development of highly selective and effective inhibitors for further practical usage, *e.g.* during tumor angiogenesis. Obtained results show high potential of computer constriction of medicinal substances for the development of low-molecular inhibitors of enzymes, which serves as a new perspective approach for the development of medicinal agents.

REFERENCES

1. E. V. Parfenova, O. S. Plekhanova, M. Yu. Men'schikov, *et al.*, *Fiziol. Zh. I. M. Sechenova*, **95**, No. 5, 442-464 (2009).
2. A. N. Romanov, O. A. Kondakova, F. V. Grigor'ev, *et al.*, *Vychislitel'nie Metody Programmirovanie*, **9**, 213-233 (2008).
3. V. A. Sadovnichii, V. B. Sulimov, E. V. Katkova, *et al.*, *Postgenomic Investigations and Technologies* [in Russian], Ed. S. D. Varfolomeev, Moscow, (2011) pp. 103-140.
4. V. A, Tkachuk, O. S. Plekhanova, I. B. Beloglazova, *et al.*, *Ukr. Biokhim. Zh.*, **85**, No. 6, 18-45 (2013).
5. H. J. Bohm, *J. Comput. Aided Mol. Des.*, 8, No. 3, 243-256 (1994).
6. M. J. Duffy, *Curr. Pharm. Des.*, **10**, No. 1, 39-49 (2004).
7. T. A. Halgren, *J. Comput. Chem.*, **17**, No. 5, 490-519 (1996).
8. O. S. Plekhanova, Y. V. Parfyonova, R. Sh. Bibilashvily, *et al.*, *J. Hypertens.*, **18**, No. 8, 1065-1069 (2000).
9. J. Rezac, P. Hobza, *Chem. Phys. Lett.*, **506**, Nos. 4-6, 266-289 (2011).
10. E. I. Sinauridze, A. N. Romanov, I. V. Gribkova, *et al.*, *PLoS One*, **6**, No. 5. doi:10.1371/journal.pone.0019969 (2011).

11. G. Spraggon, C. Phillips, U. K. Nowak, *et al*., *Structure*, **3**, No. 7, 681-691 (1995).
12. A. V. Sulimov, D. C. Kutov, I. V. Oferkin, *et al*., *J. Chem. Inf. Model*., **53**, No. 8, 1946-1956 (2013).
13. V. Tkachuk, O. S. Plekhanova, and Y. V. Parfyonova, *Can. J. Physiol. Pharmacol*., **87**, No. 4, 231-251 (2009).
14. D. O. Traktuev, Z. I. Tsokolaeva, A. A. Shevelev, *et al*., *Mol. Ther*., **15**, No. 11, 1939-1946 (2007).
15. M. Zhu, V. M. Gokhale, L. Szabo, *et al*., *Mol. Cancer Ther*., **6**, No. 4, 1348-1356 (2007).