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SYNTHESIS, BIOLOGICAL EVALUATION AND DOCKING OF NOVEL BISAMIDINOHYDRAZONES AS NON-PEPTIDE INHIBITORS OF FURIN

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A series of novel non-peptidic furin inhibitors with values of inhibitory constants (Ki) in the range of 0.74-1.54 μM was obtained by interactions of aminoguanidine hydrocarbonate with three diaryldicarbaldehydes. Correspondingly p-hydroquinone, piperazine and adipic acid were used as linkers between their benzene moieties. Docking studies of these new inhibitors into recently published 3D-structure of human furin (PDB code 4OMC) showed that they were able to interact with subsites S1 and S4 of the enzyme. The overall arrangement of bisamidinohydrazones into furin active site was similar to the position of the ligand co-crystallized with a protease. Observations obtained with molecular modeling allowed further guidance into chemical modifications of the synthesized inhibitors which improve their inhibitory activity.

K e y w o r d s: furin, non-peptide inhibitors, bisamidinohydrasones, biological evaluation, docking.

Furin, a product of gene FUR, a multidomain calcium-dependent serine enzyme belongs to the family of proprotein convertase subtilisin/kexins (PCSKs). PCSKs regulate cleaving of initially inactive polypeptide products of translation, converting them into 'mature' functionally active proteins [1]. For example, the substrates of furin are precursors of peptide hormones, growth and differentiation factors, adhesive molecules, receptors, ion channels, blood clotting factors, etc. Furin cleaves specific peptide bonds at the C-end of the segment, containing a cluster of positively charged residues of the following motif: -(K/R)-(X)n-(K/R)-Arg-↓, where n = 0, 2, 4 and X – any proteinogenic amino acid, except Cys [2-4]. Similar to other PCSKs, furin plays a large role in the initiation and development of disorders such as cancer [5], cardiovascular pathologies, obesity, diabetes, neurological and cognitive dysfunctions, viral and bacterial infections, abnormalities in the reproductive and bone calcification processes [6, 7]. Therefore, furin is a promising target for development of new medication battling various human illnesses. Discovery of the new effective and specific inhibitors of furin and other PCSKs may help to solve many biochemical, therapeutic and clinical problems [8-13]. Currently known furin inhibitors can be classified as proteins and their fragments, peptides, pseudopeptides, peptidomimetics and compounds of non-peptidic nature [8-10]. In recent years, there has been an increased interest in low-molecular-weight compounds (molecular weight below 5000 Da) of non-peptidic nature [8]. It was driven by the fact that such compounds have a higher (in comparison to peptides) proteolytic stability, high solubility in aqueous media, better pharmaco-kinetic properties, bioaccesibility and better ability to penetrate a cell. Besides they are easily accessible by synthetic routes than peptides and recombinant proteins [8, 9].

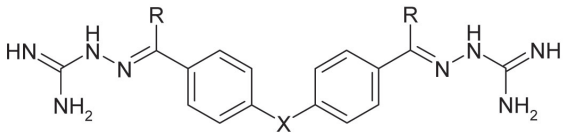
Various classes of organic compounds that inhibit furin both *in vitro* and *in vivo* were recently reported. Among them there are aromatic compounds containing positively charged groups [13–15], derivatives of naphthofluorescein, a series of carbocyclic derivatives of diterpenes [17] and dideoxitreptamines [13], azaheterocycles, such as chelating derivatives of pyridine [18], oxygen-containing heterocyclic compounds such as flavonoids and coumarins [19–21], as well as quinones and many others [8].

In our own studies we have shown that some heterocycles in a series of flavonoids and pyrazoles [22] were capable, although weakly, to inactivate

furin. Further expanding the findings of Sielaff et al [14] we synthesized compounds with weakly basic amidinohydrazone group and shown that they were inhibitors of furin with values of K_i in the range of micromoles [15].

In the present study we further advance our search for efficient non-peptide furin inhibitors into series of novel derivatives of aryl bisamidinohydrazones.

Similarly to our previous work [15] we focus on compounds **I** that contain two arylic substituents with positively charged amidinohydrazone groups. By altering the length and chemical composition of the linker X we studied the effect of hydrophobicity and structural modifications in bisamidinohydrazones on their efficacy of furin inhibition.



I

R = H, CH_3

The scheme of the synthesis is shown in Fig. 1. Bisamidinohydrazones **2** and **4** were obtained by interactions of dialdehydes **1** and **3** with aminoguanidine bicarbonate. Compound **8** was obtained by interaction of p-aminoacetophenone with the adipic acid chloride. The yield was 55-58%.

Materials and Methods

Reagents and preparations. A fluorogenic substrate Boc-Arg-Val-Arg-Arg-AMC was purchased from (Bachem, Switzerland) and recombinant human furin (2000 units/ml) was obtained from (New England BioLabs, United Kingdom) and (Sigma Aldrich, USA). Standard furin solution was diluted 20-fold with a buffer (pH 7.3; 100 mM Hepes, 1 mM $CaCl_2$, 0.5% Triton X-100 and 1 mM β-mercaptoethanol and used in enzymatic reaction. One unit of furin activity was defined as quantity of an enzyme that under standard conditions cleaved off 1 pmol of 7-amino-4-methylcoumarin (AMC) in 1 min.

EDTA, mercaptoethanol, Hepes, Triton X-100 and benzamidin hydrochloride hydrate were purchased from Sigma Aldrich and all other reagents and solvents were provided by Chimlaborreaktiv (Ukraine).

NMR-spectra were recorded using a Varian Mercuiry-400 spectrometer with DMSO-d as a solvent and TMS as the internal standard. Melting points were determined using a Fisher-Johns apparatus.

1-({[4-(4{4-[Carbamimidamidoimino)methyl]phenoxy}phenyl]methylidene}amino)-guanidine 2. Amino guanidine hydrocarbonate in the amount of 2.63 g (0.025 M) was added to the solution of 3.18 g (0.01 M) dialdehyde 1 [24] in 50 ml dimethylformamide-water (4:1) mixture, then 2.52 g (0.03 M) of $NaHCO_3$ were added. The reaction mixture was boiled for 5 h. The solvent was removed under vacuum, 50 ml of water were added to the residue, the precipitate was filtered out and crystallized from dimethylformamide. The yield was 56%. T_{mp} = 298-300 °C; NMR ^{1}H, δ, ppm: 5.43 br s (4H, 4NH), 5.91 br s (4H, 4NH), 6.95-7.72 m (12H, H arom.), 7.99 s (2H, 2CH). Elemental composition calculated, %: C 61.38; H 5.15; N 26.03; found, %: C 61.29; H 5.24; N 26.11. $C_{22}H_{22}N_8O_2$.

1-({[4-(4{4-[Carbamimidamidoimino)methyl]phenyl}-piperazine-1-yl)phenyl]methylidene}amino)-guanidine 4 was obtained using the same method as for compound **2** from aldehyde **3** [25]. The yield was 58%. T_{mp} = 300 °C; NMR ^{1}H, δ, ppm: 3.41 s (8H, $4CH_2$), 5.29 br s (4H, 4NH), 5.79 br s (4H, 4NH), 6.96-7.58 m (8H, H arom.), 7.91 s (2H, 2CH). Elemental composition calculated, %: C 59.10; H 6.45; N 34.46; found, %: C 59.19; H 6.36; N 34.51. $C_{20}H_{26}N_{10}$.

N,N′-bis(4-acethylphenyl)hexanediamide 7. Triethylamine in the amount of 2.23 g (0.022 M) was added to solution of 2.84 g (0.021 M) of p-aminoacetophenone 6 in 30 ml of non-aqueous acetonitrile, and then 1.83 g (0.01 M) of acid chlorine 5 were added. The reaction mixture was boiled for 1 h, and then was cooled and the solvent was removed under vacuum. Water in the amount of 50 ml was added to the residue, the precipitate was filtered out, and then was crystallized from ethanol. The yield was 86%. T_{mp} = 181-183 °C. NMR ^{1}H, δ, ppm: 1.61 s (4H, $2CH_2$), 2.34 s (4H, $2CH_2$), 2.46 s (6H, $2CH_3$), 7.68-7.96 m (8H, H arom.), 10.31 s (2H, 2NH). Elemental composition calculated, %: C 69.46; H 6.36; N 7.36; found, %: C 69.53; H 6.29; N 7.44. $C_{22}H_{24}N_2O_4$.

N,N′-bis({4-[1-(Carbamimidamidoimino)-ethyl]phenyl})-hexanediamide 8. The compound was obtained using the same method as for compound **2** from acetophenone **7**. Yield was 55%.

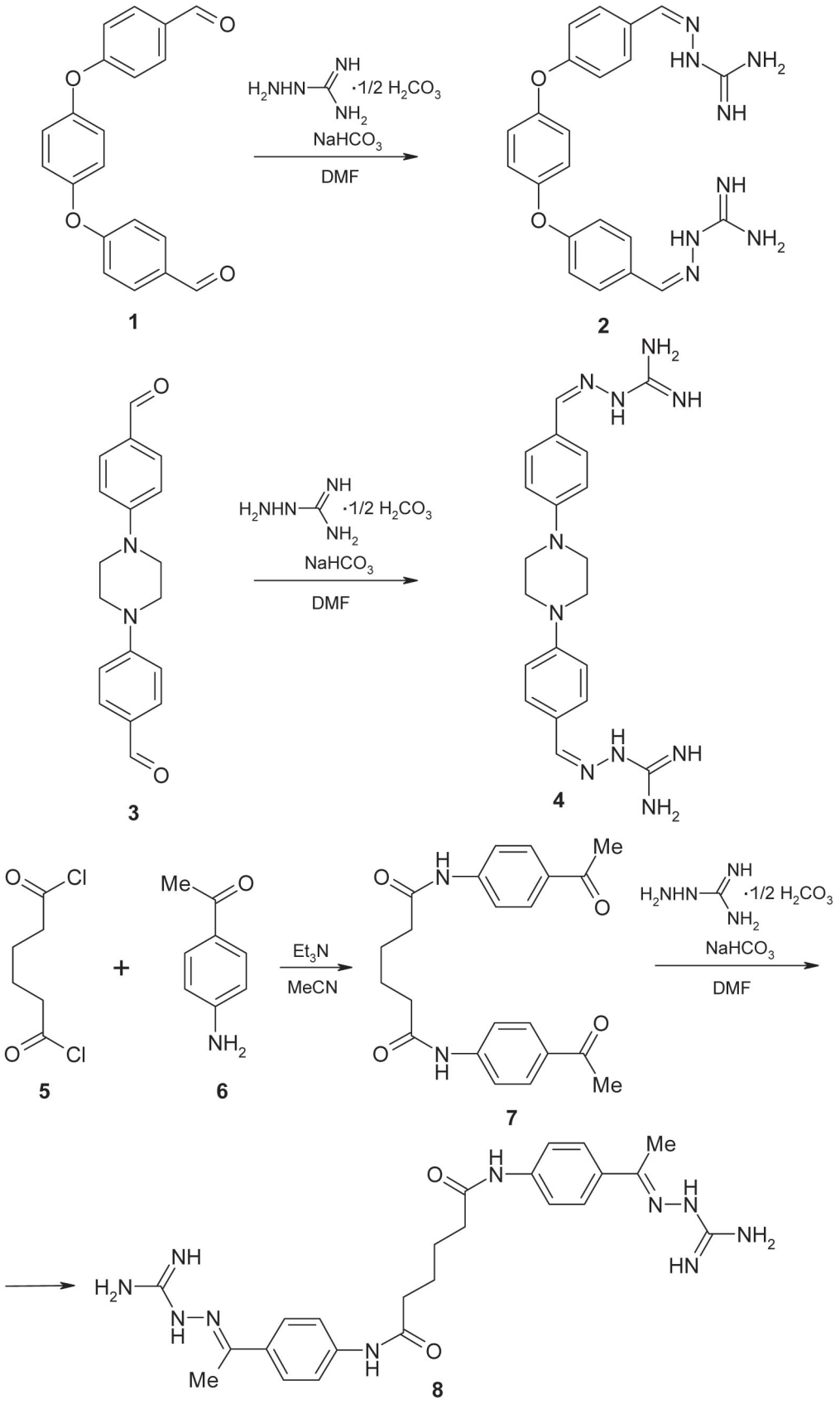


Fig. 1. Synthesis of the studied compounds

T_{mp} = 258-260 °C. NMR ^{1}H, δ, ppm: 1.65 s (4H, $2CH_2$), 2.35 s (4H, $2CH_2$), 2.47 s (6H, $2CH_3$), 5.53 br s (4H, 4NH), 5.89 br s (4H, 4NH), 7.52-7.92 m (8H, H arom.), 9.95 s (2H, 2NH). Elemental composition calculated, %: C 58.52; H 6.55; N 28.43; found, %: C 58.57; H 6.45; N 28.49. $C_{24}H_{32}N_{10}O_2$.

Determination of furin activity. 10 µl of the diluted furin solution containing 1 unit of enzymatic activity were incubated for 1 h at 37 °C with Boc-Arg-Val-Arg-Arg-AMC, (final concentration 75-250 µM) in a buffer pH 7.3 (100 mM Hepes, 1 mM $CaCl_2$, 0.5% Triton X-100 and 1 mM β-mercaptoethanol) with a total sample volume 150 µl. The reaction was terminated by adding 2 ml EDTA (initial concentration 5 mM) and relative fluorescence was detected with PTI Quanta Mastra 40 spectrofluorimeter (Canada) (excitation, 380 nm; emission, 460 nm; both band widths 2 nm). The readings were recorded over 60 seconds.

Michaels-Menten constant values were derived using Lineweaver-Burk plots from three independent experiments.

Determination of inhibitory effects of the studied compounds. To prepare a stock solution (concentration 10 mM), a sample of the corresponding compound was dissolved in DMSO. The stock solution was then diluted to the concentration required to inhibit furin. The enzyme solution (1 unit of activity), a buffer solution pH 7.3 and the studied inhibitor were incubated at room temperature for 30 min. Then a solution of fluorogenic substrate was added to reach the final concentration of 100 µM and enzymatic reaction was run for 1 h at 37 °C. The total volume of the mixture was 150 µl.

The reaction was terminated by adding 2 ml EDTA solution and the quantity of the released AMC was defined in respect to the buffer solution as previously described. Enzyme activity in the absence of the studied compounds was assumed to be 100%. Inhibition constants K_i were determined from Dixon plots. Inhibition coefficients (I_{50}) could not be determined, since the dependence of efficiency of furin inhibition (%) on concentration of the studied inhibitors was nonlinear.

Data analysis and plotting were carried out using Origin Professional 8.0 software (OriginLab). At least two measurements were used for each point. Experimental error did not exceed 10% of the measured value.

Modeling and docking of the studied compounds were carried out with the recently published 3D-structure of the catalytic domain of human furin [25]. Molecular structures of bisamidinohydrazones were prepared in MOE v.2013.08 program (Chemical Computing Group, Montreal, Canada) and then placed into furin binding pocket into place of the co-crystallized ligand – m-guanidinomethylI-Phac-RVR-Amba [25]. Reference 3D structure of furin 4OMC was taken from a PDB database. The position of water molecules was preserved as in the original cryslal complex. Induced fit docking was performed as implemented in MOE v.2013.08 program taking into account a partial mobility of side chain atoms of the furin active site. For each compound docking was limited to 1000 variations of the inhibitor positioning (so-called poses). The best 30 poses of the inhibitor based on the calculated score of its binding energy were recorded and stored for further analysis.

Results and Discussion

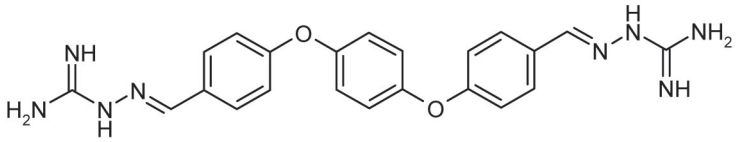
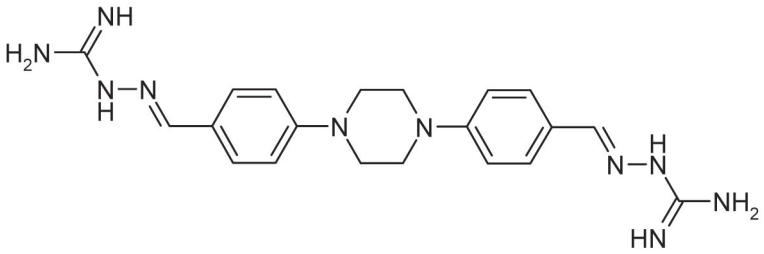
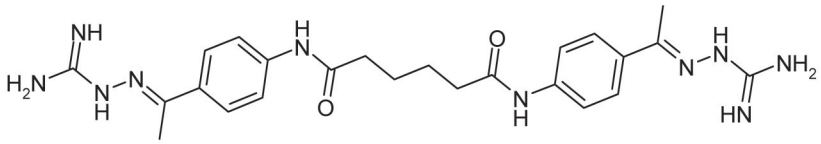
Sielaff et al. [14] first reported that aromatic compounds with weakly basic amidinohydrazone groups were able to inhibit furin. The most potent compounds were reported to have K_i values in the range of 0.46-0.59 µM.

In our previous work we synthesized symmetrical diamidinoderivatives and bisamidinohydrazones, which have linkers of varied lengths and hydrophobicity between benzene rings containing positively charged groups. K_i value of the most potent inhibitor in this series was approximately 0.50 µM. In the current work we studied how the linker structure affects inhibitory activity of bisamidinohydrazones. We have synthesized three new analogues of molecule **I**, varying in nature of the linking bridge.

Determined inhibitory constants of the studied compounds are presented in the Table. Interaction of bisamidinohydrazones with furin causes a decline of enzymatic activity in a concentration dependent manner. As an example, the process of protease inactivation by the inhibitor **2** is shown in Fig. 2. The inhibition constant values were determined using Dixon method (Fig. 3), and Lineweaver-Burk plots were used to determine the mechanism of action of the studied compounds as shown for **2** in Fig. 4. The data suggest that compound **2** inhibits furin through a mixed inhibition mechanism with K_i 0.74 µM. Since the structures of the synthesized inhibitors are similar, we assumed that compounds **4** and **8** inhibit furin activity through the same mechanism.

For better understanding of the specifics in interaction between furin and each of the type **I** bisamidinohydrazones, we conducted docking studies

Kinetic parameters of inhibitory effects of bisamidinohydrasones on furin at 37 °C and pH 7.3

Compound number in Fig. 1	Structural formula of the inhibitor	Inhibitory efficiency K_i, μM
2		0.74 ± 0.08
4		1.54 ± 0.48
8		1.21

with the active site of a recently published 3D-structure of human furin [25] co-crystallized with the first non-covalent inhibitor – guanidinomethyl-Phac-RVR-Amba. Bisamidinohydrazones **I**, which we synthesized in our lab, also form non-covalent complexes with the enzyme.

The results of the docking showed that bisamidinohydrazones **2**, **4**, **8** have the length of the linkers that allow them to interact simultaneously with furin subsites S1 and S4 (and possibly S5). Moreover, their overall arrangement in the binding pocket is similar to the position of the ligand co-crystalized with furin [25]. Due to the absence of the radicals in subsites P2 and P3 of type **I** compounds, they were not able to interact with the S2 or S3 pockets of furin. The results of docking (Fig. 5) showed that linker of compound **8** almost reaches subsite S2 of furin. We posit that introducing a substituent with a positive charge into this position will allow the new compound bind to the subsite S2 more efficiently and will increase its affinity to enzyme. It is evident that linkers of bisamidinohydrazones form a sort of "arch" over the binding site and by attaching a shorter linker at metha- (or ortho-) position to amidinohydrazone groups it may be possible to achieve more efficient interaction between the inhibitor and furin.

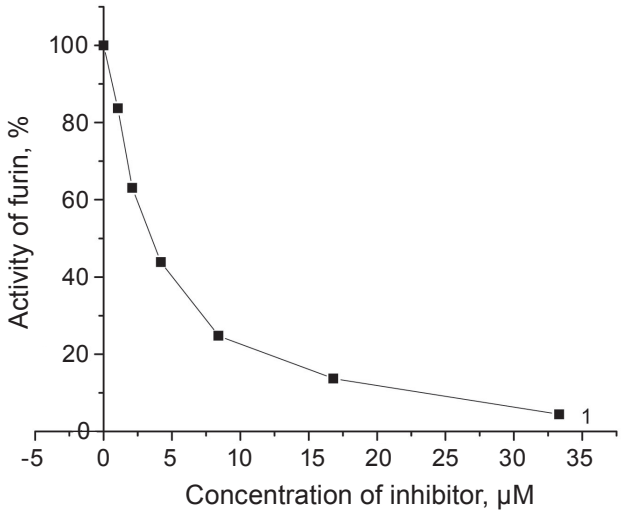


*Fig. 2. Decreasing activity of furin with increasing concentration of bisamidinohydrazone **2***

The crystallographic studies of furin [25, 26] revealed that formation of the enzyme-ligand complex is caused by predominantly electrostatic interactions, which dominate in subsites S1, S2 and S4, although a hydrogen bond network and other interactions are also important for a strong binding of the ligand. Our docking results suggest that inhibi-

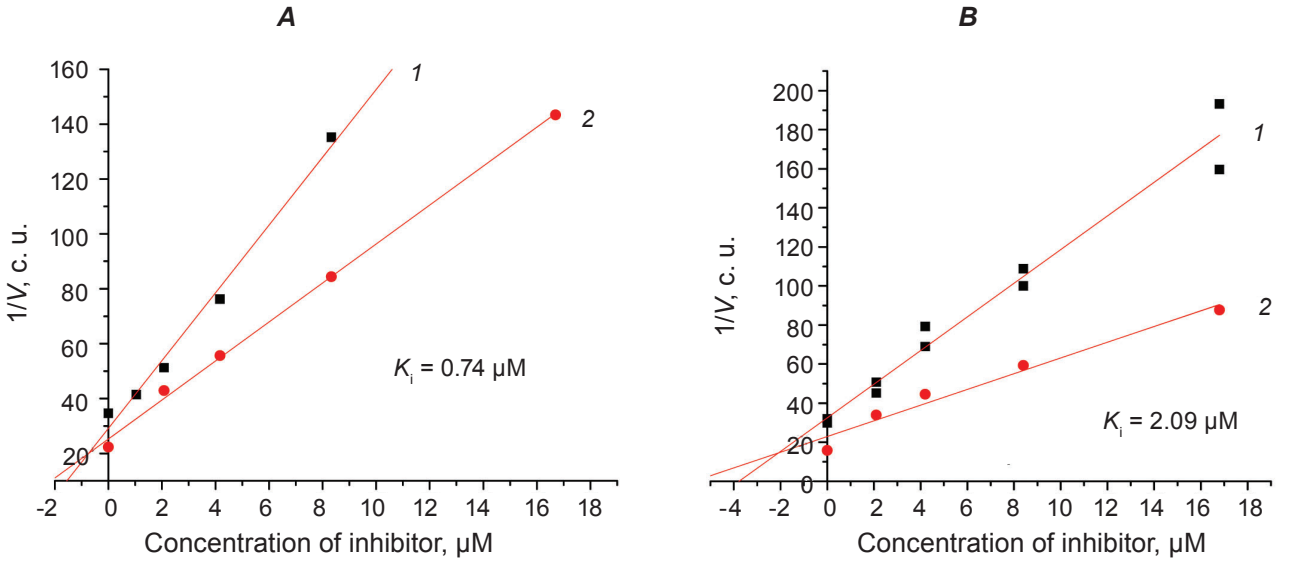


*Fig. 3. Determination (by Dixon plots) of the mixed inhibition constants of furin by compounds **2** (A) and **4** (B) at concentrations of fluorogenic substrate: 1 – 100 μM, 2 – 200 μM*

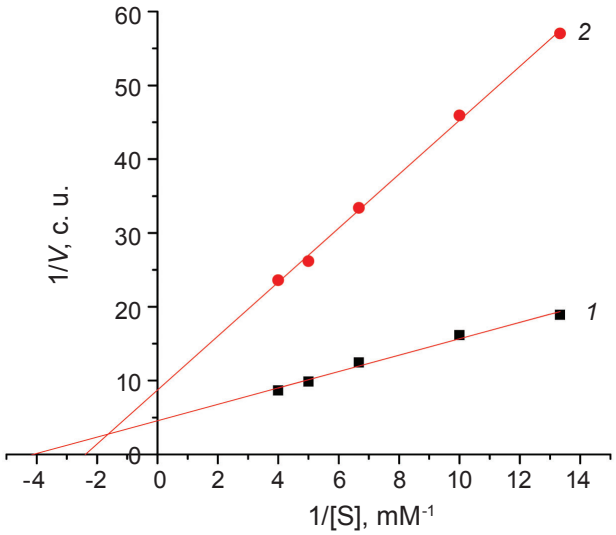


*Fig. 4. Lineweaver-Burk plot for mixed inhibition of furin at pH 7.3 by bisamidinohydrazone **2**: 1 – without the inhibitor, 2 – at concentration of inhibitor 4.2 μM*

tors **2**, **4**, **8** are "affixed" to the active site of furin due to Coulomb interactions of amidinohydrazone groups with residue Asp306 (in the subsite S1) and Asp264, Glu236 (in the subsite S4). Besides, a hydrogen bond network forms between amidinohydrazone groups and carbonyl groups of the residues Glu236 and Asp264 (in the subsite S4) of furin. Further research is required for more specific conclusions.

In summary, the design and synthesis of three novel bisamidinohydrazone analogues with positively charged groups connected with linkers of varied nature and hydrophobicity were performed. Biological evaluation of these compounds, being inhibitors of furin, was conducted and it was found that the most potent compound inhibit furin protease activity through the mixed inhibition mechanism (K_i 0.74 μM). The docking of bisamidinohydrazones showed that they were able to interact simultaneously with furin subsites S1 and S4, and their arrangement in the active site corresponds to the position of the co-crystallized ligand containing motif -RVR-Amba (PDB 4OMC).

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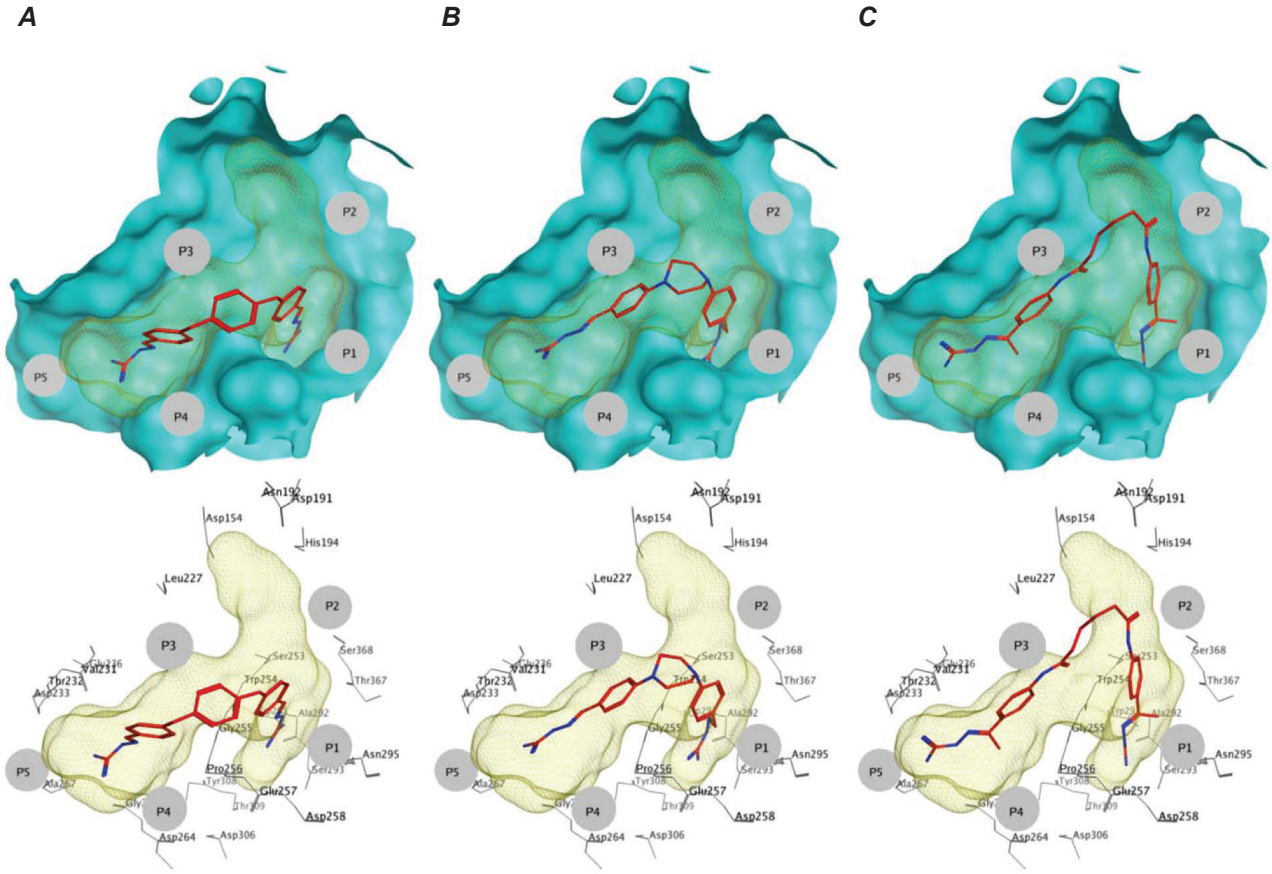

Fig. 5. Best docking conformations of compounds ***2*** *(A),* ***4*** *(B),* ***8*** *(C) in the binding pocket of human furin are labeled red. For comparison, the position of the ligand (from the crystallized PDB 4OMC structure) is shown as a yellow outline of its van der Waals surface. P1-P5 subsite locations are labeled as grey circles with the corresponding numbers. Binding pocket of furin is depicted as a blue surface. Bottom row shows amino acid residues of furin, which interact with inhibitors*

СИНТЕЗ, БІОЛОГІЧНЕ ТЕСТУВАННЯ ТА ДОКІНГ НОВИХ БІСАМІДИНОГІДРАЗОНІВ ЯК ІНГІБІТОРІВ ФУРИНУ

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Взаємодією гідрокарбонату аміногуанідину із трьома діарильними дикарбальдегідами одержано нові бісамідиногідразони – непептидні інгібітори фурину, що характеризуються величинами констант інгібування (K_i) в межах 0,74–1,54 мкМ. Їхні бензольні кільця зв'язуються між собою залишками *n*-гідрохінону, піперазину та, відповідно, адипінової кислоти. Докінг цих сполук у нещодавно опубліковану 3D-структуру фурину людини показав, що вони здатні одночасно взаємодіяти з підцентрами S1 та S4 ензиму. Загальний хід бісамідиногідразонів в активному центрі протеази є аналогічним до розташування хребта ліганду, який було кокристалізовано з фурином. Результати молекулярного моделювання дозволили намітити низку модифікацій структури синтезованих інгібіторів, які можуть поліпшити їхню антифуринову активність.

Ключові слова: фурин, непептидні інгібітори, бісамідиногідразони, біологічне тестування, докінг.

СИНТЕЗ, БИОЛОГИЧЕСКОЕ ТЕСТИРОВАНИЕ И ДОКИНГ НОВЫХ БИСАМИДИНОГИДРАЗОНОВ В КАЧЕСТВЕ ИНГИБИТОРОВ ФУРИНА

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Взаимодействием бикарбоната аминогуанидина с тремя диарильными дикарбальдегидами получены новые непептидные ингибиторы фурина, характеризующиеся величинами констант ингибирования (K_i) в пределах 0,74–1,54 мкМ. Их бензольные кольца связаны между собой остатками *n*-гидрохинона, пиперазина и, соответственно, адипиновой кислоты. Докинг этих соединений в недавно опубликованную 3D-структуру фурина человека показал, что они способны одновременно взаимодействовать с подцентрами S1 и S4 энзима. Общая «укладка» бисамидиногидразонов в активном центре протеазы аналогична ходу хребта лиганда, закристаллизованного с фурином. Результаты молекулярного моделирования позволили наметить ряд модификаций структуры синтезированных ингибиторов, которые улучшат их антифуриновую активность.

Ключевые слова: фурин, непептидные ингибиторы, бисамидиногидразоны, биологическое тестирование, докинг.

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