EXPERIMENTAL WORKS

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SYNTHESIS AND INVESTIGATION OF THE DERIVATIVES OF AMIDINOHYDRAZONELATED AROMATIC COMPOUNDS AS FURIN INHIBITORS

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The proprotein convertase furin plays a crucial role in a variety of pathogenic processes such as cancer, bacterial and viral diseases, neurodegenerative disorders and diabetes. Thus, furin inhibitors are promising therapeutics for the treatment of many diseases. In this study we synthesized some new non-peptide of furin inhibitors, with positively charged amidinohydrazone groups present in ortho-, meta- or para-positions in the benzene rings relative to the linker. From the results of biological testing it followed that the position of amidinohydrazone groups in aromatic rings was significant for the manifestation of antifurin activity. The replacement of linkers containing a propoxy group by a "bridge" with a benzene ring was found to cause an increase in the inhibitory effect of the compounds. The effect of synthesized bisamidinohydrazones on furin also depended on the substitution of the hydrogen atom in the amidinohydrazone group by the methyl group. These compounds were shown to block the enzyme activity mainly by the mechanism of mixed inhibition, and their K_i values were at the micromolar level.

K e y w o r d s: furin, amidinohydrazones, furin inhibitors, inhibition mechanism.

It is well known that many intracellular proteins in eukaryotes are initially synthesized in the form of large inactive precursors (proproteins). They are then transformed into smaller mature biologically active products: hormones, neuropeptides, enzymes and proteins as a result of controlled cleavage of the corresponding polypeptides by specialized enzymes known as proprotein convertases (PCs) [1-5]. The most characterized member of mammalian PCs is calcium-dependent serine endoprotease furin, which recognizes in its substrates a motif enriched with the residues of basic amino acids: -(Lys/Arg)-(Xxx)n-(Lys/Arg)-↓, where n = 0, 2, 4 or 6 and Xxx is any proteinogenic amino acid other than Cys [1, 3, 5]. Furin cleaves the peptide bond (indicated by the arrow) typically after a pair of basic amino acids Arg-Lys or Arg-Arg [1, 2]. This enzyme is important for embryogenesis and homeostasis; it also participates in many pathological processes, such as cancer and metastasizing, neurodegenerative pathologies, viral and bacterial infections [3-6]. In this regard, furin is considered as a promising and important target for developing of appropriate inhibitors, which could find clinical and therapeutic applications [3-7]. In works published in recent years, furin inhibitors of the protein, peptide, pseudopeptide and non-peptide nature have been discussed [7-15]. It was concluded that new research is needed to create non-peptidic low molecular weight inhibitors that, in comparison to the endogenous and

recombinant proteins would have advantages such as increased stability, enhanced bioavailability and easier production by synthetic methods [7, 8].

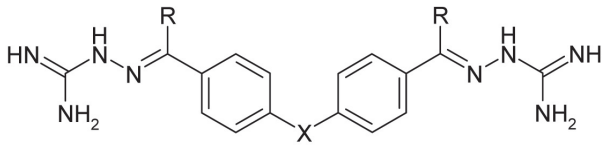
To develop the strategy for creating non-peptide inhibitors of furin, we drew attention to the article by Sielaff F. et al. [16] devoted to the synthesis and investigation of the properties of compounds containing an amidinohydrazone group. Encourages by this work, we synthesized previously non-peptidic furin inhibitors with a general formula **A** (Fig. 1) containing two aryl rings joined by a linker X, differing in their chemical nature, length and hydrophobicity [17, 18]. Synthesis of similar bisamidinohydrazones as extremely active compounds with bactericidal activity were described in the US patent [19]. In work by Borges M. N. et al. [20] some analogs were synthesized and tested as anti-*Trypanosoma cruzi* candidate.

The aim of this work was the synthesis of new bisamidinohydrazones containing positively charged groups in the *ortho*-, *meta*- or *para*-positions with respect to the linker, and testing the resulting compounds as novel furin inhibitors.

Materials and Methods

Analytical grade chemicals and solvents were purchased from commercial suppliers and used without further purification. When necessary, solvents were dried by standard techniques and distilled. Melting points (uncorrected.) were determined using Fisher Scientific apparatus IR spectra were recorded on a Bruker Vertex 70 FT/IR spectrometer. ^{1}H NMR spectra were recorded in dimethyl sulfoxide (DMSO)-d_6 using 400 MHz Varian Mercury instrument with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ scale) were expressed in parts per million (ppm) and given as s (singlet), bs (broad singlet), d (doublet), t (triplet), m (multiplet) and q (quintet). Relative fluorescence was measured using spectrofluorometer PTI Quanta Master 40 (Canada) with excitation at 380 nm and emission at 460 nm.

Chemical reagents and preparations. A fluorogenic substrate t-butyl-oxycarbonyl-L-arginyl-L-valyl-L-arginyl-L-arginine-4-methylcoumaryl-7-amide (Boc-Arg-Val-Arg-Arg-AMC) was purchased from Bachem (Switzerland). The recombinant truncated human furin (2000 units/ml) was obtained from New England BioLabs (USA). The commercial stock enzyme solution was diluted 20- to 80-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 reactions. One unit of furin activity was defined as the quantity of an enzyme that under standard conditions cleaved off 1 picamol of 7-amino-4-methylcoumarin (AMC) in 1 min.



A

R = H, CH_3

Fig. 1. The general structure of bisamidinohydrazones

Bis(2-carboxyaldehydophenoxy)propane (2a). A mixture of 2-hydroxybenzaldehyde (3.7 g, 30 mmol), 1,3-dibromopropane (1.5 ml, 15 mmol) and anhydrous potassium carbonate (8.3 g, 60 mmol) was refluxed in 30 ml anhydrous acetone for 18 h, and then the solvent was removed in a vacuum of a water jet pump. The residue was washed with distilled water (50 ml x 1) and extracted with chloroform (50 ml x 3). The combined organic layer was dried over Na_2SO_4 and, after filtration, the solvent was removed in vacuum and the residue was crystallized from 95% ethanol. Similarly, other bisaldehydes (**2b**, **c**) and bisketones (**2d-f**) were obtained.

4-[4 (4-Formylphenoxy)phenoxy]benzaldehyde (5b). 4.15 g (30 mmol) of anhydrous K_2CO_3 was added to a solution of 11.0 g (10 mmol) of hydroquinone and 2.48 g (20 mmol) of 4-fluorobenzaldehyde in 20 ml of dimethylacetamide (DMAA). The mixture was heated for 12 h at a temperature 90 °C. DMAA was poured off from insoluble K_2CO_3, and after cooling the product was precipitated with distilled water, filtered and crystallized from the mixture of isopropyl alcohol-water.

A general procedure for synthesis of bisamidinohydrazones. A mixture of 2 mmol of the corresponding dialdehyde (**2a-c**, **5a-b**) or diketone (**2d-f**) and aminoguanidine hydrochloride (0.46 g, 4.1 mmol) was refluxed for 4 h in 20 ml of 95% ethanol containing several drops of concentrated HCl. After cooling the mixture to room temperature, the solvent was removed in vacuo and the residue was triturated with chloroform. The solidified mass was filtered off and purified by crystallization from a suitable solvent.

Assay of furin activity. An aliquot of the furin solution containing 1 unit of enzyme activity was incubated with Boc-Arg-Val-Arg-Arg-AMC (final concentration 75-250 μM) in pH 7.3 buffer (100 mM Hepes, 1 mM $CaCl_2$, 0.5% Triton X-100 and 1 mM β-mercaptoethanol) for 1 hour at 37 °C in a total volume of 150 μl. The reaction was stopped by the addition of 2.0 ml of EDTA (initial concentration 5 mM). The relative fluorescence was measured on a PTI Quanta Master 40 spectrofluorometer (Canada) at an excitation wavelength of 380 nm and an emission of 460 nm. The values of the Michaelis constant were determined from the Lineweaver-Burk plots of three independent experiments.

Determination of the inhibitory effects of bisamidinohydrazones. To prepare a stock solution (concentration 10 mM), a sample of the corresponding compound was dissolved in DMSO. Then the stock solution was diluted to the required concentration. The enzyme solution (1 unit of activity), a pH 7.3 buffer and the studied inhibitor (concentration 5-10 μM) were incubated at room temperature for 30 min. Then a solution of fluorogenic substrate was added to reach its final concentration of 100 μM and the 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 of the EDTA solution and the quantity of the released AMC was assayed against the buffer solution as described above. Enzyme activity in the absence of the studied compounds was assumed to be 100%. Inhibition constants K_i were determined from Dixon or Lineweaver-Burk plots. Data analysis and plotting were carried out using Origin Professional 9.0 software (OriginLab). At least two measurements were used for each point. The experimental error did not exceed 10% of the measured value.

Results and Discussion

Chemistry. Synthesis of the investigated bisamidinohydrazones was carried out according to Scheme 1 [17-20]. The dialdehydes (**2a-c**) were easily formed by boiling hydroxybenzaldehydes (**1a-c**) with 1,3-dibromopropane in dry acetone in the presence of K_2CO_3 with yields of about 60%. The reaction of hydroxyacetophenones (**1d-f**) under similar conditions gave bisacetophenones (**2d-f**) with practically the same yields (Table 1). The reaction of 4-fluorobenzaldehyde with hydroquinone in dimethylacetamide for 12 h resulted in dialdehydes (**5a-b**) with a yield of about 70%. Bisamidinohydrazones (**3a-f** and **6a-b**) were obtained by the reaction of bisaldehydes (**2a-c**, **5a-b**) or bisketones (**2d-f**) with aminoguanidine hydrochloride in 95% ethanol. Their yields were, unfortunately, lower (Table 2). The purity of the synthesized compounds was monitored by IR and NMR spectroscopy.

1-({[2-(3-{2-[(carbamimidamidoimino)methyl]phenoxy}propoxy)phenyl]methylidene}amino) guanidine dihydrochloride (3a)

white solid; yeild 43%, m.p. 248-249 °C (dec.); IR (KBr) cm^{-1} ν_{max} 3158, 1672, 1627, 1452, 1247; ^{1}H-NMR (DMSO-d_6, 400 MHz), (δ, ppm): 11.93 (bs, 10H), 7.92 (s, 2H), 7.57 (d, 2H), 7.29 (d, 4H), 6.93 (m, 2H) 4.15 (t, 4H), 2.13 (q, 2H)

1-({[3-(3-{3-[(carbamimidamidoimino)methyl]phenoxy}propoxy)phenyl]methylidene}amino) guanidine dihydrochloride (3b)

white solid; yeild 45%, m.p. 230-231 °C (dec.); IR (KBr) cm^{-1} ν_{max} 3394, 1658, 1602, 1247, 1176; ^{1}H-NMR (DMSO-d_6, 400 MHz), (δ, ppm): 11.91 (bs, 2H), 7.99 (s, 2H), 7.79 (d, 2H), 7.61 (bs, 8H), 7.25 (m, 4H), 6.89 (d, 2H), 4.05 (t, 4H), 2.10 (q, 2H)

1-({[4-(3-{4-[(carbamimidamidoimino)methyl]phenoxy}propoxy)phenyl]methylidene}amino) guanidine dihydrochloride (3c)

white solid; yeild 40%, m.p. 298-300 °C (dec.), Lit.[20] m.p. = 300-310 °C (dec.); IR (KBr) cm^{-1}; ν_{max} 3150, 1670, 1641, 1513, 1254, 1180; ^{1}H-NMR (DMSO-d_6, 400 MHz), (δ, ppm): 11.96 (bs, 2H), 8.08 (s, 2H), 7.82 (d, 4H), 7.55 (bs, 8H), 7.15 (d, 4H), 4.22 (t, 4H), 2.20 (q, 2H)

1-({1-[2-(3-{2-[1-(carbamimidamidoimino) ethyl]phenoxy}propoxy)phenyl]ethylidene}amino) guanidine dihydrochloride (3d)

white solid; yeild 36%, m.p. 267-268 °C (dec.); IR (KBr) cm^{-1} ν_{max} 3154, 1673, 1625, 1490, 1237, 1124; ^{1}H-NMR (DMSO-d_6, 400 MHz), (δ, ppm): 11.09 (bs, 10H), 7.58 (d, 2H), 7.27 (m, 4H), 7.07 (t, 2H), 4.16 (t, 4H), 2.16 (q, 2H); 2.10 (s, 6H)

1-({1-[3-(3-{3-[1-(carbamimidamidoimino) ethyl]phenoxy}propoxy)phenyl]ethylidene}amino) guanidine dihydrochloride (3e)

white solid; yeild 47%, m.p. 278-280 °C (dec.); IR (KBr) cm^{-1} ν_{max} 3161, 1687, 1640, 1585, 1482, 1436, 1287, 1139; ^{1}H-NMR (DMSO-d_6, 400 MHz), (δ, ppm): 10.95 (bs, 2H), 9.71 (d, 8H), 7.90 (t, 4H), 7.27 (t, 2H), 7.22 (t, 4H), 6.89 (d, 2H), 4,06 (t , 4H); 2.15 (s, 6H); 2.09 (q, 2H)

1-({1-[4-(3-{4-[1-(carbamimidamidoimino) ethyl]phenoxy}propoxy)phenyl]ethylidene}amino) guanidine dihydrochloride (3f)

white solid; yeild 37%, m.p. 281-282 °C (dec.); IR (KBr) cm^{-1} 3152, 1672, 1642, 1593, 1467, 1322, 1256, 1180.

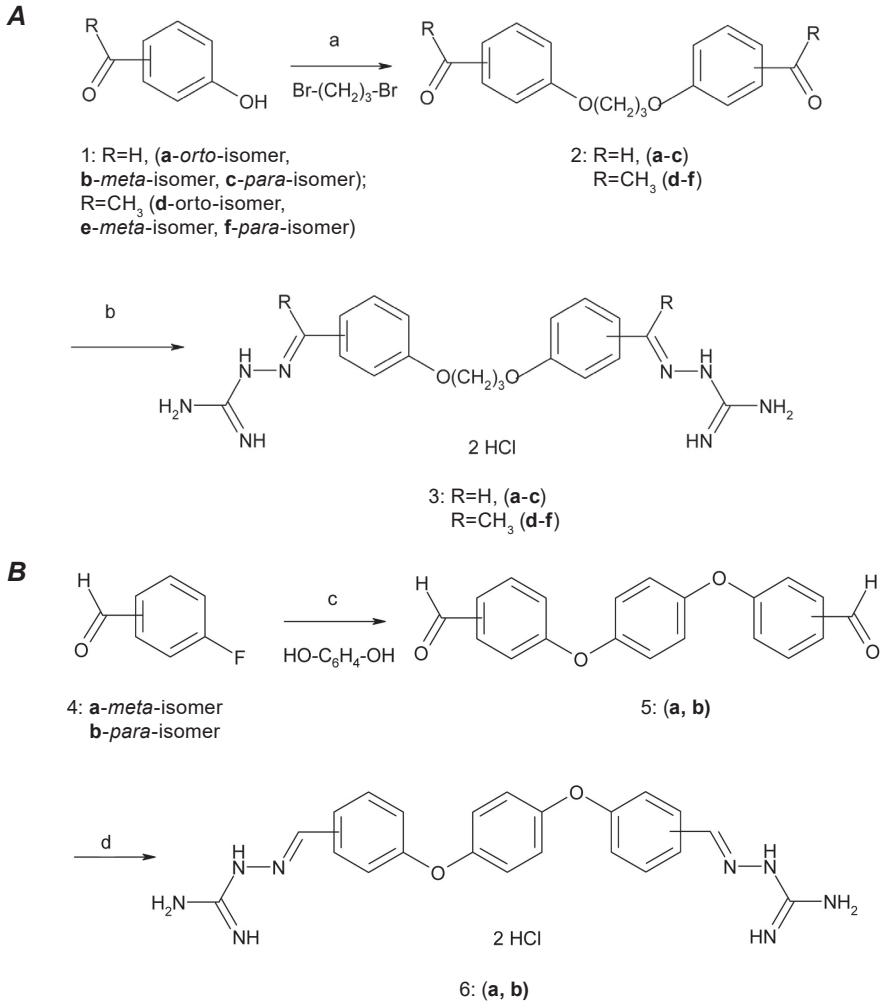
1-({[3-(4-{3-[(carbamimidamidoimino)methyl]phenoxy}phenoxy)phenyl]methylidene}amino) guanidine dihydrochloride (6a)

Reaction of 3-fluorobenzaldehyde and hydroquinone in DMAA at 90 °C gave bisaldehyde **5a** (yield 55%, m.p. 153 °C), which was condensed with aminoguanidine hydrochloride as described in the Materials and Methods without further purification. This gave bisamidinohydrazone **6a** as white solid; yeild 51%, m.p. 305 °C (dec.). According to preliminary data, this compound inhibits furin with K_i = 1.07 ± 0.23 μM.

1-({[4-(4-{4-[(carbamimidamidoimino)methyl]phenoxy}phenoxy)phenyl]methylidene}amino) guanidine dihydrochloride (6b)

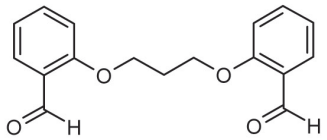
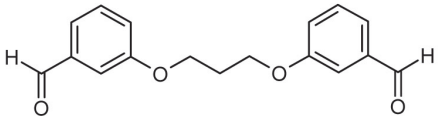
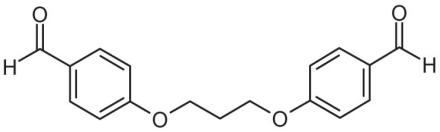
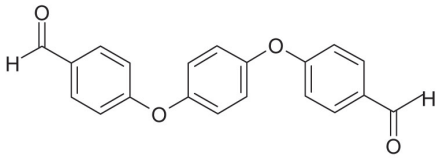
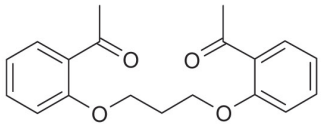
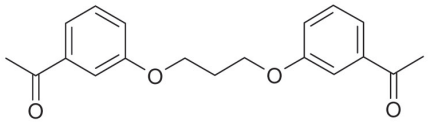
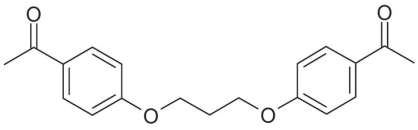
white solid; yeild 43%, m.p. 252-253 °C (dec.) This compound was synthesized in our work [18].

Biology. Inhibitory analysis. Table 2 presents data on the antifurin activity of the synthesized bisamidinohydrazones. Analysis of these data indicated that the replacement of the linkers containing the propoxy group (compounds **3b**, K_i = 1.70 μM and **3c**, K_i = 2.69 μM), on the "bridges" with the phenyl ring (compounds **6a**, K_i = 1.07 μM and **6b**, K_i = 0.74 ± 0.08 μM), led to an increase in the inhibitory effect of the compounds. This conclusion coincides with the results of studies previously pub-



*Scheme 1. Synthesis of bisamidinohydrazones with 1,3-trimethylene linker (**A**) or hydroquinone linker (**B**). Reagents and conditions: **a**) 1,3-dibromopropane, anhydrous K_2CO_3, $(CH_3)_2CO$, reflux, 18 h; **b**,**d**) aminoguanidine hydrochloride, EtOH, several drops HCl, reflux, 4 h; **c**) hydroquinone, anhydrous K_2CO_3, DMAA, heating at 90 °C, 12 h*

Table 1. Structure and physicochemical properties of synthesized bisaldehydes and bisketones

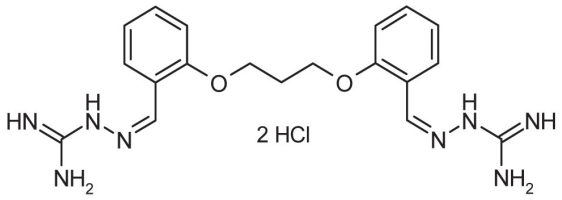
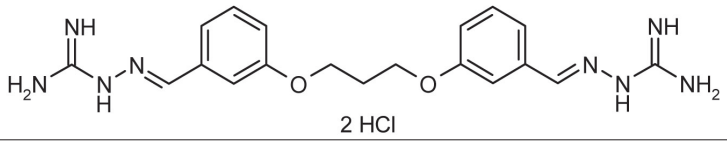
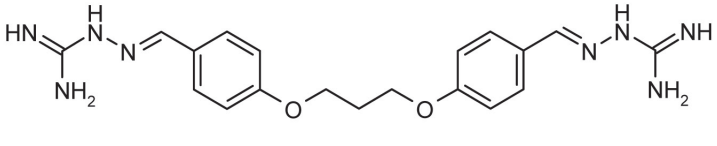
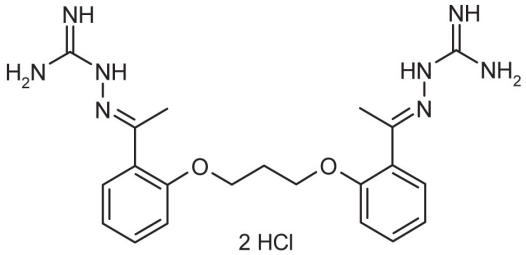
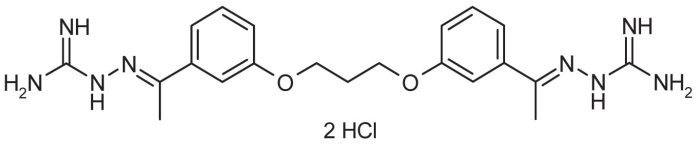
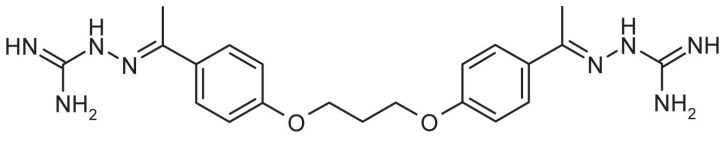
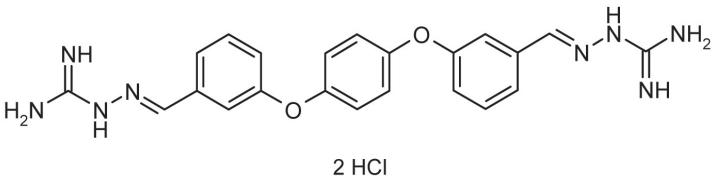
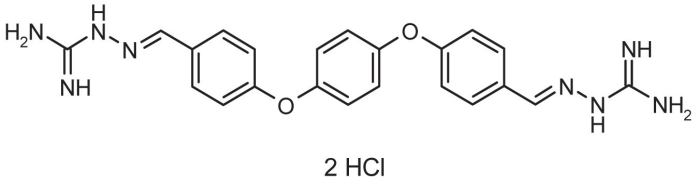
No Compound	Structure	Yield, %	M.p., °C	References
Bisaldehydes				
2a		55	98-99	[21]
2b		57	57-58	[21, 22]
2c		58	129-130	[20, 23]
5b		66	157-158	[24]
Bisketones				
2d		57	103-104	[21]
2e		62	91-92	[25]
2f		56	126-127	[20, 26]

lished by our group [17] and by other investigators of guanidilated aromatic compounds [27].

Among the derivatives (**3a-c**) with the amidinohydrazone group unsubstituted on the Me-group, the most active inhibitor was compound **3b** (K_i = 1.70 μM), which contains a positively charged group in the *meta*-position relative to the linker. The affinity to furin of *ortho*- and *para*-substituted bisamidinohydrazones **3a** and **3c** were reduced by approximately 1.6-fold in comparison with the *meta*-isomer **3b**. In a series of Me-substituted derivatives (**3d-f**), the best inhibitor of the enzyme was the *para*-isomer **3f** (K_i = 1.43 μM).

From the biological activity testing it was evident that the position of amidinohydrazone groups in the aromatic rings was essential for the antifurin activity. The effect of synthesized bisamidinohydrazones on furin depended also on the chemical nature and hydrophobicity of the linker and on the substitution of the hydrogen atom for methyl in the amidino-

Table 2. Structure and inhibition effects of synthesized bisamidinohydrazones

Compound	Structure	K_i, μM
3a		2.86 ± 0.76
3b		1.70 ± 0.51
3c		2.69 ± 0.61
3d		2.14
3e		3.29 ± 1.34
3f[a]		1.43 ± 0.46
6a[a]		1.07 ± 0.23
6b[b]		0.74 ± 0.08

[a]The preliminary data are presented; [b]the data are taken from our work [18] and are given for comparison

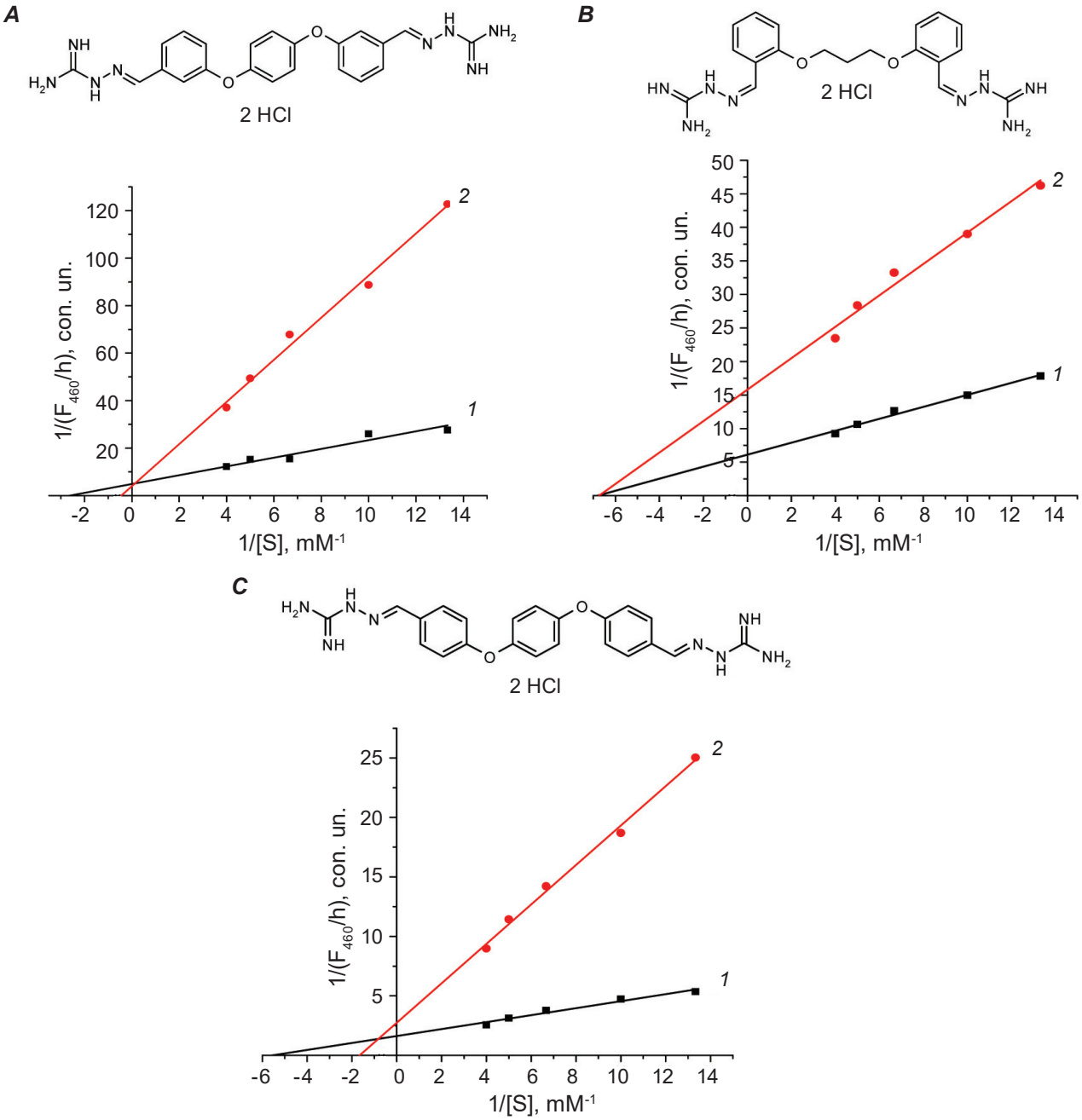


Fig. 2. Competitive (A), non-competitive (B) and mixed (C) mechanisms of furin inhibition by different bisamidinohydrazones

hydrazone group. These conclusions are supported by Sielaff F. et al. [16] and our previous publications [17, 18].

According to our data, most of the synthesized bisamidinohydrazones inactivated furin by the mechanism of mixed inhibition. Exceptions were compounds **3a** and **6a**: the first was a non-competitive inhibitor, and the second was a competitive inactivator of furin (Fig. 2). Until recently, only single examples of low-molecular non-competitive inhibitors of furin were known. Now it became clear that among bisamidinohydrazones there are many compounds that are mixed inhibitors of furin. Additional studies are needed to investigate the reasons for this phenomenon.

A series of novel non-peptide inhibitors of furin was designed and synthesized and their antifurin activities were evaluated. From the results of the in-

hibitory screening it was evident that the position of the amidinohydrazone groups in the phenyl rings was essential for the antifurin activity.

The inhibitory effect of synthesized bisamidinohydrazones on the enzyme depended also on the chemical nature and hydrophobicity of the linker and on the substitution of the hydrogen atom for methyl in the amidinohydrazone group.

It was shown that compound **3a** is a non-competitive inhibitor of furin, bisamidinohydrazone **6a** inactivates furin by the mechanism of competitive inhibition, and the remaining compounds decrease the activity of the enzyme in a mixed inhibition type.

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СИНТЕЗ І ДОСЛІДЖЕННЯ ВЛАСТИВОСТІ ПОХІДНИХ АМІДИНОГІДРАЗИЛЬОВАНИХ АРОМАТИЧНИХ СПОЛУК ЯК ІНГІБІТОРІВ ФУРИНУ

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Пропротеїнконвертаза фурин відіграє ключову роль у багатьох патологічних процесах, таких, наприклад, як рак, бактеріальні та вірусні захворювання, нейродегенеративні порушення та діабет. Тому інгібітори фурину можуть бути багатообіцяючими терапевтичними засобами для лікування цих захворювань. Нами було синтезовано низку нових непептидних інгібіторів ензиму, які містили амідиногідразовані групи, що знаходилися в бензольних кільцях в *орто-*, *мета-* або *пара*-положеннях відносно лінкера. Із результатів біологічного тестування випливає, що положення амідиногідразонових груп в ароматичних кільцях істотно впливало на антифуринову активність сполук. Знайдено, що заміна лінкерів, які містили пропокси-групу, на «місток» із бензольним кільцем, обумовлювала ріст інгібіторної активності цих сполук. Їх вплив на фурин залежав також від заміщення атома водню в амідиногідразоновому угрупованні на метильну групу. Показано, що ці сполуки блокують активність ензиму за механізмом змішаного інгібування, а значення K_i синтезованих бісамідиногідразонів знаходяться на мікромолярному рівні.

Ключові слова: фурин, амідиногідразони, інгібітори фурину, механізм інгібування.

СИНТЕЗ И ИССЛЕДОВАНИЕ СВОЙСТВ ПРОИЗВОДНЫХ АМИДИНОГИДРАЗОНИЛИРОВАННЫХ АРОМАТИЧЕСКИХ СОЕДИНЕНИЙ В КАЧЕСТВЕ ИНГИБИТОРОВ ФУРИНА

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Пропротеинконвертаза фурин играет ключевую роль в развитии многих патологических процессов, таких, например, как рак, бактериальные и вирусные инфекции, нейродегенеративные нарушения и диабет. В связи с этим ингибиторы фурина могут оказаться многообещающими терапевтическими средствами для лечения этих заболеваний. Нами синтезировано ряд новых непептидных ингибиторов фурина, содержащих амидиногидразоновые группы, находящиеся в бензольных кольцах в *орто-*, *мета-* или *пара*-положениях относительно линкера. Из данных биологического тестирования следует, что положение амидиногидразоновых групп в ароматических кольцах существенно влияло на антифуриновую активность. Найдено, что замена линкеров, содержащих пропокси-группу, на «мостик» с бензольным кольцом повышала ингибиторную активность соединений. Их влияние на фурин зависило также от замещения атома водорода в амидиногидразоновой группировке на метильную группу. Показано, что эти соединения блокируют активность энзи-

ма по механизму смешанного ингибирования, а значения K_i исследованных бисамидиногидразонов находятся на микромолярном уровне.

Ключевые слова: фурин, амидиногидразоны, ингибиторы фурина, механизм ингибирования.

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