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DESIGN AND SEARCH FOR PROSPECTIVE DIURETICS (CA II INHIBITORS) AMONG AROYLHYDRAZONES OF ESTERS QUINONE OXIME USING *IN SILICO* AND *IN VIVO* METHODOLOGY

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Ключові слова: *дизайн, молекулярний докінг, інгібітори карбоангідрази, синтез, ароїлгідразони естерів хіноноксиму, діуретична активність, аналіз SAR*

Abstract. Design and search for prospective diuretics (CA II Inhibitors) among aroylhydrazones of esters quinone oxime using *in silico* and *in vivo* methodology. Sokolova K.V., Stavytskyi V.V., Konovalova S.O., Podpletnya O.A., Kovalenko S.I., Avdeenko A.P. *The design and search for new selective inhibitors of CA II with a better pharmacological profile, which would cause minimal electrolyte disturbances in the body, remains an urgent problem of medical chemistry and pharmacology today. It is important that the discovered new classes of inhibitors do not always contain the main "pharmacophoric" function (sulfamide), which is characteristic of "classic" drugs (Acetazolamide, Methazolamide, Ethoxzolamide, Dorzolamide and others), but are derivatives of phenols, polyamines, coumarins/thiocoumarins, ureas, thioureas, hydroxamates, etc. These molecules also bind in the active site of the enzyme, but do not interact directly with the catalytic zinc ion or interact through zinc-coordinated water molecules/hydroxide ion. However, this leads to an increase in their selectivity and, as a result, pharmacological action. Continuing the search for compounds that affect urination, we were interested in aroylhydrazones of esters of quinone oxime. Firstly, they are characterized by certain structural features (dynamic and geometric isomerism); secondly, they exhibit redox properties; thirdly, the presence of aromatic fragments makes it possible to create a voluminous combinatorial library for analysis. These compounds are ligands in complexation reactions, and an additional increase in the number of hydrogen acceptors in the molecule due to structural modification will improve ligand-enzymatic interactions with carbonic anhydrase (CAII) and, as a result, reveal new promising diuretics. The aim – design and search for potential diuretics (CA II inhibitors) among aroylhydrazones of esters of quinone oxime using* in silico, *traditional synthesis and* in vivo *methodologies. Methods of organic synthesis, physico-chemical methods of analysis of organic compounds (NMR ^{1}H-spectroscopy, elemental analysis). Prediction of affinity to the biological target, prediction of toxicity and lipophilicity of the combinatorial library of benzohydrazides O-aroyl esters of quinone oxime using computer services. The study of compounds affecting the excretory function of rat kidneys was carried out according to the generally accepted method of E.B.Berkhin with water load. The investigation of the probable mechanism was carried out using flexible molecular docking, as an approach to search for molecules that have affinity for human carbonic anhydrase type II (CA II). Macromolecular data of the crystal structure of CA II (PDB ID – 3HS4) were downloaded from the Protein Data Bank*

(PDB). The design was developed and the search for diuretic agents among benzohydrazides of O-aroyl esters of quinone oximes was developed using in silico methods (prediction of affinity, lipophilicity, toxicity and enzyme-ligand interactions), traditional organic synthesis, and in vivo methods (effect on excretory function of rat kidneys). The synthesis of benzohydrazides of O-aroyl esters of quinone oxime was carried out by the interaction of aroylhydrazines with 4-[(aroylimino)]cyclohexa-2,5-dien-1-ones. The structure of the synthesized compounds was confirmed by elemental analysis and ^{1}H NMR spectra. Studies of the effect of synthesized compounds on the excretory function of rat kidneys allowed us to identify a number of promising compounds among aroylhydrazones of quinonexime esters, which increase daily diuresis by 54.2-352.8% compared to the control group. At the same time, it was established that the most active was N'-(4-[(2-chlorobenzoyloxy)imino]cyclohexa-2,5-dien-1-ylidene)-3-nitrobenzohydrazide, which increased daily diuresis by 352.8% in comparison with the control group, while exceeding the effect of "Hydrochlorothiazide" (170.8%). The developed and implemented strategy for the search for diuretics among benzohydrazides of O-aroylesters of quinone oxime allowed the identification of an effective compound, which in terms of diuretic effect exceeds the comparison drug "Hydrochlorothiazide". Visualization of the molecular docking of the active compounds showed that their geometry makes it difficult to place them in the pocket of the active site of CA II, but the pronounced diuretic effect can also be associated with their ability to form coordination bonds with the zinc cation. The obtained results justify the further targeted search for potential diuretics among this class of compounds for a more detailed understanding and study of the mechanism of action.

Реферат. Дизайн та пошук перспективних діуретиків (інгібіторів СА ІІ) серед ароїлгідразонів естерів хіноноксиму з використанням *in silico* та *in vivo* методології. Соколова К.В., Ставицький В.В., Коновалова С.О., Подплетня О.А., Коваленко С.І., Авдєєнко А.П. *Дизайн та пошук нових вибіркових інгібіторів СА ІІ із кращим фармакологічним профілем, які б викликали мінімальні електролітичні порушення в організмі, і на сьогодні залишається актуальною проблемою медичної хімії та фармакології. Важливо, що виявлені нові класи інгібіторів не завжди виконують основну «фармакофорну» функцію (сульфамідну), яка характерна для «класичних» лікарських препаратів (Acetazolamide, Methazolamide, Ethoxzolamide, Dorzolamide та інші), а є похідними фенолів, поліамінів, кумаринів/тіокумаринів, сечовин, тіосечовин, гідроксаматів тощо. Ці молекули також зв'язуються в активному центрі ферменту, але не взаємодіють безпосередньо з каталітичним іоном цинку або взаємодіють через цинк-координовану молекулу води/іону гідроксиду. Проте це приводить до підвищення їх селективності і, як наслідок, фармакологічної дії. Продовжуючи дослідження з пошуку сполук, що впливають на сечовиділення, нас зацікавили ароїлгідразони естерів хіноноксиму. По-перше, для них характерні певні особливості будови (динамічна та геометрична ізомерія), по-друге, вони виявляють окисно-відновні властивості, по-третє, наявність ароматичних фрагментів надає можливість створити об'ємну комбінаторну бібліотеку для аналізу. Ці сполуки є лігандами в реакціях комплексування, а додаткове збільшення кількості акцепторів водню в молекулі за рахунок структурної модифікації дозволить покращити ліганд-ферментативні взаємодії з карбоангідразою (СА ІІ) і, як наслідок, виявити нові перспективні діуретичні засоби. Ціль – дизайн та пошук потенційних діуретиків (інгібіторів СА ІІ) серед ароїлгідразонів естерів хіноноксиму з використанням методології* in silico*, традиційного синтезу та in vivo. Використано методи органічного синтезу, фізико-хімічні методи аналізу органічних сполук (ЯМР ^{1}Н-спектроскопія, елементний аналіз), прогнозування спорідненості до біологічної мішені, прогноз токсичності та ліпофільності комбінаторної бібліотеки бензогідразидів О-ароїлестрів хіноноксимів з використанням комп'ютерних сервісів. Дослідження сполук, що впливають на видільну функцію нирок щурів, проводили за загальноприйнятою методикою Е.Б. Берхіна з водним навантаженням. Дослідження ймовірного механізму проводилося за допомогою гнучкого молекулярного докінгу, як підходу пошуку молекул, що мають спорідненість до карбоангідрази людини ІІ типу (СА ІІ). Макромолекулярні дані кристалічної структури СА ІІ (PDB ID – 3HS4) завантажені з Protein Data Bank (PDB). Розроблено дизайн та обґрунтовано пошук діуретичних засобів серед бензогідразидів О-ароїлестерів хіноноксимів методами in silico (прогнозування спорідненості, ліпофільності, токсичності та фермент-лігандних взаємодій), традиційного органічного синтезу та методами* in vivo *(вплив на видільну функцію нирок щурів). Синтез ароїлгідразонів естерів хіноноксиму проведений шляхом взаємодії ароїлгідразинів з 4-[(ароїліміно)]циклогекса-2,5-дієн-1-онами. Структура синтезованих сполук підтверджена елементним аналізом та ^{1}Н ЯМР-спектрами. Проведені дослідження впливу синтезованих сполук на видільну функцію нирок щурів дозволили виявити ряд перспективних сполук серед ароїлгідразонів естерів хіноноксиму, які посилюють добовий діурез на 54,2-352,8% порівняно з контрольною групою. При цьому встановлено, що найбільш активним виявився* N'*-(4-[(2-хлоробензоїлокси)іміно]циклогекса-2,5-дієн-1-іліден)-3-нітробензогідразид, який підвищував добовий діурез на 352,8% порівняно з контрольною групою, перевищуючи при цьому ефект «Гідрохлоротіазиду» (170,8%). Розроблена та впроваджена стратегія пошуку діуретиків серед бензогідразидів* О*-ароїлестерів хіноноксимів дозволила ідентифікувати ефективну сполуку, яка за сечогінною дією перевищує препарат порівняння «Гідрохлоротіазид». Візуалізація молекулярного стикування активних сполук показала, що їх геометрія ускладнює розміщення в кишені активного центру СА ІІ, але виражений сечогінний ефект також можна пов'язати з їх здатністю до утворення координаційних зв'язків з катіоном цинку. Отримані результати обґрунтовують подальший цілеспрямований пошук потенційних діуретиків серед цього класу сполук для більш детального розуміння та вивчення механізму дії.*

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Modern diuretics are actively used in medical practice for hypertension, chronic heart failure, kidney disease, glaucoma, emergency conditions (edema of the brain, lungs, etc.) and demonstrate a satisfactory risk/effect ratio [1]. An important place among this class of drugs belongs to carbonic anhydrase II inhibitors (CAs), which catalyze the reversible hydration of carbon dioxide to bicarbonate and H+ ions and play a crucial role in the regulation of many physiological processes [2]. The clinical effects of these agents are an increase in diuresis and a decrease in intraocular and intracranial pressure; decrease in the excitability of brain neurons; increased excretion of potassium with urine. A decrease in the activity or non-selective inhibition of CA II leads to a decrease in the productivity of the carbonic acid synthesis reaction, and therefore to a decrease in the bicarbonate reserve of the blood and the development of hyperchloremic acidosis [1]. Along with this, losses of K+ increase and hydrogen ions H+ are retained in the body, which leads to the development of hypokalemia and acidosis.

Representatives of CA II inhibitors are sulfonamides, sulfinamides, sulfamates, and sulfondiamides, which bind in deprotonated form (anion) to the Zn (II) ion in the active center of the enzyme [2, 3] (Fig. 1A). It was established that the specified functional groups in organic molecules enhance the inhibition of enzymes of this class in the cells of nephron tubules. Therefore, in most cases, structural modification of molecules was carried out by introducing other functional groups to aromatic and heterocyclic fragments while preserving the main "pharmacophoric" function of the molecule [3, 4, 5]. These studies were aimed at increasing affinity to CA II, increasing hydrophilicity, reducing toxicity, and improving pharmacokinetic characteristics.

Despite the significant progress made in recent decades in the elucidation of molecular mechanisms, medicinal chemists and pharmacologists are still engaged in the design and directed search for new selective CA II inhibitors with a better pharmacological profile that would cause minimal electrolyte disturbances in the body. This has led to the creation of new classes of inhibitors that do not always contain the main "pharmacophoric" function (sulfonamides, sulfinamides, sulfamates, and sulfondiamides) and are derivatives of phenols, polyamines, coumarins/thiocoumarins, ureas, thioureas, hydroxamates, etc. [2, 4]. These molecules also bind in the active site of the enzyme, but do not interact (interact) directly with the catalytic zinc ion or interact through zinc-coordinated water molecules/hydroxide ion (Fig. 1B). The latter explains why inhibitors that have this interaction in the active site of the enzyme show a much better degree of selectivity or selectively inhibit only one or a limited number of isoforms among the known [2, 4].

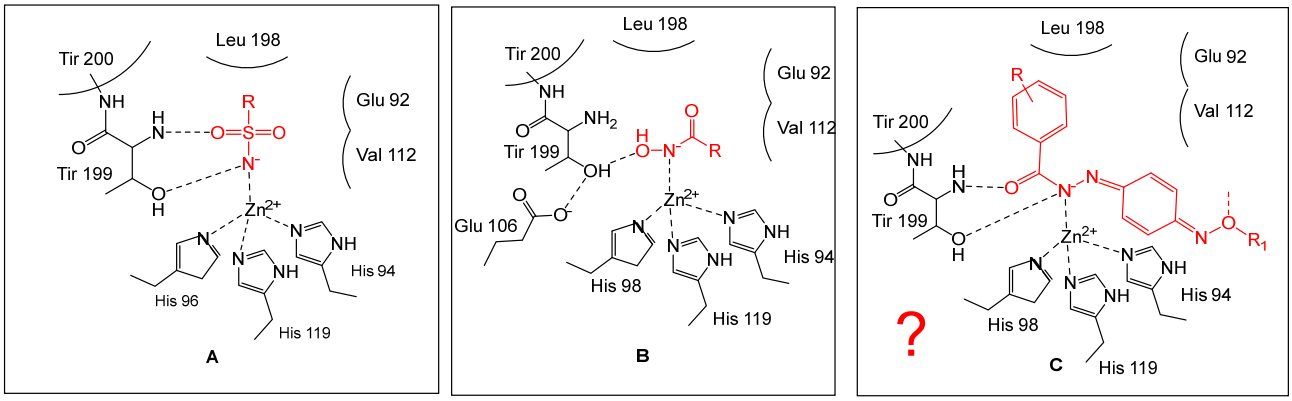

Fig. 1. Schematic images of the interaction of sulfonamides (A), hydroxamates (B) according to data [3] and the probable interaction of unknown aroylhydrazones of esters quinone oxim e with amino acids of the active center of CA II

Therefore, continuing research in the search for compounds that affect urination, we became interested in aroylhydrazines and their derivatives, which have been intensively studied due to their high reactivity and chelating ability, as well as versatile biological activity [6, 7, 8, 9, 10, 11, 12]. Moreover, their high reactivity will allow the combination of aroylhydrazines and substituted 1,4-benzoquinone oximes in one molecule, which are also characterized by redox properties and versatile biological activity [13, 14, 15, 16]. This design is interesting because it solves a number of tasks. First, to develop the synthesis of an unknown class of diuretics with an unlimited possibility of structural modification (increasing the number of hydrogen acceptors in the molecule), which is important for ligand-enzymatic interactions with CA II. The most important thing is to answer the question: "Will the created molecules

act as ligands in complexation reactions like "classical" inhibitors (Fig. 1C) and exhibit a diuretic effect?"

Therefore, *the aim of the work* is the design and search for potential diuretics (CA II inhibitors) among aroylhydrazones of esters quinone oxime using *in silico*, traditional synthesis and *in vivo* methodologies.

MATERIALS AND METHODS OF RESEARCH

Molecular docking. Research was conducted by flexible molecular docking, as an approach to finding the molecules with affinity to a specific biological target. Macromolecular data were downloaded from the Protein Data Bank (PDB) namely, the crystal structures of Human carbonic anhydrase II (PDB ID – 3HS4) [17].

Ligand preparation. The substances were drawn using MarvinSketch 20.20.0 and saved in mol format [18]. After that they were optimized by the program Chem3D, using molecular mechanical MM2 algorithm, and saved as pdb-files. Molecular mechanics was used to produce more realistic geometry values for most organic molecules, owing to the fact of being highly parameterized. Using AutoDockTools-1.5.6, the pdb-files were converted into PDBQT, number of active torsions was set as default [19].

Protein preparation. PDB files were downloaded from the protein data bank. Discovery Studio v 19.1.0.18287 was used to delete water molecules and ligands. Structures of protein were saved as pdb-files [20]. In AutoDockTools – 1.5.6 polar hydrogens were added and saved as PDBQT. The grid box was set as following: center_x=161.722, center_y=163.028, center_z=189.222, size_x=30, size_y=30, size_z=30 for Human carbonic anhydrase II (PDB ID – 3HS4). Vina was used to carry docking. For visualization Discovery Studio v 19.1.0.18287 was used.

Toxicity prognosis. Prediction of acute toxicity and calculation of octanol/water partition coefficient (logP) was made *in silico* using the service ProTox-II [21].

Chemical part. Melting points were determined in open capillary tubes in a "Mettler Toledo MP 50" apparatus and were not corrected. The elemental analyses (C, H, N) were performed using the ELEMENTAR vario EL cube analyzer (USA). Analyses were indicated by the symbols of the elements or functions within ±0.3% of the theoretical values. ^{1}H NMR spectra (400 MHz) were recorded on a Varian-Mercury 400 (Varian Inc., Palo Alto, CA, USA) spectrometers with TMS as internal standard in DMSO-d_6 solution.

Aroylhydrazines (**1**) та 4-[(aroyloxy)imino]cyclohexa-2,5-dien-1-one (**2**) and other starting materials and solvents were obtained from commercially available sources and used without additional purification.

N'-[4-(Hydroxyimino)cyclohexa-2,5-dien-1-ylidene]benzoylhydrazide (3.1). 0,05 mol 4-(hydroxyimino)cyclohexa-2,5-dien-1-one **1** was dissolved in 150 ml of methanol. The 600 ml of water and an equimolar amount of the corresponding 0.05 mol of benzoylhydrazine hydrochloride **2** were added. The reaction mixture was stirred during 1,5 h. The separated precipitate was filtered off, dried, and recrystallized from ethanol. Yield: 72%; m.p.194-195ºC (dec.). NMR ^{1}H (δ, ppm): 8.01-8.04 (dd, 1H, H^2, *J*=3,9 Hz), 7.41-7.44 (dd, 1H, H^6, *J*=3,9 Hz), 7.11-7.15 (dd, 1H, H^5, *J*=3,9 Hz), 6.73-6.76 (dd, 1H, H^3, *J*=3,9 Hz), 7.69-7.99 (m, 5H, Ph), 9.89 (s, 1H, NH), 10.76 (s, 1H, OH). 7.15 (d, 2H, H^{2,6}, *J*=9,0 Hz), 7.22 (d, 2H, H^{3,5}, *J*=9,0 Hz), 7.43-7.99 (m, 5H, Ph), 9.89 (s, 1H, NH), 10.76 (s, 1H, OH); Anal. Calcd. for: $C_{13}H_{11}N_3O_2$: C, 64.72; H, 4.60; N, 17.42. Found: C, 64.66; H, 4.52; N, 17.44.

The general methods for the synthesis of substituted *N'-(4-[(aroyloxy)imino]cyclohexa-2,5-dien-1-ylidene) benzohydrazides(4.1-4.9).* **4**-[(Aroyloxy)imino]cyclohexa-2,5-dien-1-one 4 (0.01 mol), was dissolved in 20 mL of ethanol. 60 mL of water and equimolar quantity of corresponding aroylhydrazines **1** (0.01 mol), were added. The reaction mixture was stirred during 2 h. The separated precipitate was filtered off, dried, and recrystallized from ethanol.

N'-(4-[(Benzoyloxy)imino]cyclohexa-2,5-dien-1-ylidene)-2-chlorobenzohydrazide (4.1). Yield: 85%; m.p. 210ºC (dec.); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 7.31-7.34 (dd, 1H, H^5, *J*=3,9 Hz), 7.35-7.38 (dd, 1H, H^6, *J*=3,9 Hz), 7.36-7.96 (m, 4H, 2-ClC_6H_4), 7.55-8.16 (m, 5H, Ph), 7.75-7.78 (dd, 1H, H^3, *J*=3,9 Hz), 7.89-7.92 (dd, 1H, H^2, *J*=3,9 Hz), 10.44 (s, 1H, NH); Anal. Calcd. for: $C_{20}H_{14}ClN_3O_3$: C, 63.25; H, 3.72; N, 11.06; Found: C, 62.98; H, 3.59; N, 11.15.

N'-(4-[(Benzoyloxy)imino]cyclohexa-2,5-dien-1-ylidene)-3,5-dimethoxybenzohydrazide (4.2). Yield: 81%; m.p. 207ºC (dec.). ^{1}H NMR (δ, ppm): 3.76 (s, 6H, 3,5-(MeO)$_2$-C_6H_3), 6.51 (t, 1H, H$^{4'}$, 3,5-(MeO)$_2$-C_6H_3), 7.11 (t, 2H, H$^{2'6'}$, 3,5-(MeO)$_2$-C_6H_3), 7.31-7.34 (dd, 1H, H^5, *J*=3,9 Hz), 7.36-7.39 (dd, 1H, H^6, *J*=3,9 Hz), 7.54-8.15 (m, 5H, Ph), 7.74-7.77 (dd, 1H, H^3, *J*=3,9 Hz), 7.90-7.93 (dd, 1H, H^2, *J*=3,9 Hz), 10.35 (s, 1H, NH); Anal. Calcd. for: $C_{22}H_{19}N_3O_5$: C, 65.18; H, 4.73; N, 10.37. Found: C, 65.25; H, 4.80; N, 10.32.

N'-(4-[(Benzoyloxy)imino]cyclohexa-2,5-dien-1-ylidene)-2,4-dinitrobenzohydrazide (4.3). Yield: 78 %; m.p. 218ºC (dec.). ^{1}H NMR (δ, ppm): 7.31-7.34 (dd, 1H, H^5, *J*= 3,9 Hz), 7.36-7.39 (dd, 1H, H^6, *J*=3,9 Hz), 7.54-8.15 (m, 5H, Ph), 7.74-7.77 (dd, 1H, H^3, *J*= 3,9 Hz), 7.90-7.93 (dd, 1H, H^2, *J*=3,9 Hz), 7.93 (d, 1H, H$^{6'}$, 2,4-(NO$_2$)$_2$-C_6H_3, *J*=9,0 Hz), 8.65-8.68 (dd, 1H, H$^{5'}$, 2,4-(NO$_2$)$_2$-C_6H_3), 9.03 (d, 1H, H$^{3'}$, 2,4-

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$(NO_2)_2$-C_6H_3), 10.31 (s, 1H, NH); Anal. Calcd. for: $C_{20}H_{13}N_5O_7$: C, 55.18; H, 3.01; N, 16.09. Found: C, 55.27; H, 2.89; N, 15.95.

***2-Methyl-N'-(4-[(2-methylbenzoyloxy)imino]-cyclohexa-2,5-dien-1-ylidene)benzohydrazide* (*4.4*).** Yield: 87%; m.p. 205ºC (dec.). ^{1}H NMR (δ, ppm): 2.33 (s, 3H, Me), 2.35 (s, 3H, Me), 7.19-7.91 (m, 4H, 2-MeC_6H_4), 7.21-7.95 (m, 4H, 2-MeC_6H_4), 7.30-7.33 (dd, 1H, H^5, *J*=3,9 Hz), 7.35-7.38 (dd, 1H, H^6, *J*=3,9 Hz), 7.74-7.77 (dd, 1H, H^3, *J*=3,9 Hz), 7.89-7.92 (dd, 1H, H^2, *J*=3,9 Hz), 10.44 (s, 1H, NH); Anal. Calcd. for: $C_{22}H_{19}N_3O_3$: C, 70.76; H, 5.13; N, 11.25. Found: C, 70.84; H, 4.96; N, 11.15.

***N'-(4-[(2-Bromobenzoyloxy)imino]cyclohexa-2,5-dien-1-ylidene)-4-nitrobenzohydrazide* (*4.5*).** Yield: 72%; m.p. 226ºC (dec.). ^{1}H NMR (δ, ppm): 7.29-7.32 (dd, 1H, H^5, *J*=3,9 Hz), 7.33-7.36 (dd, 1H, H^6, *J*=3,9 Hz), 7.43-7.76 (m, 4H, 2-BrC_6H_4), 7.76-7.79 (dd, 1H, H^3, *J*=3,9 Hz), 7.89-7.92 (dd, 1H, H^2, *J*=3,9 Hz), 8.08 (d, 2H, $H^{3',5'}$, 4-$NO_2C_6H_4$), 8.35 (d, 2H, $H^{2',6'}$, 4-$NO_2C_6H_4$), 10.51 (s, 1H, NH); Anal. Calcd. for: $C_{20}H_{13}BrN_4O_5$: C, 51.19; H, 2.19; N, 11.94. Found: C, 51.26; H, 2.16; N, 11.78.

N'-(4-[(2-Chlorobenzoyloxy)imino]cyclohexa-2,5-dien-1-ylidene)-3-nitrobenzohydrazide (4.6). Yield: 77 %; m.p. 220ºC (dec.). ^{1}H NMR (δ, ppm): 7.30–7.33 (dd, 1H, H^5, *J*=3,9 Hz), 7.29-7.32 (dd, 1H, H^6, *J*=3,9 Hz), 7.60-8.16 (m, 4H, 2-ClC_6H_4), 7.76-7.79 (dd, 1H, H^3, *J*=3,9 Hz), 7.90-7.93 (dd, 1H, H^2, *J*=3,9 Hz), 7.87-8.86 (m, 4H, 3-$NO_2C_6H_4$), 10.35 (s, 1H, NH); Anal. Calcd. for: $C_{20}H_{13}ClN_4O_5$: C, 56.55; H, 3.09; N, 13.19. Found: C, 56.61; H, 3.11; N, 13.26.

4-Bromo-N'-(4-[(2-iodobenzoyloxy)imino]cyclohexa-2,5-dien-1-ylidene)benzohydrazide (4.7). Yield: 83%; m.p. 202ºC (dec.). ^{1}H NMR (δ, ppm): 7.30-7.33 (dd, 1H, H^5, *J*=3,9 Hz), 7.35-7.38 (dd, 1H, H^6, *J*=3,9 Hz), 7.43-7.97 (m, 4H, 2-IC_6H_4), 7.68 (d, 2H, $H^{3',5'}$, 4-BrC_6H_4), 7.74-7.77 (dd, 1H, H^3, *J*=3,9 Hz), 7.89-7.92 (dd, 1H, H^2, *J*=3,9 Hz), 8.35 (d, 2H, $H^{2',6'}$, 4-BrC_6H_4), 10.48 (s, 1H, NH); Anal. Calcd. for: $C_{20}H_{13}BrIN_3O_3$: C, 43.66; H, 2.38; N, 7.64. Found: C, 43.71; H, 2.33; N, 7.59.

N'-(4-{[(3-Methoxybenzoyl)oxy]imino}cyclohexa-2,5-dien-1-ylidene)-2,4-dinitrobenzohydrazide (4.8). Yield: 85%; m.p. 238ºC (dec.). ^{1}H NMR (δ, ppm): 3.85 (s, 3H, MeO), 7.30-7.33 (dd, 1H, H^5, *J*=3,9 Hz), 7.34-7.85 (m, 4H, 3-MeOC_6H_4), 7.36-7.39 (dd, 1H, H^6, *J*=3,9 Hz), 7.74-7.77 (dd, 1H, H^3, *J*=3,9 Hz), 7.91-7.94 (dd, 1H, H^2, *J*=3,9 Hz), 7.93 (d, 1H, $H^{6'}$, 2,4-$(NO_2)_2C_6H_3$), 8.64-8.67 (dd, 1H, $H^{5'}$, 2,4-$(NO_2)_2C_6H_3$), 9.02 (d, 1H, $H^{3'}$, 2,4-$(NO_2)_2$-C_6H_3), 10.45 (s, 1H, NH); Anal. Calcd. for: $C_{21}H_{15}N_5O_8$: C, 54.20; H, 3.25; N, 15.05. Found: C, 54.29; H, 3.14; N, 14.95.

4-Bromo-N'-(4-{[(4-methoxybenzoyl)oxy]imino}cyclohexa-2,5-dien-1-ylidene)benzohydrazide (4.9). Yield: 79%; m.p. 208ºC (dec.). ^{1}H NMR (δ, ppm): 3.82 (s, 3H, MeO), 7.08 (d, 2H, $H^{3',5'}$, 4-MeOC_6H_4), 7.29-7.32 (dd, 1H, H^5, *J*=3,9 Hz), 7.37-7.40 (dd, 1H, H^6, *J*=3,9 Hz), 7.69 (d, 2H, $H^{3',5'}$, 4-BrC_6H_4), 7.72-7.75 (dd, 1H, H^3, *J*=3,9 Hz), 7.89-7.92 (dd, 1H, H^2, *J*=3,9 Hz), 8.07 (d, 2H, $H^{2',6'}$, 4-MeOC_6H_4), 8.34 (d, 2H, $H^{2',6'}$, 4-BrC_6H_4), 10.52 (s, 1H, NH); Anal. Calcd. for: $C_{21}H_{16}BrN_3O_4$: C, 55.52; H, 3.47; N, 9.25. Found: C, 55.59; H, 3.55; N, 9.33.

Biological part. *Study of the effect of compounds on the excretory function of the kidneys*. The initial screening was performed on 72 white male Wistar rats weighing 120-170 g, which were kept in standard conditions of the vivarium of the Dnipro State Medical University. Experimental studies were performed in accordance with the "General Ethical Principles of Animal Experiments" (Ukraine, 2001), and the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and the conclusion of the commission on issues of biomedical ethics of DSMU (protocol No. 3 16.02.2022) [22]. Screening of the new synthesized compounds, in order to identify diuretic properties in a few aroylhydrazones of esters quinone oximes, was carried out according to the generally accepted method of E.B. Berkhin [23]. Prior to the experiment, the animals were kept without food for three hours. The diuretic effect of the compounds was studied under liquid load at the rate of 5 ml per 100 g of animal weight and without. The test compounds were administered to the rats once intragastrically at a doses of 2.6 mg/kg body weight as an aqueous suspension simultaneously with the water load. Animals were placed in individual cages for urine collection at three hours and 24 hours. «Hydrochlorothiazid» in equivalent doses for rats were selected as the reference drugs [24].

The obtained data were statistically processed using the software package Statistica 6.1 (StatSoft Inc., serial number AGAR909E415822FA). The arithmetic mean values (M) and their errors (± m) were calculated. The probability of intergroup differences was determined using Student's parametric t-test and one-way analysis of variance (ANOVA). The differences were considered statistically significant at a value of $p \leq 0.05$ [25].

RESULTS AND DISCUSSION

According to the design of this study, calculations of predicted affinity to the biological target, toxicity and lipophilicity were carried out for more than 50 compounds from a virtual combinatorial library of aroylhydrazones of esters quinone oxime. Creation of the library is based on the introduction of donor-acceptor groups (methyl-, methoxy-, halogeno-,

nitro-) in various positions of the phenyl fragments of the molecule (Table 1), without taking into account the peculiarities of the structure of aroylhydrazones of esters quinone oxime, namely the hydrazide-hylazone tautomerism and geometric isomerism.

The molecular docking of the combinatorial library of aroylhydrazones of esters quinone oxime confirmed our assumptions about their high affinity to SA II (Table 1). It is true that the affinity of most of the compounds exceeded the classic inhibitors – "Acetazolamide" and "Hydrochlorothiazide." It was not possible to deduce the correct dependence of the affinity of the ligand to the enzyme on the position of the substituent in the phenyl fragment, but a certain regularity was observed, namely, most ligands with acceptor substituents have a high affinity. The IV class of toxicity (LD50 324-3000 mg/kg) is predicted for these compounds. Their lipophilicity in comparison with the reference drugs is somewhat higher and this is known to contribute to their interaction in the hydrophobic part of the active center of the enzyme. So, the method of molecular docking made it possible to conduct a preliminary assessment of their affinity to CA II, the prospects of their further synthesis and research on experimental animal models.

Table 1

Results of molecular docking and predicted toxicometric parameters of the virtual combinatorial library of aroylhydrazones of esters quinone oxime according to ProTox-II data (Fig. 1C)

Compd.*	R	R_1	Affinity (kcal/mol) to CA II (3HS4)	LD_{50}, mg/kg (Toxicity Class)**	LogP
1.	H	H	-7.2	100 (III)	2.12
2.	4-Cl	PhC(O)	-7.6	510 (IV)	4.16
3.	2-Cl	PhC(O)	-7.7	510 (IV)	4.16
4.	3-Cl	PhC(O)	-7.2	510 (IV)	4.16
5.	2,4-$(MeO)_2$	PhC(O)	-7.2	324 (IV)	3.52
6.	3,5-$(MeO)_2$	PhC(O)	-7.4	324 (IV)	3.52
7.	2,4-$(NO_2)_2$	PhC(O)	-7.7	1994 (IV)	4.37
8.	2-Me	2-$MeC_6H_4C(O)$	-7.9	510 (IV)	4.12
9.	2-Cl	2-$MeC_6H_4C(O)$	-7.3	510 (IV)	4.46
10.	3-Cl	2-$MeC_6H_4C(O)$	-7.2	510 (IV)	4.46
11.	4-Cl	2-$MeC_6H_4C(O)$	-7.7	510 (IV)	4.46
12.	2-NO_2	2-$ClC_6H_4C(O)$	-7.3	1994 (IV)	4.59
13.	3-NO_2	2-$ClC_6H_4C(O)$	-8.2	1994 (IV)	4.59
14.	4-NO_2	2-$ClC_6H_4C(O)$	-8.1	1994 (IV)	4.59
15.	4-NO_2	2-$BrC_6H_4C(O)$	-8.2	3000 (V)	4.7
16.	4-Br	2-$IC_6H_4C(O)$	-7.7	510 (IV)	4.87
17.	2-NO_2	2-$IC_6H_4C(O)$	-7.7	1994 (IV)	4.54
18.	2,4-$(NO_2)_2$	2-$IC_6H_4C(O)$	-7.4	1994 (IV)	4.97
19.	2,4-$(NO_2)_2$	3-$MeOC_6H_4C(O)$	-7.7	3000 (V)	4.37
20.	2-Me	3-$MeOC_6H_4C(O)$	-7.6	324 (IV)	3.82
21.	2-Cl	3-$MeOC_6H_4C(O)$	-7.4	324 (IV)	4.16
22.	4-Br	4-$MeOC_6H_4C(O)$	-7.4	324 (IV)	4.27
23.	2,4-$(MeO)_2$	4-$MeOC_6H_4C(O)$	-7.2	324 (IV)	3.53
24.	3,5-$(MeO)_2$	4-$MeOC_6H_4C(O)$	-7.2	2000 (IV)	3.53
Acetazolamide			-6.2	4300 (V)	1.00
Hydrochlorothiazide			-7.0	1175 (IV)	2.98

Notes: * – table shows data for compounds whose affinity is >7.0 kcal/mol; ** – Class I: fatal if swallowed ($LD_{50} \leq 5$); Class II: fatal if swallowed ($5 < LD_{50} \leq 50$); Class III: toxic if swallowed ($50 < LD_{50} \leq 300$); Class IV: harmful if swallowed ($300 < LD_{50} \leq 2000$); Class V: may be harmful if swallowed ($2000 < LD_{50} \leq 5000$); Class VI: non-toxic ($LD_{50} > 5000$).

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The next stage of this research included the synthesis of a number of aroylhydrazones of quinone oxime esters (**4**), which have high affinity and satisfactory parameters of predicted toxicity. The synthesis of compounds **4** was preliminarily worked out on the reaction of the interaction of benzoylhydrazine (**1.1**) with quinone monooxime **2.1** (Fig. 2). The nucleophilic addition-elimination reaction proceeds at room temperature, regioselectively and with a satisfactory yield of the target compound (72%). O-aroylesters of 1,4-benzoquinone oxime (**2.2-2.8**) also interact with benzoylhydrazines (**1.2-1.8**) without any particularity, forming the target compounds **4.1-4.9** with a yield of 72-87%.

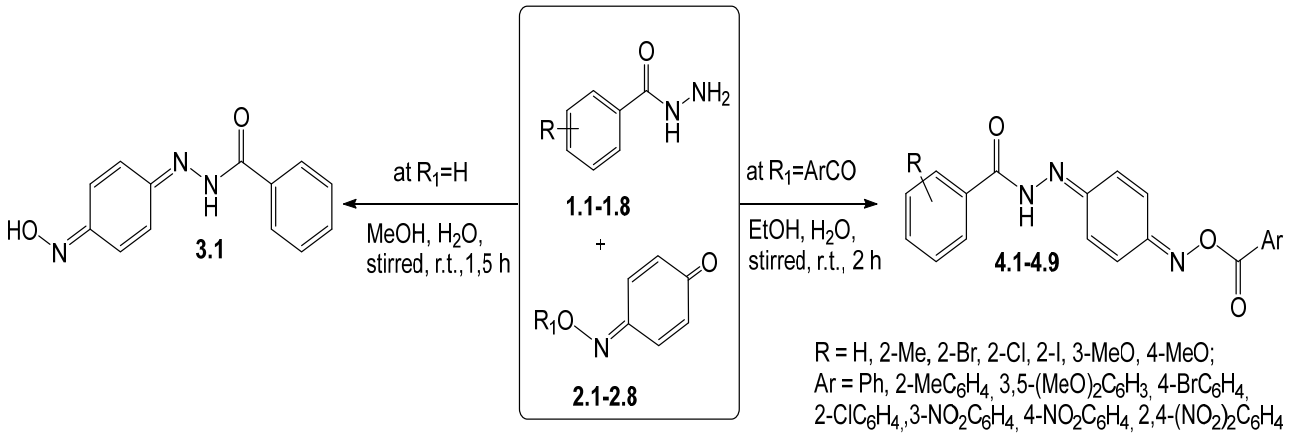

Fig. 2. Reaction of aroylhydrazines with 4-[(aroyloxy)imino]cyclohexa-2,5-dien-1-ones

The data of 1H NMR spectra indicate the benefit of their formation. Thus, N'-[4-(hydroxyimino)cyclohexa-2,5-dien-1-ylidene]benzoylhydrazide (**3.1**) in the 1H NMR spectrum has the characteristic signals of the protons of the quinoid fragment H2 and H6 and H3 and H5 resonate as doublet-doublets with a 3.9 Hz CCSW *(cis*-isomer) at 8.20, 7.43, 7.12 and 6.74 ppm, respectively [26]. The presence of signals of singlet protons -C(O)NH (9.89 ppm), =NOH groups (10.76 ppm) and signals of multiplet protons of the phenyl fragment at 7.69-7.99 ppm also supports the statement of the structure of compound **3.1**. In the 1H NMR spectra of compounds **4**, the signals of the protons of the quinoid fragment H2 and H6 and H3 and H5 have a similar multiplicity, but are observed in a weaker magnetic field (paramagnetic shift) due to the acceptor effect of the substituted benzoyl group. In addition, compounds **4** are characterized by singlet protons of the -C(O)NH group at 10.52-10.31 ppm and phenyl substituents with the corresponding multiplicity and chemical shift [26].

Screening of the synthesized compounds **3** and **4** on the process of urine excretion showed that the investigated compounds in the 2nd hour of the experiment in most cases suppress (up to 25%) or show a minor (12.9-30.7%) diuretic effect, in comparison with a control group. More significant are the results of the influence of compounds **4** on diurnal diuresis. Thus, compounds **4.1-4.4, 4.6, 4.7** increase daily diuresis by 54.2-352.8% (Table 2), which makes it possible to classify them as diuretics of medium duration of action, as well as the reference drug. At the same time, compound **4.6** was the most effective among the studied compounds, the daily diuresis of which in rats was 9.78±0.19 ml/100 g (352.8%), compared to the control (2.16±0.05 ml/100 g). The specified compound significantly exceeds the activity of the comparison drug "Hydrochlorothiazide", the diuresis of which was 5.85±0.21 ml/100 g (170.8%).

We failed to conduct a correct analysis of the "structure-activity" relationship. However, the structural modification of the benzoylhydrazone and aroyloxime fragments of compounds **4** by introducing donor (**4.4**) or acceptor (**4.1, 4.6, 4.7**) substituents has a positive effect on diuresis and significantly contributes to increasing activity. It is important that compounds with functional groups close to the phenyl substituents of the hydrazone and oxime fragments turned out to be more active.

The high diuretic activity of **4.1**, **4.4** and **4.6** and structural features (ability to complex) served as the basis for additional visualization of their interaction with the active center of CA II to assess the possible mechanism of their biological action (Table 3).

Table 2

The effect of the synthesized compounds and reference drugs on the process of urination in intact rats under water load with a single injection (M±m, n=6)*

No.	Compounds	Diuresis, ml/100 g/ 2 h	% related to control	Diuresis, ml/100 g/ 24 h	% related to control
1	Control	3.48±0.09	–	2.16±0.05	–
2	3.1	4.55±0.09	30.7	1.41±0.09*	-34.9
3	4.1	3.93±0.12	12.9	5.58±0.39*	158.3
4	4.2	2.61±0.17	-25.0	3.33±0.43	54.2
5	4.3	2.66±0.09	-23.6	3.38±0.17	56.5
6	4.4	3.36±0.14	-3.4	5.21±0.52*	141.2
7	4.5	4.13±0.25	-18.7	1.51±0.05	-30.1
8	4.6	2.85±0.09	18.1	9.78±0.19*	352.8
9	4.7	2.94±0.11	-15.5	4.62±0.38	113.9
10	4.8	2.64±0.08	-24.1	0.52±0.04*	-75.9
11	4.9	3.04±0.09	-12.6	0.70±0.05*	-67.6
12	Hydrochlorothiazide	5.39±0.07*	54.9	5.85±0.21*	170.8

Notes: # – significant changes in control ($p < 0,05$); n – the number of animals in the group.

At the same time, it was established that the standard ligand (Hydrochlorothiazide) with the active site CA II (Fig. 3A) forms two hydrogen bonds of the sulfamide group with amino acid residues $THR200^{A}$ (2,14Å), $GLN92^{A}$ (2,39Å), hydrophobic π- interactions of the aromatic fragment with amino acid residues $GLN92^{B}$ (4.17Å), $VAL121^{B}$ (4.96Å), π- interactions of Oxygen and Sulfur of the sulfamide group and Chlorine with $VAL143^{B}$ (4,24Å), $VAL143^{B}$ (5.07Å), $HIS96^{C}$ (5,37Å), $HIS94^{B}$ (5.48Å), $HIS119^{C}$ (5,07Å), $LEU198^{B}$ (4.67Å), $VAL121^{B}$ (3.84Å), $LEU198^{B}$ (4.67Å), $VAL121^{B}$ (4,96Å), $LEU198^{B}$ (4,67Å). In addition, in the active site there is a coordination bond of the sulfamide group of hydrochlorothiazide with Zn^{2+} CA II (ZN301 2.62Å).

Table 3

The main types of interactions of synthesized compounds and pharmacological standards with amino acid residues of human carbonic anhydrase II (PDB ID – 3HS4)

Compd.	The main interaction types between compounds, pharmacological standards and amino acid residues of enzymes*
3.1	$PRO202^{A}$, $ASN62^{A}$, $THR200^{A}$, $LEU198^{F}$, $THR200^{F}$, $HIS94^{F}$, $VAL121^{F}$, $ALA65^{F}$
4.1	$GLN92^{A}$, $HIS94^{A}$, $ZN301^{H}$, $ASN62^{A}$, $THR200^{A}$, $LEU198^{F}$, $HIS119^{F}$, $HIS94^{F}$, $VAL121^{F}$, $VAL143^{F}$, $LEU198^{F}$
4.2	$THR200^{A}$, $PRO201^{A}$, $HIS64^{H}$, $TRP5^{A}$, $PHE231^{F}$, $TRP5^{F}$, $TRP5^{F}$
4.3	$PRO201^{A}$, $LYS170^{H}$, $HIS64^{F}$, $PHE231^{F}$, $HIS64^{F}$, $LYS170^{F}$
4.4	$ASP72^{H}$, $GLN92^{A}$, $ILE91^{F}$, $LEU198^{F}$, $HIS94^{F}$, $ILE91^{F}$, $VAL121^{F}$, $LEU198^{F}$, $PHE131^{F}$, $VAL121^{F}$
4.5	$GLN92^{A}$, $ASN67^{A}$, $HIS94^{A}$, $ASN62^{A}$, $THR200^{A}$, $HIS94^{A}$, $LEU198^{F}$, $HIS94^{F}$, $HIS96^{F}$, $VAL121^{F}$, $VAL143^{F}$, $LEU198^{F}$
4.6	$GLU69^{A}$, $THR199^{A}$, $THR200^{F}$, $ZN301^{G}$, $GLU69^{H}$, $LEU198^{F}$, $PHE131^{F}$, $VAL121^{F}$
4.7	$GLU69^{H}$, $ASN67^{A}$, $GLN92^{A}$, $LEU198^{F}$, $HIS94^{F}$, $PHE131^{F}$, $VAL121^{F}$, $VAL143^{F}$, $HIS94^{F}$, $HIS119^{F}$, $TRP209^{F}$, $ILE91^{F}$, $VAL121^{F}$
4.8	$GLU239^{H}$, $PHE231^{A}$, $PRO201^{A}$, $GLU239^{A}$, $LYS170^{H}$, $HIS64^{F}$, $PHE231^{F}$, $HIS64^{F}$, $LYS170^{F}$
4.9	$GLU69^{A}$, $GLN92^{A}$, $ILE91^{F}$, $LEU198^{F}$, $HIS94^{F}$, $PHE131^{F}$, $HIS94^{F}$, $HIS119^{F}$, $TRP209^{F}$, $ILE91^{F}$, $VAL121^{F}$
Hydrochloro-thiazide	$THR200^{A}$, $THR199^{A}$, $ZN301^{G}$, $GLN92^{B}$, $HIS96^{C}$, $HIS119^{C}$, $VAL121^{B}$, $VAL143^{B}$, $LEU198^{B}$, $HIS94^{B}$, $VAL121^{B}$, $LEU198^{B}$

Notes: * A – Conventional Hydrogen Bond; B – Pi-Sigma, C - Alkyl, D – Pi-Alkyl, E – Sulfur; F – Hydrophobic; G- Metal; H – Electrostatic.

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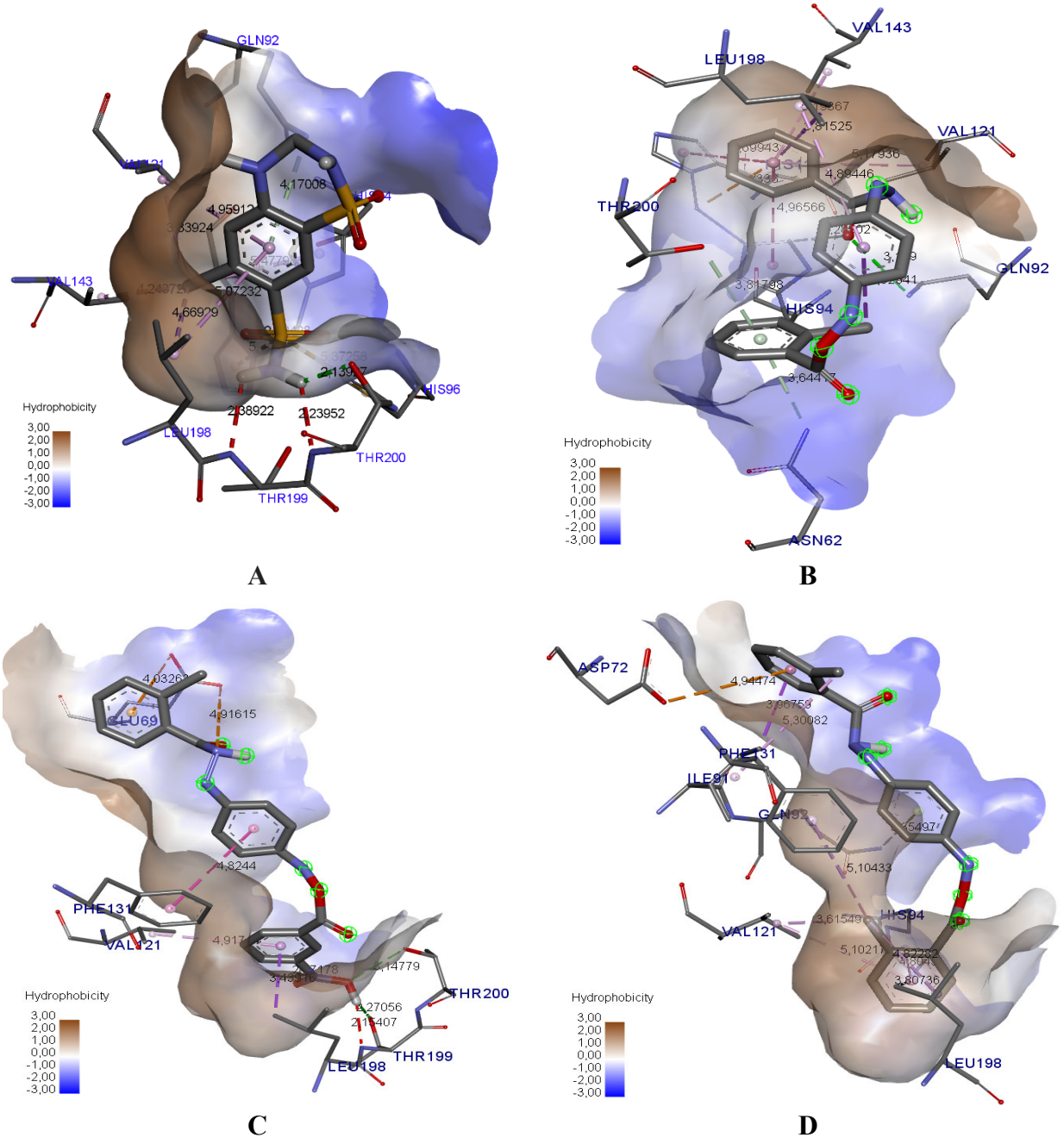

Fig. 3. Types of ligand-enzyme interactions of Hydrochlorothiazide (A), compounds 4.1 (B), 4.6 (C) and 4.4 (D) with CA II 3D by docking visualization

Visualisation of the results of molecular docking of aroylhydrazones of esters of quinone oxime (**4**) (Table 3) showed that the geometry of the molecules of these compounds somewhat complicates their displacement in the pocket of the active center of CA II. Thus, in compounds **4.1, 4.4,** and **4.6**, part of the molecule is located in the hydrophilic part of the enzyme due to the aroylhydrazone fragment, as in the case of the standard ligand (Hydrochlorothiazide). Whereas the other part of the molecule is located in the lipophilic part of the enzyme due to the benzoyloxime fragment. This arrangement of molecules in the enzyme pocket provides slightly different ligand-enzymatic interactions (Table 3). Thus, in the case of compound **4.1**, the 2-chloro-benzoylhydrazone fragment forms two conventional hydrogen bonds with amino acid residues GLN92 (3.14Å), HIS94 (3,24Å, Fig. 3B). In addition, the visualization predicts two hydrogen bonds of the oxime group with ASN62 (3,64Å) and THR200

(3,82Å) and additional hydrophobic interactions of the aromatic fragment with LEU198 (3,82Å), HIS119 (5,70Å), HIS94 (4,97Å), VAL121 (5,18Å), VAL143 (5,19Å) and LEU198 (4,89 Å, Fig. 3B). It is important that the excess electron density of the benzohydrazide fragment ensures the interaction of compound **4.1** with the zinc cation CA II (ZN301, 3.83Å) in contrast to the classical ligand.

Molecular docking analysis of CA II with compound **4.6** (Fig. 3C) showed that the molecule has a similar location in the center of the enzyme. The indicated position of compound **4.6** in the center of the enzyme is provided by two conventional hydrogen bonds of the 2-chlorobenzoylhydrazone group of the molecule with amino acid residues GLU69 (3.15 Å), THR199 (2.27 Å) and additional hydrophobic and electrostatic interactions with THR200 (4,03 Å), GLU69 (4.92 Å), LEU198 (3.44 Å), PHE131 (4.82 Å) та VAL121 (4.92 Å). Interestingly, compound **4.6** also visualizes a coordination bond between the nitro group of the benzoyloxime fragment and the zinc cation (ZN301 2.57 Å) of the enzyme.

As for the visualisation of the interaction of CA II with compound **4.4** (Fig. 3D), only one hydrogen bond of the 2-methylbenzoylhydrazone group of the molecule with the amino acid residue GLN92 (3.35 Å) and a large number of hydrophobic and electrostatic interactions with ASP72 (4.94 Å), ILE91 (3.97; 5.30 Å), LEU198 (3.81; 4.82 Å), HIS94 (4.80 Å), VAL121 (3.62; 5.10 Å), PHE131 (5.10 Å) and VAL121 (3.52 Å) are traced. In addition, compound **4.4** does not form a coordination bond with the zinc cation of the enzyme.

So, the results of the initial pharmacological screening confirmed the presence of a diuretic effect in the little-known N'-(4-[(aroyloxy)imino]cyclohexa-2,5-dien-1-ylidene)benzohydrazides and showed the prospects of their further study on the excretory function of the kidneys. At the same time, according to the results of molecular docking, it was established that the binding of the studied molecules to the zinc cation in the active center of the enzyme leads to an increase in the diuretic effect.

CONCLUSIONS

A strategy for the search for diuretics among aroylhydrazones of esters of quinone oxime using molecular docking, traditional synthesis and in vitro methodology (experimental animals) was developed and implemented. The synthesis of N'-(4-[(aroyloxy)imino]cyclohexa-2,5-dien-1-ylidene)benzohydrazides was carried out by the reaction of nucleophilic addition-cleavage of benzo-hydrazides to O-aroylesters of quinone oxime. Among the synthesized compounds, compound **4.6** was found, which according to daily diuresis (9.78±0.19 ml/100 g; 352,8%) exceeds the reference drug "Hydrochlorothiazide" (5.85±0.21 ml/100 g; 170,8%). Visualization of the results of molecular docking of aroylhydrazones of esters of quinone oxime showed that the geometry of the molecules of these compounds somewhat complicates their placement in the pocket of the active center of CA II. However, the binding of the studied molecules to the zinc cation in the active center of the enzyme leads to an increase in the diuretic effect. The obtained results justify further targeted search and more detailed study of the mechanism of action of potential diuretics among this class of compounds.

Recommendations. The results of the research confirmed the presence of a diuretic effect in aroylhydrazones of esters of quinone oxime and revealed the prospects for further study of their influence on the urinary system. First, it is a versatile structural modification by introducing additional sulfur-containing functional groups to the aromatic fragments, as carriers of the astringent effect. Secondly, conducting *in vitro* studies on the ability of the synthesized compounds to inhibit CA II, which will serve as a selection method for their further *in vivo* studies.

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