

UDC 547.562'367:615.33].03/.04.057

## ***1-Cycloalkanecarbonyl-substituted thioureas and thiosemicarbazides as effective dihydrofolate reductase inhibitors with antibacterial activity***

O. V. Kholodniak, M. Tniguer, I. S. Nosulenko, A. O. Kinichenko,  
K. I. Kandybey, O. M. Antypenko, S. I. Kovalenko

Zaporizhia State Medical University  
26, Mayakovsky avenue, Zaporizhzhia, Ukraine, 69035  
[antypenkoan@gmail.com](mailto:antypenkoan@gmail.com)

**Aim.** Search for new antibacterial agents with dihydrofolate reductase-inhibitory activity among *N*-(*R*-carbamoethyl)cycloalkylcarboxamides using *in silico* and *in vitro* methodology, SAR analysis to optimize the synthesis of new potential antimicrobials. **Methods.** Molecular docking, *in vitro* DHFR inhibition assay, antimicrobial evaluation, SAR analysis, statistical methods. **Results.** According to the results of molecular docking to the active center of dihydrofolate reductase (DHFR), namely affinity, the main types of interactions and arrangement in the active center of the enzyme, several *N*-(*R*-carbamoethyl)cycloalkylcarboxamides were selected for their inhibitory effect. Based on *in vitro* screening, few promising compounds with high ability to inhibit DHFR were identified. It was found, that diacylsemicarbazides are more effective inhibitors of DHFR compared to acylthioureas. The studies on antibacterial activity have revealed several promising compounds, namely *N*-(2-*R*-hydrazine-1-carbonothioyl)cycloalkanecarboxamides, as highly active antimicrobial agents against *E. coli* and *St. aureus* (MIC 3.125–25.0 µg/ml) with high DHFR-inhibitory effect, the activity of which competes with the comparison drug “Nitrofurazone”. This justifies the continuation of systematic research in this direction. **Conclusions.** A well-founded search among *N*-(*R*-carbamoethyl)cycloalkylcarboxamides for new antibacterial agents with dihydrofolate reductase-inhibitory activity, using *in silico* and *in vitro* methodology, established relationship between the chemical structure and activity aimed at further design of new potential drug agents.

**Keywords:** *N*-(*R*-carbamoethyl)cycloalkylcarboxamides, dihydrofolate reductase, molecular docking, inhibitors, antibacterial activity, SAR analysis

### **Introduction**

Pathogenic microorganisms are a danger to humanity since its inception, being the leading cause of morbidity and mortality. During the 20<sup>th</sup> century, antibacterial drugs (ADs) have played an important role in the treatment of infectious diseases, however, in the last decade

---

© 2022 O. V. Kholodniak *et al.*; Published by the Institute of Molecular Biology and Genetics, NAS of Ukraine on behalf of Biopolymers and Cell. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited

there has been an increase in the number of dangerous microorganisms resistant to ADs. The resistance to ADs could be natural or acquired caused by various mechanisms. The natural resistance is characterized by the absence of target microorganisms or inaccessibility of the target due to low permeability or enzymatic inactivation of drugs. In the presence of bacteria of natural resistance, ADs are clinically ineffective. The acquired stability is the property of individual strains of microorganisms to remain viable at the concentrations of ADs that suppress the bulk of the microbial population. The situations are possible when only a part of the microbial population shows acquired resistance. The appearance of acquired resistance in bacteria is not necessarily accompanied by a decrease in the clinical efficacy of the antibiotic. The formation of resistance in all cases is due to genetics: the acquisition of new genetic information or changes in the level of expression of their own genes. Overcoming such a problem is a difficult task. The causes of resistance to ADs include irresponsible or excessive use of drugs in the areas such as medicine, veterinary medicine and agriculture. Moreover, the range of new ADs is constantly depleted, whereas the resistance of microorganisms increased.

One of the mechanisms of ADs activity is the effect on folate metabolism, which was long ago recognized as an attractive chemotherapeutic target. Progress in understanding the biochemical basis of the mechanisms responsible for the enzyme selectivity and anti-proliferative effects, the discovery of aminopterin and methotrexate, a detailed study of their metabolism, were the impetus for the introduction of a group of “classical” antifolates

(Raltitrexed, Pralatrexate, Leucovorin, Pemetrexed, *etc.*) and “non-classical” antifolates (Trimetrexate, Piritrexim, Nolatrexed, Talotrexime, Trimethoprim, *etc.*) into medical practice [1–7]. These drugs are widely used as antitumor, antibacterial, antimalarial, antifungal agents, *etc.* [1–7].

Nowadays, more than 60 structures of antibacterial agents with DHFR inhibitory activity are known, but Trimethoprim is the “gold” standard for the treatment of urinary tract infections (UTIs) in women caused by *E. coli*, *Proteus mirabilis*, *Klebsiella spp.* and other microorganisms (*Enterobacteriaceae* and *Staphylococcus saprophyticus*) [8]. Interestingly, the combination of trimethoprim and sulfamethoxazole (“Co-trimoxazole”) is synergistic and is used to treat airways (bronchitis, pneumonia, pulmonary abscess, pulmonary abscess, empyema) meningitis, brain abscess, genitourinary system (pyelonephritis, urethritis, salpingitis, prostatitis), including gonorrhea, gastrointestinal (typhoid, paratyphoid, diarrhea), skin and soft tissues (pyoderma, furunculosis and others). However, the search for antibacterial agents has not been very successful in recent years, only a small number of effective antimicrobials with this mechanism of activity was introduced in the last 20 years. “Trimethoprim” was highly effective against *E. coli*, *S. aureus*, *St. pneumonia* [9] and against methicillin, trimethoprim- and vancomycin-resistant strains of *S. aureus*.

However, despite the proven effectiveness, confirmed mechanism of activity and identified biotargets, the design and search for antibacterial agents continue among various classes of compounds. We paid attention to functionalized acylthioureas and acylthiosemicarbazides,

among which the compounds with antibacterial, fungicidal, antitumor and other activities were found [10].

**The aim** of the study is to search for new antibacterial agents with the dihydrofolate reductase-inhibitory activity among *N*-(*R*-carbamoithiol)cycloalkylcarboxamides, using *in silico* and *in vitro* methodology, to establish relationship between chemical structure and antimicrobial activity and to optimize the synthesis of new potential substances.

## Materials and Methods

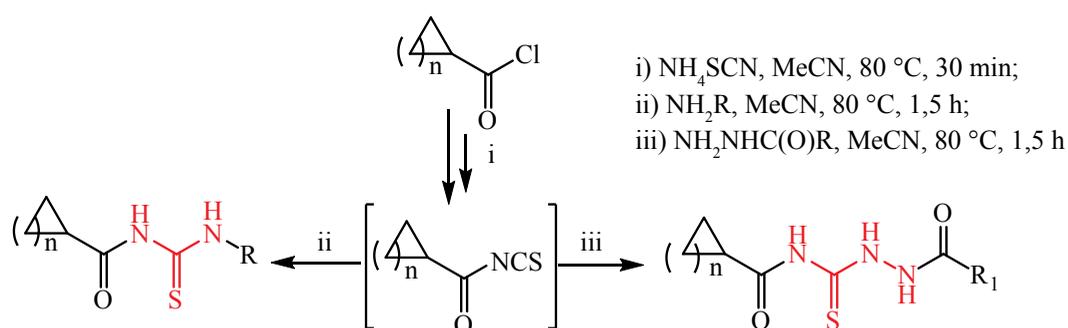
To study DHFR-inhibitory and antibacterial activity, *N*-(*R*-carbamoithiyl)- and *N*-(*R*-carbonyl)hydrazine-1-carbonothioyl)-cycloalkanecarboxamides were selected, the synthesis methods of which are known [11–13] and shown in Figure 1 and Table 1.

**Molecular docking.** The research was conducted by flexible molecular docking, as an approach of finding the molecules with affinity to a specific biological target. The macro-

molecules from Protein Data Bank (PDB) were used as biological targets, namely DHFR (PDB ID — 1RG7) (*Protein Data Bank*. <http://www.rcsb.org/pdb/home/home.do>. Accessed March, 2022). The choice of biological targets was due to the literature about the mechanism of antibacterial drugs activity [14].

**Ligand preparation.** Substances were drawn using MarvinSketch 20.20.0 and saved in mol format (*MarvinSketch version 20.20.0*, *ChemAxon* <http://www.chemaxon.com>). After that they were optimized by program Chem3D, using molecular mechanical MM2 algorithm, and saved as pdb-files. Molecular mechanics were 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, the number of active torsions was set as default [15].

**Protein preparation.** The PDB files were downloaded from the protein data bank. Discovery Studio v 19.1.0.18287 was used to



$\text{R} = \text{Ph}$ , 3- $\text{MeC}_6\text{H}_4$ , 4- $\text{MeC}_6\text{H}_5$ , 2- $\text{MeOC}_6\text{H}_4$ , 2- $\text{ClC}_6\text{H}_4$ , 4- $\text{ClC}_6\text{H}_4$ , 3-( $\text{COOH}$ ) $\text{C}_6\text{H}_4$ , 4-( $\text{COOH}$ ) $\text{C}_6\text{H}_4$ , 2,5-( $\text{COOMe}$ ) $\text{C}_6\text{H}_3$ , 4- $\text{NH}_2\text{SO}_2\text{C}_6\text{H}_4$ ;  $\text{R}_1 = \text{Cpr}$ ,  $-\text{CH}_2\text{OPh}$ ,  $-\text{CH}_2\text{SPh}$ ,  $-\text{Ph}$ , 2- $\text{NH}_2\text{C}_6\text{H}_4$ , *rfuryl*-2, *pyridyl*-4,  $n = 1-3$

**Fig. 1.** Approaches for the synthesis of *l*-cycloalkanecarbonyl-3-arylthioureas and cycloalkanecarboxylic acid (*N'*-*R*-carbonyl-hydrazinocarbothiyl)-amides

delete water molecules and ligands. Structures of proteins were saved as pdb files (*Discovery Studio Visualizer v19.1.018287*, Accelrys Software Inc., <https://www.3dsbiovia.com>). In AutoDockTools-1.5.6 polar hydrogens were added and saved as PDBQT. Grid box was set as following: center\_x = -1.657, center\_y = 22.030, center\_z = 23.080, size\_x = 22, size\_y = 22, size\_z = 22 for DHFR (1RG7). Vina was used to carry docking. For visualization, Discovery Studio v 19.1.0.18287 was used.

### ***In vitro* DHFR inhibition assay**

#### *Reagents*

Dihydrofolate Reductase Assay Kit (Sigma-Aldrich, Catalog Number CS0340, Batch Number 067M4065V) was used for evaluation of DHFR-inhibitory activity of synthesized compounds. The protein content in supplied dihydrofolate reductase was 0.032 mg/ml and the activity of enzyme was 3.75 U/mgP.

#### *The procedure of estimation of DHFR-inhibitory activity of studied compounds*

To the microcentrifuge tube (volume 2 ml) 966 µl of diluted 1:10 assay buffer we added. Then sequentially 13 µl of dihydrofolate reductase and 10 µl of 100 µM solution of studied compound in DMSO were added. The tube was sealed, intensively shaken, and the formed mixture was transferred to the 1.4 ml quartz cuvette. 6 µl of 10 mM solution of the NADPH were added to the formed mixture, the cuvette was sealed by parafilm and shaken. 5 µl of 10 mM solution of dihydrofolic acid were added to the formed mixture, the cuvette was sealed by parafilm, shaken, and immediately transferred to spectrophotometer ULab 108 UV. The

absorption of sample at 340 nm was measured each 15 second[s] during 150 seconds.

The activity of enzyme was calculated according to the following formula:

$$\text{Activity (Units/mg P)} = \frac{\Delta\text{OD}/\text{min}_{\text{sample}} - \Delta\text{OD}/\text{min}_{\text{blank}}}{12.3 \times 0.013 \times 0.032}$$

where:

$\Delta\text{OD}/\text{min}_{\text{blank}}$  =  $\Delta\text{OD}/\text{min}$  for the blank, from the spectrophotometer readings;

$\Delta\text{OD}/\text{min}_{\text{sample}}$  =  $\Delta\text{OD}/\text{min}$ . for the reaction, from the spectrophotometer readings;

12.3 = extinction coefficient ( $\epsilon$ ,  $\text{mM}^{-1}\cdot\text{cm}^{-1}$ ) for the DHFR reaction at 340 nm;

0.013 = enzyme volume in ml (the volume of enzyme used in the assay);

0.032 = enzyme concentration of the original sample.

The value of DHFR-inhibitory activity in % was calculated according to the formula:

$$\text{DHFR - inhibitory activity (\%)} = \frac{3.75 - \text{Activity (Units/mg P)}}{3.75} \times 100 \%$$

Methotrexate was used as a reference compound.

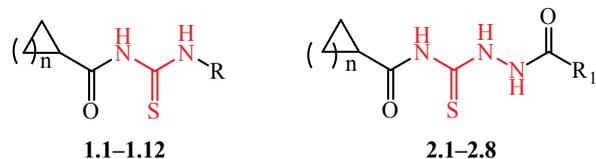
**Antimicrobial activity.** The sensitivity of the microorganisms to the synthesized compounds was evaluated according to the described methods [16]. The assay was conducted on Mueller-Hinton agar by two-fold serial dilution of the compound in 1 ml. After this, 0.1 ml of microbial seeding ( $10^6$  cells/ml) was added. Minimal inhibition concentration of the compound was determined by the absence of visual growth in the test tube with a minimal concentration of the substance. Minimal bactericide/fungicide concentration was determined by the absence of growth on

agar medium after inoculation of the microorganism from the transparent test-tubes. DMSO was used as a solvent, initial solution concentration was 1 mg/ml. For preliminary screening of the abovementioned standard the test cultures were used: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853. All test strains were received from bacteriological laboratory in Zaporizhzhia Regional Laboratory Center of State Sanitary and Epidemiological Service of Ukraine. Nitrofurantoin ((*E*)-2-((5-nitrofuranyl)methylene)hydrazine-1-carboxamide) was used as reference compound with proved antibacterial activity. Additional quality control of the culture media and solvents was conducted by commonly used methods [16].

## Results and Discussion

Molecular docking was used as a tool to determine the affinity, the main types of interactions of *N*-(*R*-carbamothioyl)cycloalkylcarboxamides (**1**, **2**) to the active site of DHFR and to predict the ability to inhibit it. The results of molecular docking of more than 50 compounds showed that compounds **1** and **2** do not have a high affinity for the enzyme, in this case lower than Methotrexate (Table 1). Noteworthy, the affinity for the enzyme in 20 selected compounds **1** and **2** is practically independent of the size of the cycloalkane fragment, and is mostly determined by the nature of the arylthiourea or aryl-(hetaryl)-thiosemicarbazide residue. A more detailed analysis showed that the compounds containing acyl residues with donor properties should be more effective DHFR inhibitors.

Table 1. Results of molecular docking of synthesized compounds to DHFR (1R7)\*



Compound	R(R <sub>1</sub> )	n	Affinity (kcal/mol)
<b>1.1</b>	Ph	1	-7.6
<b>1.2</b>	4-MeC <sub>6</sub> H <sub>4</sub>	1	-7.3
<b>1.3</b>	2-MeOC <sub>6</sub> H <sub>4</sub>	1	-7.0
<b>1.4</b>	4-MeC <sub>6</sub> H <sub>4</sub>	2	-7.8
<b>1.5</b>	4-MeC <sub>6</sub> H <sub>4</sub>	3	-7.7
<b>1.6</b>	2-ClC <sub>6</sub> H <sub>4</sub>	3	-6.9
<b>1.7</b>	4-ClC <sub>6</sub> H <sub>4</sub>	3	-7.5
<b>1.8</b>	2-MeOC <sub>6</sub> H <sub>4</sub>	3	-7.1
<b>1.9</b>	3-(COOH)C <sub>6</sub> H <sub>4</sub>	1	-7.2
<b>1.10</b>	4-(COOH)C <sub>6</sub> H <sub>4</sub>	1	-7.5
<b>1.11</b>	2,5-(COOMe) <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	1	-7.7
<b>1.12</b>	4-NH <sub>2</sub> SO <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	1	-7.3
<b>2.1</b>	Cpr	1	-7.3
<b>2.2</b>	-CH <sub>2</sub> OPh	1	-7.9
<b>2.3</b>	-CH <sub>2</sub> SPh	1	-7.6
<b>2.4</b>	Ph	1	-7.5
<b>2.5</b>	2-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub>	1	-7.1
<b>2.6</b>	furyl-2	3	-7.9
<b>2.7</b>	Pyridyl-4	1	-7.1
<b>2.8</b>	Pyridyl-4	2	-7.2
<b>MT</b> <sup>#</sup>	—	—	-8.7

Note: \* — data of compounds whose affinity is > -6.9 kcal/mol; # — MT – Methotrexate

Subsequently, we determined a probable mechanism of activity of this class of compounds. To do this, we visualized the molecular docking of several compounds (compounds **2.2**, **2.3**, **2.6**) and ligand (MT) to DHFR. Visualization of MT interaction in the active

center of DHFR showed (Table 2, Fig. 1) that the ligand has a significant number of hydrogen bonds, which are formed by electron-donor interactions of amino groups of 2<sup>nd</sup> and 4<sup>th</sup> positions of the pteridine cycle with amino acids ASP A: 27 (2.64 Å) and ILE A: 5 (2.77 Å), ILE A: 94 (2.90 Å), TYR A: 100 (3.23 Å), respectively. Attractive charge interaction bonds and hydrogen bonds of MTX with the enzyme are formed due to the interactions of the carboxyl group with ARG A: 57 (2.96 Å), ARG A: 57 (2.56 Å), ARG A: 52 (4.20 Å), LYS A: 32 (4.40 Å) and methylamine groups of the *p*-methylaminobenzoylglutamate moiety with ASN A: 18 (3.36 Å). The pteridine cycle of the molecule is characterized by  $\pi$ - $\sigma$ -interactions with ALA A: 19 (3.65 Å), ILE A: 5 (5.23 Å), ASN A: 18 (3.60 Å) and  $\pi$ -alkyl interactions with ALA A: 19 (4.15 Å) and ALA A: 7 (4.49 Å). Additionally, the MT molecule has hydrogen bonds between the amino group of 2<sup>nd</sup> position and the carboxyl group of the

glutamine residue with water molecules (HOH A: 2.98 Å and HOH200 (3.20 Å, respectively)) in the active site of the enzyme.

Compound **2.2** has the interaction with the enzyme due to hydrogen bonds with GLU A: 17 (3.69 Å), ASN A: 23 (3.29 Å), ASN A: 18 (2.28 Å) with thioamide and amide groups and SER A: 49 (3.06 Å) with the Oxygen atom of the phenoxyacyl fragment of the molecule (Table 2, Fig. 1). Other types of interactions, such as hydrophobic  $\pi$ -alkyl,  $\pi$ - $\sigma$ - and alkyl interactions with PHE A: 31 (4.38 Å), LEU A: 28 (4.99 Å), ALA A: 7 (4.49 Å), ALA A: 19 (4.15 Å), LEU A: 28 (4.88 Å), ILE A: 50 (4.17 Å), ILE A 50 (3.59 Å), ALA A: 7 (4.99 Å) and ALA A: 19 (3.77 Å) are carried out due to benzene and alpha fragments of the molecule.

Visualization of the interactions of compound **2.3** showed that the replacement of the oxygen atom in the molecule (compound **2.2**) by sulfur did not lead to other interactions (Fig. 2). These interactions occur due to hy-

**Table 2. The main types of interactions of synthesized compounds and pharmacological standard with amino acid residues of DHFR**

Compound	Types of interactions between compounds, pharmacological standard and amino acid residues of DHFR
<b>MT</b>	ARG57 <sup>a</sup> (2.96 Å), LYS32 <sup>j</sup> (4.40 Å), ARG52 <sup>j</sup> (4.20 Å), HOH200 <sup>a</sup> (3.20 Å), HOH262 <sup>a</sup> (2.98 Å), ARG57 <sup>a</sup> (2.56 Å), ASP27 <sup>a</sup> (2.64 Å), ILE5 <sup>a</sup> (2.77 Å), ILE94 <sup>a</sup> (2.90 Å), TYR100 <sup>a</sup> (3.23 Å), ASN18 <sup>b</sup> (3.36 Å), ASN18 <sup>b</sup> (3.60 Å), ALA19 <sup>d</sup> (3.65 Å), ILE5 <sup>f</sup> (5.23 Å), ALA7 <sup>f</sup> (4.49 Å), ALA19 <sup>f</sup> (4.15 Å), LEU28 <sup>f</sup> (4.88 Å), ILE50 <sup>f</sup> (4.17 Å)
<b>2.2</b>	GLU17 <sup>a</sup> (3.69 Å), ASN23 <sup>a</sup> (3.29 Å), ASN18 <sup>a</sup> (2.28 Å), SER49 <sup>b</sup> (3.06 Å), ILE50 <sup>d</sup> (3.59 Å), ALA7 <sup>c</sup> (4.99 Å), ALA19 <sup>c</sup> (3.77 Å), PHE31 <sup>f</sup> (4.38 Å), LEU28 <sup>f</sup> (4.99 Å), ALA7 <sup>f</sup> (4.49 Å), ALA19 <sup>f</sup> (4.15 Å), LEU28 <sup>f</sup> (4.88 Å), ILE50 <sup>f</sup> (4.17 Å)
<b>2.3</b>	ALA7 <sup>a</sup> (3.22 Å), TYR100 <sup>a</sup> (3.08 Å), TYR100 <sup>a</sup> (3.75 Å), ILE94 <sup>a</sup> (2.04 Å), ASN18 <sup>a</sup> (2.67 Å), ALA6 <sup>b</sup> (3.34 Å), ALA19 <sup>b</sup> (3.36 Å), SER49 <sup>b</sup> (3.78 Å), LEU28 <sup>d</sup> (3.88 Å), PHE31 <sup>k</sup> (4.34 Å), ALA7 <sup>c</sup> (4.31 Å), ALA19 <sup>c</sup> (3.78 Å), PHE31 <sup>f</sup> (4.72 Å), ILE50 <sup>f</sup> (5.14 Å)
<b>2.6</b>	TYR100 <sup>a</sup> (2.97 Å), ALA19 <sup>d</sup> (3.91 Å), PHE31 <sup>h</sup> (4.49 Å), LEU28 <sup>e</sup> (5.00 Å), ILE50 <sup>e</sup> (4.16 Å), ALA7 <sup>f</sup> (4.68 Å)

Notes: \* — a — Conventional Hydrogen Bond; b — Carbon Hydrogen Bond, c — Pi-Donor Hydrogen Bond, d — Hydrophobic ( $\pi$ - $\sigma$ ); e — Hydrophobic (Alk); f — Hydrophobic ( $\pi$ -Alk); g — Hydrophobic ( $\pi$ -Orbitals); h — Hydrophobic (amide- $\pi$  stacked); i — Hydrophobic ( $\pi$ - $\pi$  T-shaped), j — Electrostatic, k —  $\pi$ -Sulfur

drogen bonds ALA A: 7 (3.22 Å), TYR A: 100 (3.08 Å), TYR A: 100 (3.75 Å), ILE A: 94 (2.04 Å), ASN A: 18 (2.67 Å), ALA A: 6 (3.34 Å), ALA A: 19 (3.36 Å), SER A: 49 (3.78 Å) with thioamide and amide groups of molecules. Additionally, the molecule is characterized by other interactions with amino acid residues of the enzyme, namely hydrophobic  $\pi$ - $\sigma$ -,  $\pi$ -Alk-, Alk- and  $\pi$ -Sulfur-interactions with LEU A: 28 (3.88 Å), PHE A: 31 (4.34 Å), ALA A: 7 (4.31 Å), ALA A: 19 (3.78 Å), PHE A: 31 (4.72 Å) and ILE A: 50 (5.14 Å). For compound **2.6**, the hydrogen bond between TYR100a (2.97 Å) and the amide moiety of the molecule is visualized. Other interactions are rather weak and are realized through hydrophilic  $\pi$ - $\sigma$ -,  $\pi$ -Alk-interactions of the electron-rich heterocycle with ALA A: 19 (3.91 Å), ALA A: 7 (4.68 Å), PHE A: 31 (4.49 Å) and Alk-interaction of the cyclopropane cycle with LEU A: 28 (5.00 Å) and ILE A: 50 (4.16 Å) (Fig. 2.2).

Thus, it is found that compounds **2.2**, **2.3** and **2.6** in most cases have similar interactions with the amino acid residues as the classical inhibitor of MT and this leads to their close location in the active site of the enzyme. Undoubtedly, this is a clear argument for their further *in vitro* study of the ability to inhibit DHFR.

*In vitro* studies have shown (Table 3) that compounds **1.4**, **1.6–1.9**, **1.11** do not inhibit DHFR, and compounds **1.1–1.3**, **1.5**, **1.10**, **1.12** do not show a significant inhibitory effect on the enzyme (3.16–20.59 %). However, compounds **2.1–2.6** were found to be more active and inhibited DHFR by 28.34–90.32 %, except for *N*-(2-isonicotinoylhydrazine-1-carbothiyl) cyclopropanecarboxamide (**2.7**). The analysis

of the structure-activity relationship has shown that diacylsemicarbazides (**2**) are more effective inhibitors of DHFR than acylthioureas (**1**). It is important that the modification of cyclopropanecarbonyl isothiocyanates with acylhydrazide moieties (**2.1–2.6**) has a positive effect on the enzyme inhibition and, importantly, in most cases is determined by the nature (donor-acceptor properties) of acyl residues. Thus, the introduction of cyclopropane (**2.1**), phenoxy- (**2.2**), phenylthiol- (**2.3**) and furyl (**2.6**) fragments as electron donors leads to high activity (Table 3). However, the replacement of the above fragments by phenyl- (**2.4**), *o*-aminophenyl- (**2.5**) and isonicotinic- (**2.7**, **2.8**) substituents as electron acceptors leads to a significant loss of the ability to inhibit DHFR. An increase in the size of the cycloalkyl moiety from cyclopropane to cyclohexane has a positive effect on the DHFR- inhibitory activity with the additional introduction of a “pharmacophore” furan cycle (**2.6**). Compound **2.6** inhibits the enzyme by 90.32 %, exceeding the reference compound “Methotrexate” (82.57 %). The disubstituted thioureas (**1**), regardless of the size of the cycloalkanecarboxamide moiety and the nature of the aryl group, are not effective DHFR inhibitors. The highest inhibitory activity among the compounds of this class is characteristic of *N*-[(4-(methylphenyl(carbamothioyl))]cyclopropanecarboxamide (**2.2**, 20.59 %), which contains a donor substituent in the thioureide group.

The antimicrobial activity was investigated for the compounds that had a high inhibitory effect on DHFR (Table 4). It was found, that *N*-substituted cycloalkanecarbonylthioureas (**1.1–1.3**, **1.5**, **1.10**, **1.12**) with poor ability to inhibit DHFR will show moderate antimicro-



**Table 3. Results of molecular docking and inhibitory activity of synthesized compounds to DHFR**

Compound	DHFR activity, units/mg protein	Inhibitory activity,%	Compound	DHFR activity, units/mg protein	Inhibitory activity,%
<b>1.1</b>	5.61	3.16	<b>1.12</b>	5.38	7.03
<b>1.2</b>	4.59	20.59	<b>2.1</b>	1.01	72.89
<b>1.3</b>	5.72	1.22	<b>2.2</b>	1.57	82.57
<b>1.4</b>	6.95	0	<b>2.3</b>	1.57	72.89
<b>1.5</b>	5.49	5.10	<b>2.4</b>	6.50	69.01
<b>1.6</b>	6.50	0	<b>2.5</b>	3.14	45.77
<b>1.7</b>	6.95	0	<b>2.6</b>	0.56	90.32
<b>1.8</b>	6.95	0	<b>2.7</b>	6.84	0
<b>1.9</b>	5.83	0	<b>2.8</b>	4.15	28.34
<b>1.10</b>	5.15	10.91	<b>MT*</b>	1.01	82.57
<b>1.11</b>	7.62	0			

Note: \* – MT - Methotrexate

6.25–100 µg/ml) and *St. aureus* (MIC 6.25–100 µg/mL, MBC 12.5–100 µg/mL). However, compounds **2** show moderate antimicrobial

activity against *Ps. aeruginosa* (MIC 50 µg/mL, MBC 100 µg/mL).

An important issue of this study is to establish the relationship of antibacterial activity

**Table 4. Antibacterial activity of synthesized compounds**

Compound	Strains					
	<i>E. coli</i>		<i>St. aureus</i>		<i>Ps. aeruginosa</i>	
	MIC, µg/ml	MBC, µg/ml	MIC, µg/ml	MBC, µg/ml	MIC, µg/ml	MBC, µg/ml
1	2	3	4	5	6	7
<b>1.1</b>	100	200	50	100	50	100
<b>1.2</b>	100	200	50	100	50	100
<b>1.3</b>	100	200	50	100	50	100
<b>1.5</b>	100	200	50	100	50	100
<b>1.10</b>	100	200	50	100	50	100
<b>1.12</b>	100	100	50	100	50	50
<b>2.1</b>	3.125	6.25	6.25	12.5	50	100
<b>2.2</b>	50	100	25	50	50	100
<b>2.3</b>	12.5	25	12.5	25	50	100
<b>2.4</b>	6.25	12.5	6.25	12.5	50	100
<b>2.5</b>	50	100	12.5	25	50	100
<b>2.6</b>	6.25	12.5	6.25	12.5	50	100
<b>2.8</b>	100	100	100	200	100	200
Nitrofurazone	1.5	–	6.25	–	6.25	–

with the DHFR-inhibitory activity (Tables 3, 4). It is true that compounds **2.1–2.6**, which showed a high enzyme-inhibiting effect at the level of 45.77–90.32 %, inhibit bacterial growth with MIC of 3.125–100 µg/ml.

Thus, the studies on the antibacterial activity revealed several promising compounds, namely *N*-(2-*R*-hydrazine-1-carbonothioyl)cycloalkanecarboxamides (**2.1–2.6**), as highly active antimicrobial agents against *E. coli* and *St. aureus* (MIC 3.125–25.0 µg/ml) with the DHFR-inhibitory action, which justifies the continuation of systemic studies in this direction.

## Conclusion

Using *in silico* and *in vitro* methodology, the well-founded search for new antibacterial agents with dihydrofolate reductase-inhibitory activity was performed among *N*-(*R*-carbamo-thiol)cycloalkylcarboxamides, the regularities between chemical structure and antimicrobial activity were established to optimize chemical synthesis. A few *N*-(*R*-carbamo-thioyl)cycloalkylcarboxamides were selected for their inhibitory effect, according to the results of molecular docking to the active center of dihydrofolate reductase (DHFR), namely affinity, the main types of interactions in the active center of the enzyme. Based on traditional *in vitro* screening, several promising compounds with high ability to inhibit DHFR have been identified. It was found, that diacylsemicarbazides are more effective inhibitors of DHFR compared to acylthioureas. The studies on antibacterial activity have revealed several promising compounds, namely *N*-(2-*R*-hydrazine-1-carbonothioyl)cycloalkanecarboxamides to be highly active antimicrobial agents

against *Es. coli* and *St. aureus* (MIC 3.125–25.0 µg/ml) with high DHFR-inhibitory activity, which competes with the compared drug “Nitrofurazone”. This justifies the continuation of systematic research in this direction.

## Acknowledgements

The work was carried out on the research topic of the Ministry of Health of Ukraine «Cycloalkylcarbonylisothiocyanates — effective precursors for the synthesis of substituted thioureas and the construction of heterocyclic systems» (problem «Pharmacy», state registration № 0118U 004261, period of study 2017–2022).

The synthetic part of the work was performed with the financial support of Enamine Ltd (Kyiv, Ukraine).

## REFERENCES

1. Raimondi M, Randazzo O, La Franca M, Barone G, Vignoni E, Rossi D, Collina S. DHFR inhibitors: reading the past for discovering novel anticancer agents. *Molecules*. 2019; **24**(6):1140.
2. Sharma M, Chauhan PMS. Dihydrofolate reductase as a therapeutic target for infectious diseases: opportunities and challenges. *Future Med Chem*. 2012; **4**(10):1335–65.
3. Gangjee A, Jain HD, Kurup S. Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents: part I. *Anti-Cancer Agents Med Chem*. 2007; **7**(5):524–42.
4. Gangjee A, Jain H, Kurup S. Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents: part II. *Anti-Cancer Agents Med Chem*. 2008; **8**(2):205–31.
5. Wright DL, Anderson AC. Antifolate agents: a patent review (2006-2010). *Expert Opin Ther Pat*. 2011; **21**(9):1293–308.
6. Anderson AC, Wright DL. Antifolate agents: a patent review (2010-2013). *Expert Opin Ther Pat*. 2014; **24**(6):687–97.

7. Wyss PC, Gerber P, Hartman PG, Hubschwerlen CH, Marty L, Martin S. Novel dihydrofolate reductase inhibitors. Structure-based versus diversity-based library design and high-throughput synthesis and screening. *J Med Chem.* 2003; **46**(12):2304–12.
  8. Patel RB, Welling PG. Clinical Pharmacokinetics of Co-trimoxazole (trimethoprim-sulphamethoxazole). *Clin Pharmacokinet.* 1980; **5**(5): 405–23.
  9. Schneider P, Hawser S, Islam K. Iclaprim, a novel diaminopyrimidine with potent activity on trimethoprim sensitive and resistant bacteria. *Bioorg Med Chem Lett.* 2003; **13**(23):4217–21.
  10. Kholodniak O, Kovalenko S. Substituted acylthioureas and acylthiosemicarbazides: synthesis and biological activity (minireview). *ScienceRise: Pharmaceut Sci,* 2022; **2**(36):56–71.
  11. Antypenko L, Meyer F, Kholodniak O, Sadykova Z, Jirásková T, Troianova A, Buhaiova V, Cao S, Kovalenko S, Garbe LA, Steffens KG. Novel acyl thiourea derivatives: synthesis, antifungal activity, gene toxicity, drug-like screening and molecular docking. *Arch Pharm (Weinheim).* 2018; **352**(2):e1800275.
  12. Kholodniak OV, Kazunin MS, Meyer F, Kovalenko SI, Steffens KG. Novel N-cycloalkylcarbonyl-N $\epsilon$ -arylthioureas: synthesis, design, antifungal activity and gene toxicity. *Chem Biodivers.* 2020; **17**(7):52000212.
  13. Kholodniak OV, Stavyskyi VV, Kazunin MS, Bukhtiyarova NV, Berst GG, Belenichev IF, Kovalenko SI. Design, synthesis and anticonvulsant activity of new diacylthiosemicarbazides. *Biopolym Cell.* 2021; **37**(2):125–142.
  14. Filman DJ, Bolin JT, Matthews DA, Kraut J. Crystal structures of Escherichia coli and Lactobacillus casei dihydrofolate reductase refined at 1.7 Å resolution. II. Environment of bound NADPH and implications for catalysis. *J Biol Chem.* 1982; **257**(22):13663–62.
  15. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem.* 2010; **31**(2):455–61.
  16. Wayne PA. Performance standards for antimicrobial disk susceptibility tests, Approved Standard 9th Edition. 2006. 52 p.
- N*-(*R*-карбамотіол)циклоалкілкарбоксаміди ефективні інгібітори дегідрофолатредуктази з антибактеріальною активністю**
- О. В. Холодняк, М. Тнігуер, І. С. Носуленко, А. О. Кініченко, К. І. Кандибей, О. М. Антипенко, С. І. Коваленко
- Мета.** Пошук нових антибактеріальних агентів з дигідрофолатредуктазо-інгібуючою дією з використанням методології *in silico* та *in vitro* серед *N*-(*R*-карбамотіол)циклоалкілкарбоксамідів, встановлення закономірностей між хімічною будовою та протимікробною активністю для оптимізації синтезу нових потенційних біологічно активних речовин. **Методи.** Молекулярний докінг, аналіз інгібування ДГФР *in vitro*, антимикробний тест, SAR-аналіз, статистичні методи. **Результати.** За результатами молекулярного докінгу до активного центру дигідрофолатредуктази (ДГФР), а саме спорідненості, основних типів взаємодій та розміщення в активному центрі ферменту, відібрано ряд *N*-(*R*-карбамотіол)циклоалкілкарбоксамідів для їх дослідження інгібуючої дії. На основі традиційного *in vitro* скринінгу виявлено ряд перспективних сполук з високою здатністю інгібування ДГФР. При цьому встановлено, що більш ефективними інгібіторами ДГФР є диацилсемікарбазиди у порівнянні до ацилтіосечовинами. Проведені дослідження на антибактеріальну активність дозволили виявити ряд перспективних сполук, а саме *N*-(2-*R*-гідразин-1-карбонотіол)циклоалканкарбоксамідів, як високоактивних протимікробних агентів щодо *E. coli* та *St. aureus* (МІК 3.125–25.0 мкг/мл) з високою ДГФР-інгібуючою дією, активність яких конкурує з препаратом-порівняння «Нітрофуразон». Зазначене обґрунтовує продовження системних досліджень у наведеному напрямку. **Висновки.** Проведено обґрунтований пошук нових антибактеріальних агентів з дигідрофолатредуктазо-інгібуючою дією з використанням методології *in silico* та *in vitro* серед *N*-(*R*-карбамотіол)циклоалкілкарбоксамідів, встановлені закономірності між хімічною будовою та активністю для подальшої оптимізації синтезу нових потенційних лікарських засобів.
- Ключові слова:** *N*-(*R*-карбамотіол)циклоалкілкарбоксаміди, дегідрофолатредуктаза, молекулярний докінг, інгібітори, антибактеріальна активність, SAR-аналіз

Received 10.01.2022