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RESEARCH ARTICLE

Inhibition of glutathione *S*-transferases A1-1 and P1-1 by pyrazolone-containing 4-(5-substituted furan-2-yl)benzoic acids

Yurii V. Shulha, Oleksandr L. Kobzar, Stepan G. Pilyo, Andriy I. Vovk*

V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry of the NAS of Ukraine, Kyiv, Ukraine

Abstract: 4-(5-Substituted furan-2-yl)benzoic acids with a pyrazolone moiety were evaluated *in vitro* as inhibitors of human cytosolic glutathione *S*-transferases A1-1 and P1-1, which are involved in cellular mechanisms of drug resistance and carcinogenesis. When inhibiting GSTA1-1, the compounds demonstrated micromolar and nanomolar IC_{50} values depending on the nature of the substituent at position 3 of the pyrazolone ring. In particular, the inhibition was increased when the methyl group was replaced by a trifluoromethyl, phenyl, 4-fluorophenyl, thiophen-2-yl, or pyridin-4-yl substituent. The effect of the 4-(5-substituted furan-2-yl)benzoic acids on GSTP1-1 was approximately an order of magnitude lower than that on GSTA1-1. In both cases, the compound bearing a 1-phenyl-3-(4-nitrophenyl)-substituted pyrazolone moiety demonstrated the best inhibitory activity among the derivatives studied. Molecular docking results indicated that the inhibitors interact with GSTA1-1 mainly due to the participation of amino acid residues from the G-site, while in the case of GSTP1-1 the compounds bind to the H-site.

Keywords: 4-(furan-2-yl)benzoic acids; pyrazolones; glutathione *S*-transferases; inhibition.

Introduction

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) represent a superfamily of phase II detoxification enzymes that catalyze the conjugation of reduced glutathione with different endogenous and exogenous electrophilic compounds [1, 2]. This reaction leads to glutathione *S*-conjugated products, which have better water solubility and lower cytotoxicity and can be further metabolized *via* the mercapturic acid pathway [3]. The widely distributed cytosolic GSTA1-1 (liver, intestine, kidney, adrenal gland, and testis) and GSTP1-1 (brain, heart, lung, testis, skin, kidney, pancreas, erythrocytes) [4, 5] play diverse biological roles. In addition to the detoxification functions, these enzymes are also involved in cellular signaling [5, 6]. The regulation of signaling can occur through the mitogen-activated protein (MAP) kinase pathway and includes inhibition of kinases JNK1, ASK1, and MEKK1 [7]. Thus, the role of GSTP1-1 and GSTA1-1 makes these enzymes promising targets for drug design to overcome resistance mechanisms to anticancer agents as well as to influence signaling pathways in cancer cells. In this connection, the new bioactive compounds can be developed to be used solely or together with chemotherapeutic drugs [8-10].

Various organic compounds such as derivatives of glutathione [11], 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)-hexanol [12, 13], coumarins [14, 15], benzisoselenazolones [16] were studied as inhibitors of GSTs. Glutathione-based ezatiostat (TLK199) and canfosfamide (TLK286) were found to be useful as prodrugs targeting GSTP1-1 [17, 18]. Ethacrynic acid, used as a diuretic, as well as its derivatives and structural analogues can exhibit inhibitory activity towards GSTP1-1 [19, 20]. Recently, methyl 3-amino-4-nitrobenzoate [21], methyl 4-amino-2-nitrobenzoate, and methyl 4-amino-3-bromo-5-fluorobenzoate [22] were found to be effective inhibitors of GST isolated from human erythrocytes. 3,4-Dihydroxyphenylacetic acid, being a metabolite of dopamine, can *in vitro* inhibit the glutathione *S*-transferase isolated from N27 dopaminergic cells [23].

In the presented work, the objects of our research were

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* Corresponding author. Tel.: +380-44-558-5388;
e-mail: vovk@bpci.kiev.ua (A.I. Vovk)
ORCID: 0000-0001-6167-076X

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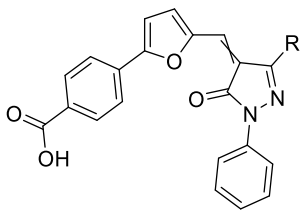
4-(5-substituted furan-2-yl)benzoic acids. Similar compounds are known to be inhibitors of SARS-CoV and MERS-CoV 3C-like proteases [24], heptosyltransferase WaaC [25], and neuraminidase [26]. It was recently shown that 4-(furan-2-yl)benzoic acids with a pyrazolone or rhodanine part can inhibit xanthine oxidase [27, 28]. This study aimed to evaluate 4-(5-substituted furan-2-yl)benzoic acids with different substituents in position 3 of the pyrazolone moiety (Figure 1) as inhibitors of human glutathione *S*-transferases A1-1 and P1-1.



1-8

R = Me (**1**); CF_3 (**2**); Ph (**3**); 4-FC_6H_4 (**4**); 4-ClC_6H_4 (**5**); 4-$O_2NC_6H_4$ (**6**); thiophen-2-yl (**7**); pyridin-4-yl (**8**)

Figure 1. Pyrazolone-containing 4-(5-substituted furan-2-yl)benzoic acids studied as GSTP1-1 and GSTA1-1 inhibitors.

Results and Discussion

According to the results obtained (Table 1), compounds **2-8** exhibited submicromolar and nanomolar IC_{50} values against recombinant GSTA1-1 *in vitro*. Compound **1**, which contains a 3-methyl-5-phenylpyrazolone ring, was characterized by an IC_{50} value of 3.7 μM, while 4-(5-formylfuran-2-yl)benzoic acid exhibited lower activity (IC_{50} value >25 μM). This indicates the involvement of the pyrazolone moiety in the binding of the inhibitor to the enzyme.

The inhibitory efficacy of compounds **1-8** against GSTA1-1 increased when the methyl group at position 3 of the pyrazolone moiety was replaced by trifluoromethyl, phenyl, 4-fluorophenyl, thiophen-2-yl, or pyridin-4-yl group, varying in the range from 0.45 to 0.18 μM. A further decrease in IC_{50} values occurs when going from phenyl (compounds **3**) to 4-chlorophenyl or 4-nitrophenyl substituents at position 3 of the pyrazolone ring (compounds **5** and **6**).

The inhibitory effects of 4-(5-substituted furan-2-yl)benzoic acids **1-8** on GSTP1-1 were approximately an order of magnitude worse than those on the A1-1 isoform. Among the derivatives **1-8**, the significant decrease in inhibitory activity was observed for compound **5** with a 4-chlorophenyl substituent. As in the case of isoform A1-1, the derivative **6** bearing 4-nitrophenyl substituents was the most effective, showing IC_{50} values for GSTP1-1 inhibition in the low micromolar range. The similar effect of compound **6** was also observed in a system with the enzyme isolated from human placenta, with activity significantly exceeding that of ethacrynic acid used as a reference compound [29] (IC_{50} value of 4.3 ± 0.6 μM). The inhibition of GSTHP by compounds **2**, **4**, and **8** was found to be almost the same as the effect of ethacrynic acid, while the influence of derivatives **1**, **3**, **5**, and **7** was noticeably weaker.

Molecular docking by AutoVina was performed to assess the possible interactions between the inhibitors and amino acid residues at the active sites of GSTA1-1 and GSTP1-1. According to the values of the docking scores (Table 1), the binding energy between the ligand and enzyme for the most effective inhibitor **6** was -9.3 kcal/mol (GSTA1-1) and -9.7 kcal/mol (GSTP1-1). Compound **1**, with low inhibitory potential against GSTA1-1 and GSTP1-1, is characterized by docking scores of -8.7 kcal/mol and -8.6 kcal/mol, respectively. The data obtained suggest that inhibitor **6** has stronger binding interactions within the active site as compared to compound **1**.

The GSTA1-1 is known to possess three possible ligand binding sites. The conserved G-site for glutathione binding is located in the *N*-terminal domain I. H-Site at the C-terminal domain II binds the diverse electrophilic substrates with participation of the amino acid residues of the 1-1 loop, the C-terminal part of helix 4, and the C-terminus. Non-catalytic site of the human GSTA1-1, located at the dimer interface, can be involved in the binding of various non-substrate ligands [30, 31]. The results in Table 1 show that mainly amino acid resi-

Table 1 IC_{50} values (μM) of 4-(5-substituted furan-2-yl)benzoic acids as inhibitors of glutathione *S*-transferases [a].

Compound	GSTA1-1	GSTP1-1	GSTHP[b]
1	3.7 ± 1.1	>25	16.7 ± 1.4
2	0.45 ± 0.04	6.1 ± 0.4	2.4 ± 0.9
3	0.45 ± 0.15	6.7 ± 1.1	17.1± 4.0
4	0.29 ± 0.04	2.5 ± 0.4	5.5 ± 2.1
5	0.035 ± 0.021	2.4 ± 0.7	13.9 ± 4.0
6	0.018 ± 0.006	0.20 ± 0.05	0.38 ± 0.10
7	0.30 ± 0.11	1.6 ± 0.7	10.9 ± 3.8
8	0.18 ± 0.05	2.5 ± 0.9	4.3 ± 0.7

[a] IC_{50} values were determined from 2-3 series of experiments and shown as an average value ± standard deviation. [b] Glutathione *S*-transferase from human placenta.

Table 2. The docking scores and amino acid residues of GSTA1-1 and GSTP1-1 involved in binding the 4-(5-substituted furan-2-yl)benzoic acids [a].

Enzyme	Inhibitor	Docking score, kcal/mol	Amino acid residues	
			Subunit A	Subunit B
GSTA1-1	**1**	-8.7	Tyr9, Phe10, Gly14, Arg15, Gln54, Val55, Gln67, Thr68, Glu104, Met208, Ala216, Phe220	Asp101
	2	-9.1	Tyr9, Phe10, Gly14, Arg15, Gln54, Val55, Pro56, Gln67, Thr68, Glu104, Met208, Ala216, Phe220	Asp101
	3	-9.2	Arg15, Arg45, Gln54, Gln67, Thr68, Arg69, Val111, Phe220, Phe222	Asp101, Arg131
	4	-9.2	Tyr9, Phe10, Arg15, Arg45, Gln54, Val55, Ala216, Phe220, Phe222	Asp101, Glu104, Arg131
	5	-9.1	Tyr9, Phe10, Arg15, Arg45, Gln54, Val55, Arg69, Val111, Ala216, Phe220, Phe222	Asp101
	6	-9.3	Tyr9, Phe10, Arg15, Arg45, Gln54, Gln67, Thr68, Arg69, Ala216, Phe220, Phe222	Glu97, Asp101, Lys127, Arg131
	7	-8.6	Tyr9, Phe10, Arg45, Gln54, Val55, Met208, Ala216, Phe220, Arg221, Phe222	Asp101, Arg121
	8	-9.3	Tyr9, Arg15, Arg45, Gln54, Val55, Gln67, Thr68, Arg69, Val111, Phe220, Phe222	Leu123, Lys127, Arg131
GSTP1-1	**1**	-8.6	Tyr7, Phe8, Val10, Arg13, Val35, Gln51, Leu52, Gln64, Ile104, Tyr108, Gly205	Asp98
	2	-9.2	Tyr7, Phe8, Val10, Gly12, Arg13, Gln51, Leu52, Ile104, Tyr108, Ile203, Asn204, Gly205	Asp98, Lys102
	3	-9.1	Tyr7, Phe8, Pro9, Val10, Gly12, Arg13, Val35, Arg100, Tyr103, Ile104, Tyr108, Ile161, Ile203, Asn204, Gly205, Asn206	
	4	-9.3	Tyr7, Phe8, Pro9, Gly12, Arg13, Val33, Thr34, Val35, Arg100, Tyr103, Ile104, Tyr108, Ile161, Ile203, Asn204, Gly205, Asn206	
	5	-9.3	Tyr7, Phe8,Val10, Arg13, Gln51, Leu52, Gln64, Cys101, Ile104, Ser105, Tyr108, Gly205	Asp98
	6	-9.7	Tyr7, Phe8,Val10, Arg13, Val35, Gln51, Leu52, Gln64, Ile104, Ser105, Tyr108, Thr109, Gly205, Asn206	Asp98
	7	-9.0	Tyr7, Phe8, Val10, Arg13, Gln51, Leu52, Gln64, Ser65, Cys101, Ile104, Tyr108, Gly205	
	8	-9.0	Tyr7, Phe8, Val10, Arg13, Gln51, Leu52, Gln64, Cys101, Ile104, Tyr108, Gly205	Asp98

[a] There are shown the amino acid residues surrounding the ligand at a distance not more than 4.0 Å.

dues from the G-site of GSTA1-1 can provide the enzyme-inhibitor complex formation. Among them, Arg15 is known as a conserved residue stabilizing the thiolate anion of substrate [32], and Arg131 from the other subunit coordinates the carboxylate group of the glycine part of glutathione [2]. In addition, the residues Phe220 and Phe222 belonging to the H-site are involved in the formation of a lid over the active site.

In contrast, the inhibitors of GSTP1-1 can bind to a region close to the H-site. This site, consisting of both hydrophobic and hydrophilic residues, is not such hydrophobic as in GSTA1-1 [30, 33]. Key amino acid residues that determine the binding of pyrazolone-tethered 4-(5-substituted furan-2-yl)benzoic acids are also involved in the mechanisms of ethacrynic acid binding [34].

In particular, Phe8 coordinates to the chlorine atoms of ethacrynic acid, and Arg13 forms a hydrogen bond with its carboxyl group [35]. There is also the G-site Tyr108 residue nearby, which can participate in the interaction of ethacrynic acid with glutathione [34].

As shown in Figure 1, compound **6** can occupy the region of glutathione binding to GSTA1-1. The residue Arg15, which plays an important role in the glutathione binding and its ionization, can interact with the oxygen of pyrazolone and the benzene ring of 4-(furan-2-yl)benzoic acid fragment through a hydrogen bond and π-cationic interactions. Gln54 possesses a hydrogen interaction with the oxygen atom of the furan-2-yl linker. The residues Thr68 and Arg69 can be crucial for the fixation of the carboxylic group, while Arg45 as well as Lys127 and Arg131 of the B subunit are involved in the hydrogen bonds with the nitro group of compound **6**. The 5-phenyl substituent of the pyrazolone part of the inhibitor shows π-π stacking interaction with Phe220 and hydrophobic contacts with Phe10 and Phe222.

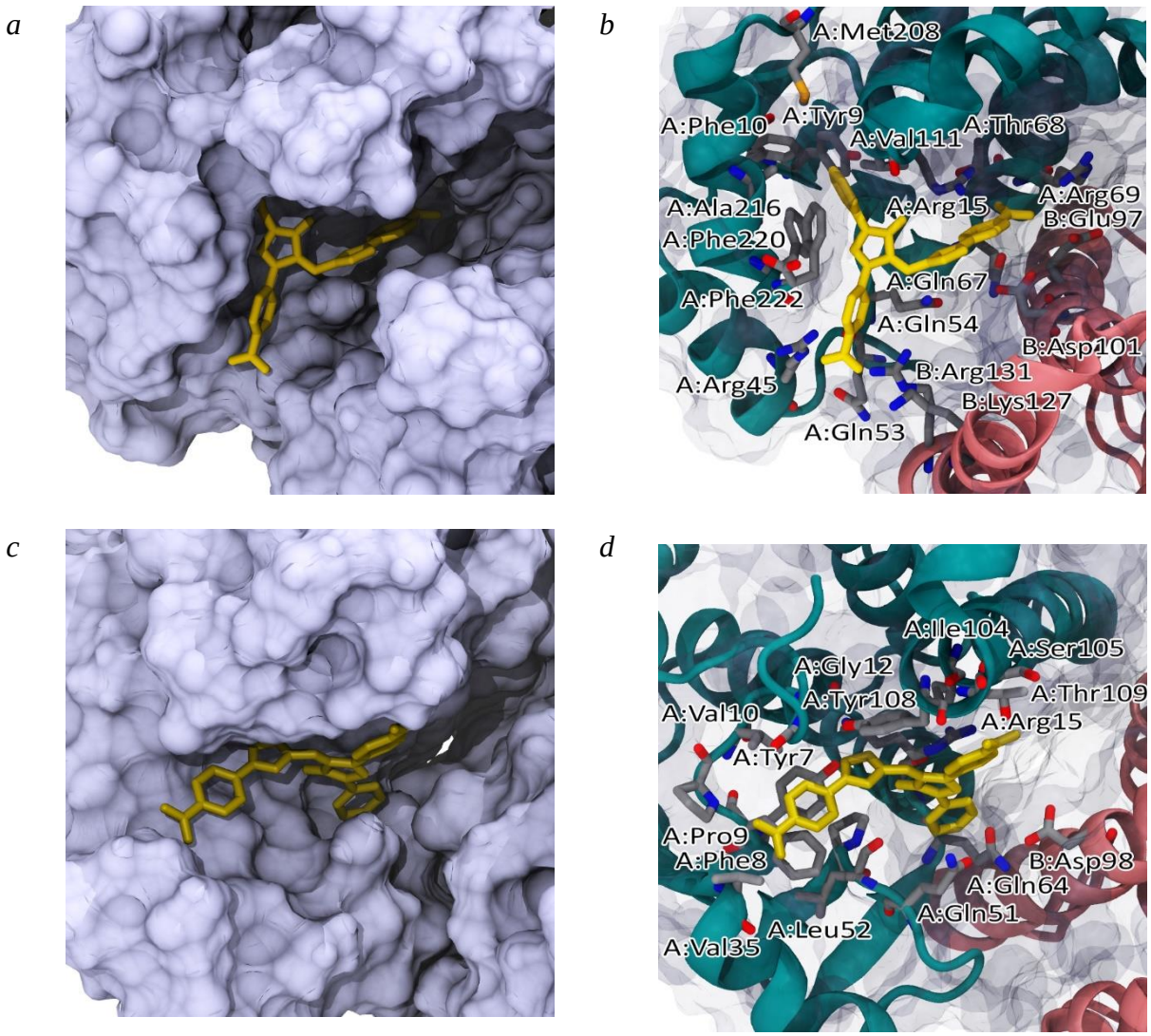


Figure 1. Predicted binding modes of compound **6** in the active site of subunit A of GSTA1-1 (*a*, *b*) and GSTP1-1 (*c*, *d*). Amino acid residues surrounding the compound are shown in panels *b* and *d*.

Compound **6** binds in the H-site of GSTP1-1 similarly to binding ethacrynic acid. This binding shows the contacts of Arg13 with the pyrazolone and phenyl substituent in position 1 through π-cation interactions. The residue Tyr7 forms a hydrogen bond with the oxygen of pyrazolone, while Phe8 participates in π-π stacking with the benzene ring of the benzoic acid fragment. Tyr108 has a π-π stacking interaction with the benzene ring of the 4-nitrophenyl substituent, and Thr109 possesses a hydrogen bond with the nitro group. Van der Waals contacts and hydrophobic interactions are also important for stabilizing the position of inhibitor **6** at the H-site of GSTP1-1.

Conclusions

This study expands the possible biological functions of 4-(5-substituted furan-2-yl)benzoic acids with a pyrazolone moiety as potent inhibitors of GSTA1-1 and GSTP1-1. The structure and *in vitro* activity of a series of compounds with different substituents at position 3 of the pyrazolone ring were analyzed. Based on molecular docking modeling, the distinct locations were suggested for the binding of the inhibitors to GSTA1-1 and GSTP1-1. Thus, the compounds of this class can be considered a basis for further development of the pyrazolone-containing 4-(5-substituted furan-2-yl)benzoic acids as inhibitors of GSTA1-1 and GSTP1-1.

Experimental section

Synthesis of 4-(5-substituted furan-2-yl)benzoic acids

Compounds **1-8** were synthesized as previously described [27]. Compounds **2** and **7** were obtained as *Z*-isomers, and the *E*/*Z* ratio was 1/3 for compounds **1** and **5**. This ratio was 1/5 for compounds **3**, **4**, **6**, and **8**.

In vitro inhibition assays

The human recombinant GSTA1-1 and GSTP1-1, as well as GST from human placenta, were purchased from Sigma-Aldrich. Before using in experiments, GST from human placenta was diluted in bidistilled water. GSTA1-1 or GSTP1-1 were diluted in 1 ml of a solution consisting of 50 mM Tris-HCl buffer (pH 7.5), 50 mM NaCl, 1 mM DTT, 5 mM EDTA, and 50 vol.% glycerol. The compounds tested were dissolved in pure DMSO. The assay system containing

0.1 M sodium-phosphate buffer (pH 6.5), 0.1 mM EDTA, 2.5 vol.% DMSO, 20 μL of enzyme solution, and inhibitor was incubated for 5 min at 25 ± 2 ºC, and then the enzymatic reaction was started by the addition of GSH and CDNB (concentrations of 1 mM in the reaction mixture). The enzyme activity was monitored spectrophotometrically by the accumulation of glutathione conjugate at 340 nm. The IC_{50} values were determined from the dose-dependent inhibition curves as the concentration of compounds at which the rate of the enzymatic reaction decreased by 50%.

Molecular docking

The PDB crystals of human GSTA1-1(PDB code 6ATO) and human GSTP1-1 (PDB code 6GSS) were downloaded from the RCSB PDB server (https://www.rcsb.org) [36]. The ligands, water molecules, and amino acids conformers were removed from these files before docking calculations performed by the program AutoDock Vina 1.1.2 [37]. The pdbqt files of the enzymes were saved from AutoDockTools (version 1.5.6) software [38] after adding hydrogen atoms and Gasteiger charges. The coordinates 82.939, 28.292, and 16.07 of the grid box center and box size of 20 Å × 20 Å × 20 Å were used for docking the 4-(5-substituted furan-2-yl)benzoic acids into the active site of subunit A of GSTA1-1. The coordinates 10.412, 5.923, and 25.019 of the grid box center and box size 20 Å × 20 Å × 20 Å were used for molecular docking of the compounds into the active site of subunit A of GSTP1-1.

The structures of pyrazolone-containing 4-(5-substituted furan-2-yl)benzoic acids with a carboxylic group in ionized form were drawn using the MarvinSketch program [39] and optimized with the MMFF94s force field in Avogadro 1.2.0 [40]. The conversion of the obtained mol2 files to pdbqt format, which is used by AutoDock Vina, was performed using AutoDockTools software. Discovery Studio 3.5 visualizer (Accelrys Software Inc., San Diego, CA, USA) and Visual Molecular Dynamics 1.9.3 [41] software were used for analysis of binding modes of the compounds.

Notes

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The authors declare no conflict of interest.

Author contributions. **Yu.V.S.**: *in vitro* and *in silico* experiments, preparation of data. **O.L.K.**: *in silico* studies, data analysis, and writing the manuscript. **S.G.P.**: synthesis of compounds. **A.I.V.**: conceptualization and writing the manuscript.

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Інгібування глутатіон *S*-трансфераз A1-1 та P1-1 піразолоновмісними 4-(5-заміщеними фуран-2-іл)бензойними кислотами

Ю.В. Шульга, О.Л. Кобзар, С.Г. Пільо, А.І. Вовк*

Інститут біоорганічної хімії та нафтохімії ім. В.П. Кухаря НАН України, Київ, Україна

Резюме: 4-(5-Заміщені фуран-2-іл)бензойні кислоти з піразолоновим фрагментом були оцінені *in vitro* як інгібітори цитозольних глутатіон *S*-трансфераз людини A1-1 та P1-1, які беруть участь у клітинних механізмах лікарської стійкості та канцерогенезу. При інгібуванні GSTA1-1 сполуки демонстрували мікромолярні та наномолярні значення IC_{50}, які залежали від природи замісника в положенні 3 піразолонового кільця. Зокрема, ефективність інгібування GSTA1-1 зростала, коли метильна група в положенні 3 піразолонового циклу була замінена трифлуорометильним, фенільним, 4-флуорофенільним, тіофен-2-ільним або піридин-4-ільним замісником. Вплив 4-(5-заміщених фуран-2-іл)бензойних кислот на GSTP1-1 був приблизно на порядок нижчим, ніж на GSTA1-1. При цьому в обох випадках сполука, що містить 1-феніл-3-(4-нітрофеніл)-заміщений піразолоновий фрагмент, демонструвала найкращу інгібувальну активність серед досліджених похідних. Результати молекулярного докінгу вказують на те, що інгібітори можуть взаємодіяти з GSTA1-1 переважно за участю амінокислотних залишків з G-сайту, тоді як у випадку GSTP1-1 сполуки зв'язуються в H-сайті.

Ключові слова: 4-(фуран-2-іл)бензойні кислоти; піразолони; глутатіон *S*-трансферази; інгібування.