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Anti-inflammatory activity study of the 3-amino-2-thioxo-2,3-dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-one-6-carboxylic acid derivatives and their fused heterocyclic systems

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ABSTRACT

The study of anti-inflammatory and analgesic activity of some 3-amino-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one-6-carboxylic acid derivatives, and the fused heterocyclic systems obtained by their modification allowed to find the compounds with the pharmacological effects similar to the effects of the drug "Ortofen" (diclofenac sodium). The ulcerogenity indices for the compounds obtained were found to be less than the index of the reference-drug; the acute toxicity study showed that the tested compounds are practically non-toxic. The results of docking study disclosed that some of the tested compounds may bind the active site of COX-2.

Key words: thiophene, pyrimidine, carboxylic acids, inflammation, analgesia.

INTRODUCTION

The derivatives of 3-amino-2-thioxo-2,3-dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-ones and more complex compounds obtained via their chemical modification attract researchers as the potent anti-inflammatory and analgesic agents. The series of the papers were published about the anti-inflammatory activity screening study for *N*-[2-(alkyl/arylthio)-4-oxothieno[2,3-*d*]pyrimidin-3(4*H*)-yl]methanesulfonamides [1,2]. The results of molecular modeling for these compounds allowed determining of the substances with the high affinity to the active site of cyclooxygenase-2 [3]. Anti-inflammatory properties were also discovered for 3-amino-2-thioxo-2,3-dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-ones with amino group at position 3 modified with the fragment of thiourea; the activity for some of these compounds was higher than activity of diclofenac sodium in the experiment [4]. The authors [5] discovered the anti-inflammatory properties for thiadiazolo[2,3-*b*]-6,7,8,9-terahydrobenzo(*b*)thieno[3,2-*e*]pyrimidin-5(4*H*)-ones obtained by cyclization of the starting 3-amino-2-mercapto-5,6,7,8-tetrahydro[1] benzothieno[2,3-*d*] pyrimidin-4(3*H*)-one, while the products of its S-alkylation with phenacyl bromides were inactive.

The recent papers also report the anti-inflammatory properties not only for the core alkyl 3-amino-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidin-6-carboxylates [6], but also for the structures with the sulfur atom at position 2 modified with monosaccharide unit [7]. Regarding the known anti-inflammatory properties of 3-amino-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidin-6-carboxylic acid derivatives, together with the fused systems of heterocycles obtained starting from these building blocks, we studied anti-inflammatory and analgesic activities for some compound from this class.

MATERIALS AND METHODS

In the course of the work synthetic organic chemistry methods and instrumental methods of chemical analysis (^{1}H NMR, chromato-mass spectrometry and elemental analysis) were used. Melting points were determined with Kofler

melting point apparatus and were not corrected. ^{1}H NMR spectra were measured with Varian Mercury (200 MHz) device using TMS as an internal standard; chemical shifts (δ) are reported in ppm. LC-MS spectra were obtained by PE SCIEX API 150EX mass-spectrometer. Elemental analyses were within ± 0.4% of the theoretical value. All of the reagents were obtained from the commercial sources and used without additional purification. The starting derivatives 3-amino-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidin-6-carboxyxlic acids **1a-c** were obtained by the previously reported methods [8,9].

Ethyl 3-amino-5-methyl-2-[(4-methylbenzyl)thio]-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-6-carboxylate 2.
To the solution of ethyl 3-amino-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidin-6-carboxylate **1a** (3.0 g) and 1.5 ml of thriethylamine in 20 ml of dimethylformamide (DMF) 1.5 ml of 4-methylbenzyl chloride was added. The reaction mixture was stirred and heated at 50-70°C for 5 hours. Then the reaction mixture was diluted with water and the precipitate formed was filtered of and crystallized from 2-propanol-DMF mixture.

Yield 78%. M.p. > 172-174 ˚C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.29 (3H, t, $COOCH_2CH_3$); 2.22 (3H, s, $ArCH_3$); 2.79 (3H, s, CH_3); 4.30 (4H, q, $COOCH_2CH_3$ + SCH_2Ar); 5.74 (1H, s, NH_2), 7.12 (2H, d, 3'H+5'H); 7.31 (2H, d, 2'H+6'H). LC-MS, m/z 390.6 ($[M]^+$). Found, %: C 55.32, H 5.48, N 10.90. $C_{18}H_{19}N_3O_3S_2$. Calculated, %: C 55,22, H 5.41, N 10.73. M.w. 391.51.

General method for synthesis of alkyl 8-oxo-8*H*-[1,3,4]thiadiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidine-6-carboxylates 3 and 4.
The suspension of ethyl 3-amino-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidin-6-carboxylate **1a** (3.0 g) in the mixture of the corresponding carboxylic acid (25 ml) and polyphosphoric acid (25 ml) was heated at 170°C for 5-7 hours. Then the cool reaction mixture was diluted with water and neutralized with concentrated solution of sodium hydroxide. The precipitate formed was filtered off and washed with plenty of water. Compounds **3** and **4** were additionally purified by boiling in ethanol.

Methyl 2,7-dimethyl-8-oxo-8*H*-[1,3,4]thiadiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidine-6-carboxylate 3: Yield 55%. M.p. > 255-257 ˚C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.81 (3H, s, CH_3); 2.69 (3H, s, $C\underline{H}_3$); 3.82 (3H, s, $COOC\underline{H}_3$). LC-MS, m/z 296.3 ($[MH]^+$). Found, %: C 44.82, H 3.15, N 14.12. $C_{11}H_9N_3O_3S_2$. Calculated, %: C 44.74, H 3.07, N 14.23. M.w. 295.34.

Methyl 2-*iso*-propyl-7-methyl-8-oxo-8*H*-[1,3,4]thiadiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidine-6-carboxylate 4:
Yield 58%. M.p. > 210-212 ˚C. ^{1}H ЯМР (200 MHz, DMSO-d_6) δ м.д.: 1.34 (6H, m, $CH(C\underline{H}_3)_2$); 2.81 (3H, s, CH_3); 3.45 (1H, m, $C\underline{H}(CH_3)_2$), 3.82 (3H, s, $COOC\underline{H}_3$). LC-MS, m/z 324.3 ($[MH]^+$). Found, %: C 48.35, H 4.18, N 12.88. $C_{13}H_{13}N_3O_3S_2$. Calculated, %: C 48.28, H 4.05, N 12.99. M.w. 323.39.

***N*-(2-Chlorophenyl)-8-methyl-9-oxo-3-phenyl-3H,9H-thieno[2',3':4,5]pyrimido[2,1-*b*][1,3,4]thiadiazine-7-carboxamide 5.**
The mixture of 5.0 g of 3-amino-*N*-(2-chlorophenyl)-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidine-6-carboxamide **1c** and 2.27 g of phenacyl bromide in 20 ml of dimethylformamide was heated at 130°C for 3 hours. The precipitate formed was filtered and washed with ethanol, till the transparent ethanol passed the filter pad.

Yiled 73 %. M.p. > 296-298 ˚C. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.79 (3H, s, CH_3); 4.37 (2H, s, CH_2); 7.34 (2H, m, 3H+4H); 7.6 (5H, m, 2H+5H+3'H+4'H+5'H); 8.04 (2H, d, 2'H+6'H); 9.97 (1H, s, NH). Found, %: C 56.70, H 3.19, N 11.92. $C_{22}H_{15}ClN_4O_2S_2$. Calculated, %: C 56.59, H 3.24, N 12.00. M.w. 466.97.

Analgesic activity. The influence of the compounds obtained on the nociceptive system was studied using the acetic acid induced writhing response in mice [10]. For the assessment of analgesic activity both male and female mice (18-20 g, six animals in each group) were used. "Ortofen" (diclofenac sodium) was used as the reference drug.

Analgesic activity for the tested compounds was estimated by their ability to reduce the number of writhes and stretching in the experimental group of animals against the control and the percentage of protection was calculated by the formula:

$$AA = \frac{(C_c - C_e)}{C_c} \times 100\%,$$

where AA – percentage of analgesic activity %;

C_c – the average number of writhes and stretching in the control group;
C_e – the average number of writhes and stretching in the experimental group.

For the comparative estimation of the analgesic effect ED_{50} and their confidence intervals using the least square method were calculated [11].

Anti-inflammatory activity. The influence of the synthesized compounds on the exudative phase of inflammation was studied using carrageenan-induced rat paw edema model. The white albino rats (180-220 g, six animals in each group) were used in the experiment [12,13].

Evaluation of ulcerogenicity index. Ulceration in rats was studied as described by Marazzi-Uberti [14]; the reference drug "Ortofen" (diclofenac sodium) was applied. For the experiment the white albino rats (180-220 g, seven animals in each group) were used. Before the experiment animals were fasted and treated only with water for 24 hours; after the animals of the experimental group were orally administered with the Tween 80 suspension of the compounds tested (dosage 1 mg/kg). "Ortofen" (diclofenac sodium) was administered in dosage 8 mg/kg according to its ED_{50} for antiexudative effect. The control group animals were treated with equivalent amount of distilled water. After the 3 hour of experiment animals were decapitated and the stomach mucosa was investigated. The following criteria were used to evaluate the damage: 0,5 point – edema, hyperemia tiny petechiae, 1 point – 2 or 3 small ulcers; 2 points – more than 3 small ulcers; 3 points – sizable ulcer; 4 points – few sizable ulcers; 5 points – perforated ulcer.

Acute toxicity. In order to investigate the symptoms of acute poisoning and to determine the lethal dosages (LD_{50}) of the tested compounds express method of T.V. Pastushenko and *co-authors* was applied [12,15]. The toxicity study was carried out using male and female albino mice weighing 18 – 25 g each; the single oral dose of each compound was administered.

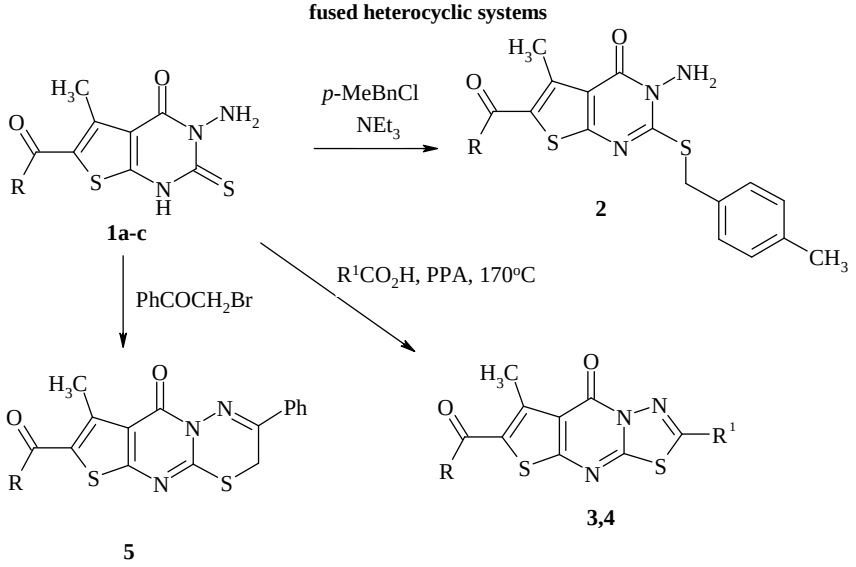
Computer modeling and docking studies were performed using the following computer programs: ISISDraw 2.3, Discovery studio Visualizer 4.0, Python molecule viewer and Autodock Vina. Docking was calculated for the flexible ligands and rigid models of proteins. Crystallographic data for COX-1 (PDB ID 1EQG) and COX-2 (PDB ID1CX2) proteins were obtained from the Protein Data Bank (http://www.rcsb.org/pdb). Water molecules and ligands were removed from the protein molecule along with the chains B, C and D. The calibration of the system was performed using the crystallographic data for Ibuprofen complex with COX-1 and results of docking calculations for Ibuprofen molecule model and COX-2.

RESULTS AND DISCUSSION

The compounds used in the screening experiments for analgesic, anti-inflammatory and ulcerogenic activity were obtained starting from the derivatives of 3-amino-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidin-6-carboxylic acid **1a-c**, prepared according to the previously reported methods [8,9]. The modification of the building blocks **1a-c** *via* S-alkylation or cyclization with carboxylic acids and phenacyl bromide resulted in the target compounds **2-5** (scheme). The structures of the compounds obtained were assigned by ^{1}H NMR data. For all of the compounds in ^{1}H NMR spectra the signals of thiophene methyl group are observed in the range 2.79 – 2.82 ppm. The spectrum of the compound **2** contains the signals of ethyl radical as a triplet of CH_3 group at 1.29 ppm and a signal of CH_2 at 4.30, which forms a multiplet signal resulted from its overlap with the CH_2 group peak of the benzyl fragment. The signal of amino protons is observed at 5.74 ppm as an intensive singlet.

The ^{1}H NMR spectra of compounds **3** and **4** contain the typical signals of carbmethoxy group protons at 3.82 ppm and also the signals of the substituents at position 2 of heterocyclic system (for the derivative **3** it is a singlet of CH_3 at 2.69 ppm, for 4 – the signals of *iso*-propyl radical as 1.34 [6H, m] and 3.45 [1H, m]). ^{1}H NMR spectrum of the amide **5** contains the signal of NH proton at 9.97 ppm and the signals of nine aromatic protons.

Scheme: Preparation of 3-amino-2-mercapto-5-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-6-carboxylic acid derivatives and their fused heterocyclic systems



1a R = -OEt, **1b** R = -OMe, **1c** R = -NH(2-ClPh); **2** R = -OEt; **3** R = -OMe, R^1 = Me; **4** R = -OMe, R^1 = i-Pr; **5** R = -NH(2-ClPh).

Table 1 The results of analgesic and anti-inflammatory activity study for the derivatives of 5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid 1a, 2-5

Compnd. №	dosage, mg/kg	analgesic activity, %	ED_{50}, mg/kg	dosage, mg/kg	mean anti-inflammatory activity, % (5 hours)	ED, mg/kg
1a	0.1	54.45±1.15	1.11	-	-	–
	1.0	30.10±3.87		1.0	-	
	5.0	61.79±8.48		10.0	18.57±4.62	
2	0.1	66.36±3.33	0.70	0.1	21.47±4.76	ED_{40} = 1.10
	1.0	20.25±14.84		1.0	47.58±2.65	
	5.0	26.79±14.51		5.0	42.57±3.23	
	10.0	89.01±1.20		10.0	47.04±2.59	
	15.0	79.07±9.98		15.0	30.41±6.82	
3	1.0	43.98±2.62	1.42	0.1	42.06±3.43	ED_{40} = 1.07
	5.0	34.65±15.37		0.5	5.26±3.37	
				1.0	34.1±1.76	
	10.0	70.68±3.14		5.0	27.5±3.69	
				10.0	18.72±2.17	
4	1.0	71.34±3.45	0.70	1.0	16.39±39	–
	5.0	62.31±3.22		5.0	-	
	10.0	68.85±3.39		10.0	-	
5	1.0	52.15±2.95	1.03	1.0	-	–
	5.0	52.96±2.97				
	10.0	74.45±3.52		10.0	13.69±4.93	
"**Ortofen**"	5.0	77.14±9.34	5.00	8.0	77.14±9.34	ED_{50} = 8.00

The results of the screening study showed that most of the compounds displayed significant analgesic activity as to their ED_{50} (table 1). All of the tested compounds **1a** (ED_{50}=1,11 mg/kg), **2** (ED_{50}=0.7 mg/kg), **3** (ED_{50}=1.42 mg/kg), **4** (ED_{50}=0.7 mg/kg), **5** (ED_{50}=1.03 mg/kg) showed higher analgesic effect than the reference drug "Ortofen" ($ЕД_{50}$=5 mg/kg). The derivatives **1a**, **4** and **5** have not displayed sufficient anti-inflammatory effect and were determined as non-competitive with the reference drug.

The results obtained showed that compounds **2** and **3** were determined as the most active derivatives of 5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid among the tested ones.

Considering the compounds **2** and **3** as potent non-steroidal anti-inflammatory drugs (NSAIDs), which mechanism of action includes suppression of prostaglandins synthesis, and also in the stomach mucosa, which results in ulceration, we decided to study their influence on the state of stomach mucosa using "Ortofen" as the reference drug. The results showed that the compounds **2** and **3** tested have no influence on the stomach mucosa.

The results of the preliminary acute toxicity study and the lethal dosages (LD_{50}) of the compounds **2** and **3** were obtained by express method in mice [12,15]. The LD_{50} values were determined till the minimal dose which

administration gave the lethal effect. The results of this experiment were evaluated using the standard classification [12,16].

To investigate the mechanism of the compounds tested we calculated the binding of the compounds **1a**, **2-5** with the active site of both isoforms of cyclooxygenase (COx-1 and COX-2). It is known that this enzyme is the target for most of the modern NSAIDs.

Table 2 The results of ulcerogenicity and acute toxicity investigation for the derivatives of 5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid 2 and 3

Comp'd. №	dosage, mg/kg	degree of ulceration, points	LD_{50}, mg/kg
control	–	–	–
2	1.0	0.05 ± 0.03*	5860 (4840÷6880)
3	1.0	0.07 ± 0.07*	5860 (4840÷6880)
"Ortofen"	8.0	2.29 ± 0.46	

**– statistically significant differences between the "Ortofen" treated group, p<0,05.*

The previously reported investigations showed that most of NSAIDs bind with the pocket formed with the following amino acids His-90, Arg-120, Tyr-355, Tyr-385, Arg-513, Val-523 and Ser-530 [17-20]. For aspirine it was determined that it reversibly inhibits cyclooxygenase by acetylation of Ser-580 [21].

The docking results showed that most of the tested compounds do not bind the active site of COX-1. But the compounds **1a** and **3** were determined as the ligands that effectively binded the site of COX-2. It is interesting that for the compound **2**, which showed valuable results in the experiment (anti-inflammatory ED_{40} = 1.10 mg/kg and analgesic ED_{50} = 0.70), the docking results showed no effective binding with COX-2 active site. This can be explained by the possible metabolism of the compound **2**; probably not this compound but its metabolite shows high activity. The analysis of docking studies evidently confirms that diminution of the subsistent size at positions 2 and 3 of thieno[2,3-*d*]pyrimidine system improves the binding properties of the ligands for COX-2 active site.

Table 3 The results of docking studies of derivatives of 5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid 1a, 2-5 with COX-1 and COX-2*

Compnd. №	COX-1	COX-2
1a	– His-90**	+ Arg-120 Tyr-355 Tyr-385 Val-523 Ser-530**
2	– His-90**	– Arg-120
3	– His-90**	+ Arg-120 Tyr-355 Tyr-385 Ser-530**
4	–	– Arg-120**
5	– His-90**	– Arg-120**

** + - ligand engagement to the active site; – - no ligand engagement to the active site;*
*** - amino acids of the active site interacting with the ligand.*

CONCLUSION

The study of anti-inflammatory and analgesic activity of some 3-amino-2-thioxo-2,3-dihydrothieno[2,3-*d*]pyrimidin-4(1*H*)-one-6-carboxylic acid derivatives, and the fused heterocyclic systems obtained by their modification allowed to find the compounds with the analgesic and anti-inflammatory activities similar to the effects of the reference drug "Ortofen" (diclofenac sodium). The ulcerogenity indices for the most active compounds were found to be less than the index of the reference-drug; the acute toxicity study showed that the tested compounds are practically nontoxic. The results of docking study displayed that ethyl 3-amino-5-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydrothieno[2,3-*d*]pyrimidine-6-carboxylate **1a** and methyl 2,7-dimethyl-8-oxo-8H-[1,3,4]thiadiazolo[3,2-a]thieno[2,3-*d*]pyrimidine-6-carboxylate **3** bind the active site of COX-2, while the docking result for the other active compound — ethyl 3-amino-5-methyl-2-[(4-methylbenzyl)thio]-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-6-carboxylate **2** showed no effective binding with COX-2 active site. This can be explained by the possible metabolism of the compound **2**; probably not this compound, but its metabolite shows high activity.

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