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Original article

Antihelminthic activity of some newly synthesized 5(6)-(un)substituted-1*H*-benzimidazol-2-ylthioacetylpiperazine derivatives

Anelia Ts. Mavrova [a,*], Kamelya K. Anichina [a], Dimitar I. Vuchev [b], Jordan A. Tsenov [c], Pavletta S. Denkova [c], Magdalena S. Kondeva [d], Mitka K. Micheva [d]

[a] *Department of Organic Synthesis, University of Chemical Technology and Metallurgy, 8 Kliment Ohridski Blvd., 1756 Sofia, Bulgaria*
[b] *National Center of Infectious and Parasitic Diseases, 26 Yanko Sakazov Blvd., 1504 Sofia, Bulgaria*
[c] *Institute of Organic Chemistry, Bulgarian Academy of Science, 1113 Sofia, Bulgaria*
[d] *Department of Pharmacology and Toxicology, Medicinal University of Sofia, 2 Dunav street, 1504 Sofia, Bulgaria*

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Abstract

Piperazine derivatives of 5(6)-substituted-(1*H*-benzimidazol-2-ylthio)acetic acids were synthesized by using two methods and studied for antihelminthic activity.

The antiparasitic screening showed that compounds **18–24** exhibited higher activity against *Trichinella spiralis* in vitro in comparison to methyl 5-(propylthio)-1*H*-benzimidazol-2-yl-carbamate (albendazole). Most active were compounds 2-({2-[4-(4-chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio)-1*H*-benzimidazole **21** and 2-{[2-oxo-2-(4-benzhydrylpiperazin-1-yl)ethyl]thio}-5(6)-methyl-1(*H*)-benzimidazole **19** as well as 2-({2-[4-(4-chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio)-5(6)-methyl-1(*H*)-benzimidazole **23** with efficacy of 96.0%, 98.4% and 100%, respectively.

The tested derivatives **15–19** and **20–23** were less active against *Syphacia obvelata* in vivo than albendazole and exhibited the same efficacy as piperazine, but in twice lower concentration.Compounds 2-({2-[4-(4-chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio)-1*H*-benzimidazole **21**, 1,4-bis[(5(6)-methyl-1(*H*)-benzimidazol-2-ylthio)acetyl]piperazine **17** and 2-({2-[4-(4-chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio)-5(6)-methyl-1(*H*)-benzimidazole **23** had higher efficacies of 73%, 76%, and 77%, respectively.

Keywords: Benzimidazoles; 1*H*-Benzimidazol-2-ylthioacetylpiperazines; Antitrichinellosis activity; Antinematode activity; Hepatotoxicity

1. Introduction

The benzimidazole compounds possess broad spectrum of biological properties and were investigated for their antiviral (anti-HIV), anticancer and antibacterial activities [1–10]. Some of them found application in the above-mentioned fields of the medicine praxis. Many benzimidazole derivatives are widely used for the treatment of parasitic diseases. Their efficacy against helminthes and fungi resulted from their mode of action, which is expressed in the interruption and consequent blockade of microtubule assembly in nematode, cestode and trematode cells as well as in the violation of the glycose transport and fumarase inhibition [11–13]. Some of the benzimidazole drugs used in the parasitological praxis belong to the group of 2-amino-1*H*-benzimidazole analogues as benzimidazole carbamates, while the others contain 2-mercapto-1-*H*-benzimidazole moiety. It appeared that the benzimidazoles, which have been used in the chemotherapy of trichinellosis were unable to kill *Trichinella spiralis* larvae at an advanced stage of development. This fact indicates that the search for better drugs is still necessary.

The pathway that has been exploited successfully in the search for antiparasitic agents is the one for the synthesis of

* Corresponding author. Tel.: +359 2 8163207; fax: +359 2 9238401.
E-mail addresses: anmav@abv.bg (A.Ts. Mavrova), dvutchev@yahoo.com (D.I. Vuchev), magdalenakondeva@hotmail.com (M.S. Kondeva).

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polyamines playing essential roles in cell proliferation and cell differentiation. Many diamine analogues, which interfere with polyamines' metabolism have been synthesized and their antiparasitic effects against protozoa have been proved [14–17]. Among the diamines we choose piperazine because it is one of the earliest drugs used against ascaridosis and enterobiosis and which is in the structure of many antihelminthic pharmaceutical drugsadministered today. The piperazine compounds applied in the parasitological praxis exert hyperpolarization of muscle membrane and act as chloride channel opener and GABA agonist. From the other side the piperazine cycle participates as pharmacophore in the structure of many drugs, belonging to different pharmacological groups.

On that reason the synthesis of new benzimidazoles containing piperazine cycle and the investigation of their antihelminthic activity in order to compare it to the efficacy of albendazole and to the activity of the most important commercial antiparasitic drug ivermectin are of pharmacological interest.

In this paper we describe the synthesis of new piperazine derivatives of (1*H*-benzimidazol-2-ylthio)acetic acid and their activity in vivo against *Syphacia obvelata* and *T. spiralis* as well as their hepatotoxicity. In our previous paper we reported the synthesis and antitrichinellosis activity of some 2-substituted-[1,3]-thiazolo[3,2-*a*]benzimidazol-3(2*H*)-ones and consecutively this paper is a continuation of our work [18].

2. Chemistry

The synthesis of the piperazine derivatives of (1*H*-benzimidazol-2-ylthio)acetic acids is carried out as depicted in Figs. 1 and 2.

1*H*-Benzimidazole-2-thiols **1–3** were commercially available and compound **4** was prepared by refluxing ethanol–water solution of sodium hydroxide, carbon disulphide and 4-chloro-1,2-diamino-benzenes [19,20].

The 5(6)-(un)substituted-(1*H*-benzimidazol-2-ylthio)acetic acids (**5–8**) were obtained through William's reaction of 5(6)-(un)substituted-1*H*-benzimidazole-2-thiols (**1a–d**) with chloroacetic acid in the presence of sodium hydroxide by refluxing [20]. The yields were in the range of 59–75%.

1,3-Thiazolo[3,2-*a*]benzimidazol-3(2*H*)ones **9–10** and **12** were synthesized by heating 5(6)-(un)substituted-(1*H*-benzimidazol-2-ylthio)acetic acids **5**, **6**, **8** with acetic anhydride in pyridine medium on steam bath [20]. The cyclization of (benzimidazol-2-ylthio)acetic acids **7** in the presence of dicyclohexyl carbodiimide (DCC) at room temperature resulted in compound **11**.

The hydrolysis of [1,3]-thiazolo[3,2-*a*]benzimidazol-3(2*H*)-ones with an appropriated *N*-monosubstituted piperazine in ethanol medium under refluxing conditions for 3 h resulted in the piperazine derivatives of (benzimidazol-2-ylthio)acetic acids **15–26** (Method A). Compounds **13** and **14** were obtained from 2-(2-hydroxy-ethylamino)-ethanol and the corresponding 4-substituted-anilines through the procedure given in Ref. [21]; piperazine and benzhydrylpiperazine were commercially available.

Compounds **16**, **17**, **19**, **22**, **24** and **25** were prepared also through direct condensation of (benzimidazol-2-ylthio)acetic acid with the corresponding piperazine at 5–10 °C in the presence of DCC for 20–25 h (Method B).

The chemical structures of compounds **15–26** were established by elemental analyses, IR, ^{1}H NMR and ^{13}C NMR spectra as well as mass spectral data for compounds **15**, **16**, **20** and **21** and the results are presented in Section 7. The elemental analyses indicated by the symbols of the element were within ±0.4% of theoretical values. The IR spectra of piperazine derivatives **15–26** were quite similar. The characteristic absorption bands at 1640–1660 were observed in the IR spectra.

The analysis of the NMR spectra was done taking into consideration the electronic effect of the respective substituents and the structure numbering used for the description of the ^{1}H NMR spectra is given in Fig. 1. For compounds **21** and **22** the 2D heteronuclear ^{1}H–^{13}C correlation spectra (HMQC technique – heteronuclear multiple quantum correlation) were recorded to confirm the assignment of the signals in ^{1}H and ^{13}C spectra.

3. Pharmacology

3.1. Antihelminthic activity

The 1*H*-benzimidazol-2-ylthioacetylpiperazine derivatives **18–24** were evaluated for their activity against *T. spiralis* in vitro as well as their antinematode activity against *S. obvelata* in vivo. The hepatotoxicity effect of compounds **15**, **17**, **18**, and **20–23** on the viability of isolated rat hepatocytes in vitro was studied.

4. Results

4.1. Antitrichinellosis activity

The in vitro parasitological study showed that most of the tested compounds exhibited higher activity than albendazole's effect against *T. spiralis* and comparable to that of ivermectin (Table 1). Compounds **21**, **23** and **19** demonstrated 96.0%, 98.2% and 100% activities, respectively, at a dose of 200 μg/mL after 48 h, expressed in suppressing motor activity of larvae and opening of their spiral form. In the control samples with physiological solution and the control samples with DMSO all *T. spiralis* larvae had spiral form i.e. are vital [11,12].

4.2. Activity against S. obvelata

The results for antinematode activity determined three days after finishing the three-day-treatment course of invasion with *S. obvelata* white mice are systematized and the average number of parasites of each experimental group is given in Table 2. The data indicated that the tested compounds exhibit different antinematode effect which is less than the effect of the referent albendazole. The most active compounds were **17**, **21** and **23** with 76%, 73% and 77% activities, respectively [22].

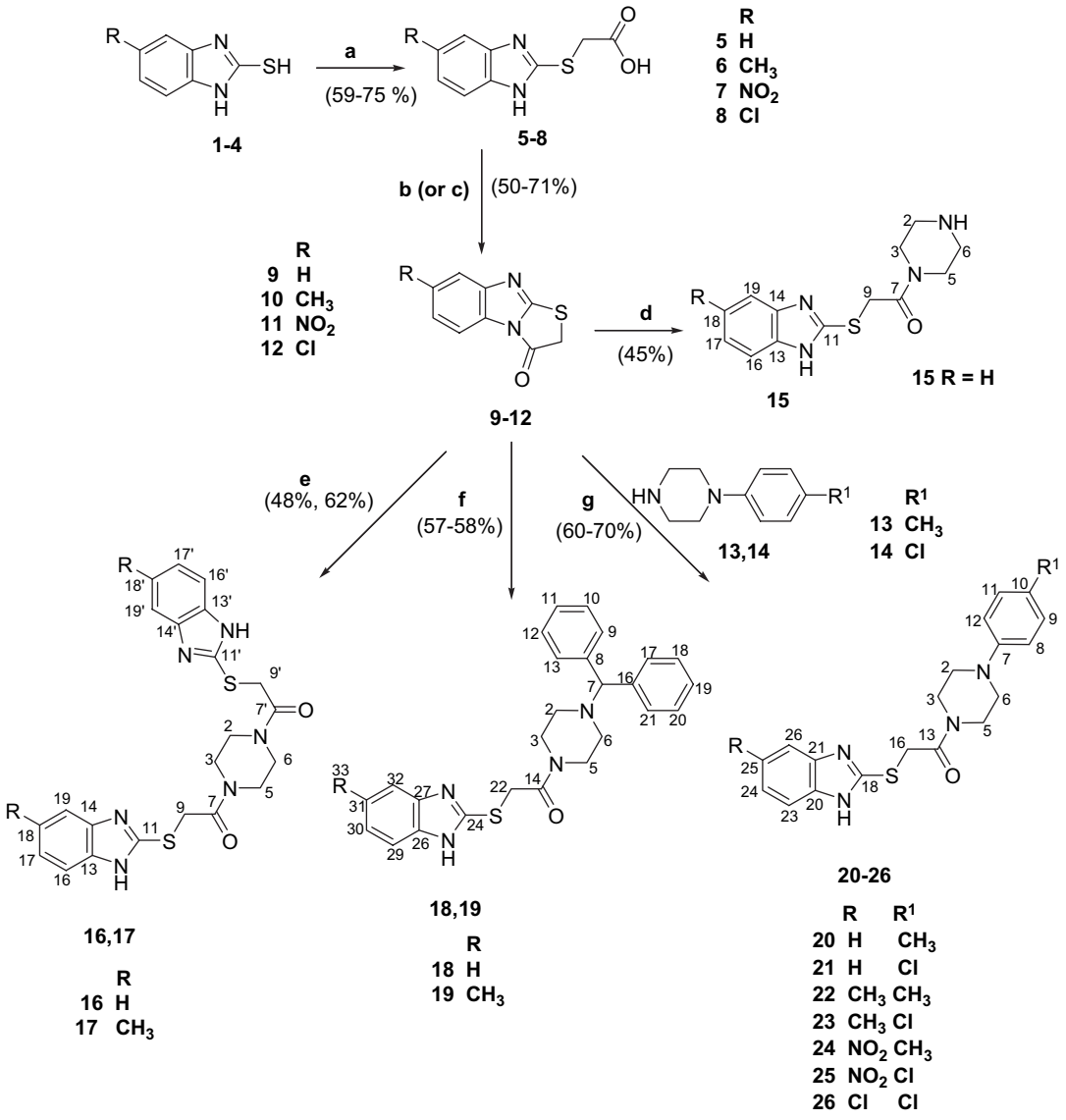

Fig. 1. Synthesis of 1*H*-benzimidazol-2-ylthioacetylpiperazine derivatives **15**–**26** (Method A) and structure numbering used in NMR spectra description. Reagents and conditions: (a) $ClCH_2COOH$, NaOH, ethanol; (b) Ac_2O/Py, reflux; (c) DDC/Py, reflux; (d) piperazine hexahydrate, C_2H_5OH, reflux; (e) **9**, **10**, piperazine hexahydrate; (f) benzhydrylpiperazine ethanol, reflux; (g) C_2H_5OH, reflux.

4.3. Hepatotoxicity

The hepatotoxicity of compounds **15**, **17**, **18**, and **20**–**23** on the viability of isolated rat hepatocytes was determined towards the control group (untreated cells) as well as compared to the hepatotoxicity of albendazole and the results are given in Table 3 [23,24].

5. Discussion

5.1. Chemistry

In a basic medium the condensed thiazole cycle underwent a cleavage [25]. Because of these properties of the above-mentioned compounds we choose the reaction between [1,3]-thiazolo[3,2-*a*]benzimidazol-3(2*H*)-one and amines among the different methods for synthesis of (benzimidazol-2-ylthio)acetamides (Method A). This non-traditional reaction offers the possibility for preparing large number of compounds by means of a simple procedure through introduction of various substituents both in the 5(6)-position of thiazolobenzimidazole ring and in the second place of thiazole cycle. Some 2-[(2-oxo-2-piperazin-1-ylethyl)thio]-1*H*-benzimidazoles were obtained by direct condensation between 5(6)-(un)substituted-(1*H*-benzimidazol-2-ylthio)acetic acids and *N*-substituted piperazines in the presence of DCC in pyridine medium (Method B). Irrespective of the less number of reaction stages of Method B, Method A was preferred because of the simple work-up procedure and the higher purity of the synthesized substances.

5.2. Antitrichinellosis activity

The most piperazine derivatives, excluding **18** and **24**, exhibited significant larvocide activity against *T. spiralis* larvae.

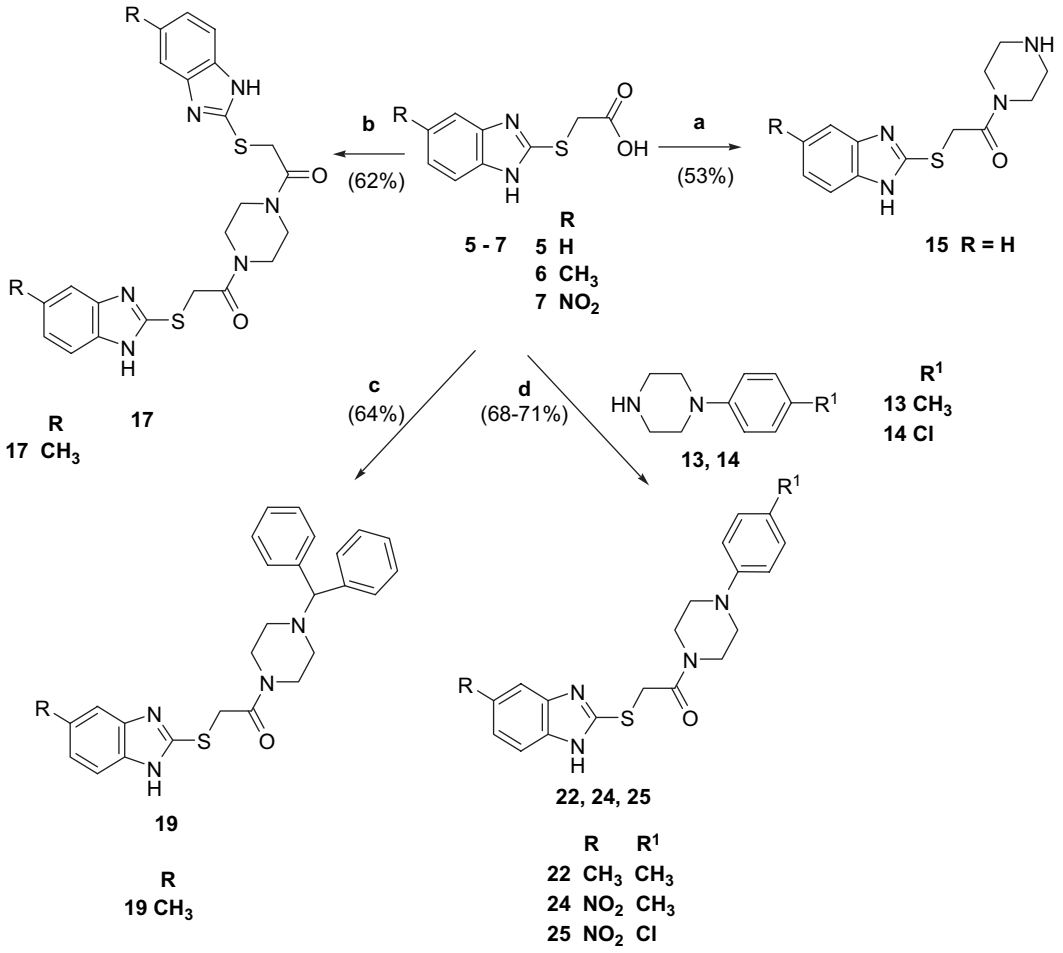

Fig. 2. Synthesis of compounds **15**, **17**, **19**, **22**, **24** and **25** (Method B). Reagents and conditions: (a) piperazine hexahydrate, DCC/Py, 5−10 °C; (b) compound **6**, piperazine hexahydrate, DCC/Py, 5−10 °C; (c) compound **6**, benzhydrylpiperazine, DCC/Py, 5−10 °C; (d) DCC/Py, 5−10 °C.

In parasitological experiment in vitro for antitrichinellosis activity it was ascertained that the tested compounds **18**, **19**, and **20**−**24** exert antihelminthic effect, expressed in suppressing motor activity of larvae and opening of their spiral form. In the first control group with physiological solution and in the second with DMSO practically all larvae were in spiral form.

All piperazine derivatives of (benzimidazol-2-ylthio)acetic acid exhibited higher activity against *T. spiralis* larvae in regards to the activity of albendazole, moreover compounds **19**, **21** and **23** manifested antitrichinellosis activity, which surpassed five times the activity of albendazole. Compounds **19**−**23** demonstrated higher effect against *T. spiralis* larvae at a concentration of 200 μg/mL than ivermectin after 24 h. At the same concentration the efficacy of compounds **19**, **21**, and **23** was also higher than the efficacy of ivermectin after 48 h.

Compounds **22**−**24** containing methyl substituent at 5(6)-position of benzimidazole ring showed higher antihelminthic activity in comparison to the unsubstituted compounds **18**, **20** and **21**. Compound **19** having methyl group in the 5(6)-position of benzimidazole ring was distinguished for 92.7% efficacy at a concentration of 200 μg/mL, while compound **18** exhibited 13.6% larvocide effect at the same concentration after 24 h. The products possessing chlorine atom as substituent at fourth position of benzene cycle (**21** and **23**) were more active than those containing methyl group at the same place (**20** and **22**).

Due to the obtained good results antitrichinellosis effect in vitro, further research is currently in progress for the antitrichinellosis activity in intestinal and muscle phase.

5.3. Antihelminthic activity against S. obvelata

The analysis of the results obtained by the study of the tested and the control groups ascertained statistically significant difference in the level of parasites' invasion ($p < 0.05$). Albendazole and piperazin hexahydrate were used as referent compounds. The control test carried out with DMSO as well as the control group of white mice (without treatment) did not indicate real reduction of the infection and the number of parasites was equal. All tested products excluding **15** and 1**8** showed a significant antihelminthic effect. The efficacy of each compound was established according to Ref. [22] (Eq. (1)).

$$E = [(I_c - I_t)/I_c] \times 100 \quad (1)$$

Table 1
Antihelminthic activity of compounds **18**–**24** against *Trichinella spiralis*

Compound no.	Efficacy (%) after 24 h			Comp. no.	Efficacy (%) after 48 h		
	50 μg/mL	100 μg/mL	200 μg/mL		50 μg/mL	100 μg/mL	200 μg/mL
18	11.2	13.6	13.6	**18**	19.5	19.7	53.9
19	37.4	75.8	92.7	**19**	44.0	88.0	100
20	19.5	48.0	78.8	**20**	21.0	63.0	80.0
21	29.6	64.7	82.6	**21**	33.5	83.0	96.0
22	26.5	56.8	80.6	**22**	34.7	63.0	82.1
23	35.8	72.0	86.5	**23**	41.0	92.7	98.4
24	11.4	15.6	31.2	**24**	14.3	16.0	37.8
Albendazole	10.6	10.7	13.3	Albendazole	14.8	15.1	17.4
Ivermectin	45.4	48.7	54.2	Ivermectin	62.1	78.4	88.3

E − efficacy, I_c − intensity of invasion in the control group e.g. the number of parasites; I_t − intensity of invasion in the treatment group.

Effective compound was accepted compound, which possesses an efficacy higher than 50%.

The highest activity against *S. obvelata* manifested compounds **17**, **21** and **23** ($E = 73\%$, 70% and 71% in a dose of 20 mg/kg mw). The antinematode activity of compounds **16**, **20** and **19** in a dose of 20 mg/kg mw was similar to the activity of piperazine hexahydrate in a dose of 100 mg/kg mw, but in comparison to albendazole all tested compounds were less active. The five times increase of the dose exerts insignificant influence on the therapeutic effect.

The obtaining of an acceptable dose–response curve depends on the biological characteristic of the parasites and on how much is previously known about the biological activity and the safety of the newly tested compounds, therefore additional experiments are required using lower or higher concentrations.

At a concentration of 0.200 μg/kg mw ivermectin showed an efficacy of 64% that is lower than the efficacy of compounds **17**, **21** and **23** at a concentration of 20 mg/kg mw.

By the establishment of the number of parasites after treatment, a large number of nematode larvae were observed by the microscopic study of the intestinal content both in the experimental animals and in the control groups. On the grounds of this observation we accepted that the tested compounds exhibited antihelminthic activity, expressed only against imaginal forms of nematode but not against larvae.

5.4. Hepatotoxicity

The purpose of our study using isolated rat hepatocytes was to compare whether the new compounds showed more toxic effect than albendazole. As it is well known that albendazole exerts hepatotoxic effect, we used it as a control, versus which, to trace the effect of the new compounds (more toxic/less toxic) [26,27]. The effect of the compounds on cell viability was investigated by using a trypan blue test [23,24].

The results obtained by the study showed that albendazole reduced the cells vitality by 56%. The piperazine containing benzimidazole **20** was less hepatotoxic than albendazole and preserved with 15% the cell viability regarding to albendazole, while the most active compounds **23** and **19** showed hepatotoxicity comparable to that of albendazole (7%, respectively, 2%). Compounds **20** and **19** comprising methyl group at 5(6)-position of benzimidazole cycle were less hepatotoxic

Table 2
Efficacy (E) of compounds **15**–**23** against *Syphacia obvelata*

Compound no.	20 mg/kg mw		100 mg/kg mw	
	Average number of parasites in groups[a]	E (%)	Average number of parasites in groups[a]	E (%)
15	84.4 ± 20.58	11	81.4 ± 9.2	14
16	37.0 ± 10.5	61	34.2 ± 11.6	64
17	25.2 ± 26.7	73	22.8 ± 21.2	76
18	83.4 ± 11.9	12	79.0 ± 15.8	17
19	36.6 ± 42.2	63	29.9 ± 30.1	69
20	32.0 ± 7.87	66	30.2 ± 14.7	68
21	34.8 ± 25.6	70	25.6 ± 13.9	73
22	28.2 ± 14.1	68	28.4 ± 15.1	70
23	29.4 ± 27.4	71	21.8 ± 8.9	77
Albendazole	14.2 ± 7.36	85	0.8 ± 1.3	99
Piperazine hexahydrate	–		37.0 ± 22.1	61
Ivermectin[b]	33.87 ± 24.7	64		
Control group	94.0 ± 44.9		94.0 ± 44.9	

[a] $p < 0.05$.
[b] At concentration of 0.200 μg/kg mw.

Table 3
Effect of benzimidazole derivatives on the cell viability

Compounds no.	Cell viability	Effect versus control (%)	Effect versus albendazole (%)
Control	93 ± 3.5[a]	100	
Albendazole	41 ± 2.0[a]	56↓	
15	27 ± 4.3[a,b]	71↓	34↓
17	34 ± 2.9[a,b]	63↓	17↓
18	20 ± 1.6[a,b]	78↓	51↓
19	42 ± 2.9[a]	55↓	
20	47 ± 4.5[a]	49↓	15↑
21	29 ± 2.5[a,b]	69↓	29↓
22	28 ± 4.0[a,b]	70↓	32↓
23	44 ± 2.9[a]	53↓	7↑

[a] $p < 0.05$ versus control.
[b] $p < 0.05$ versus albendazole.

than their unsubstituted analogues **21** and **18** (R = H), but this relation was not affirmed by the derivatives **20** and **22**. Compound **18**, which showed the lowest activity both against *T. spiralis* larvae and *S. obvelata* was most hepatotoxic compared to albendazole.

6. Conclusion

New piperazine derivatives of 5(6)-substituted-(1*H*-benzimidazol-2-ylthio)acetic acids were synthesized by using two methods and studied for antihelminthic activity.

The in vitro and in vivo screening for antinematode effect showed that the compounds exhibit significant antiparasitic activity. All tested piperazine derivatives of (1*H*-benzimidazol-2-ylthio)acetic acids exhibited higher activity against *T. spiralis* larvae in comparison to albendazole, furthermore some of the compounds (**21**, **23** and **19**) demonstrated antitrichinellosis activity, which surpassed five times the activity of albendazole. With respect to ivermectin compounds **20**, **21** and **19**, **22–23** demonstrated higher effect against *T. spiralis* larvae at a concentration of 200 μg/mL after 24 h. At the same concentration the efficacy of compounds **19**, **21** and **23** was also higher than the efficacy of ivermectin after 48 h.

The pharmaco-therapeutic study in vivo showed that the tested compounds were less active than albendazole against *S. obvelata* and have equal efficacy in a twice lower concentration related to piperazine. Moreover it was ascertained that the 5(6)-substituted-1*H*-benzimidazol-2-ylthioacetylpiperazine derivatives were active against the mature nematodes but not against the larvae. Nevertheless all tested compounds exhibited 60–77% efficacy and as it is known effective compounds were accepted compounds which demonstrate efficacy higher than 50%.

The most active 5(6)-(un)substituted-1*H*-benzimidazol-2-ylthioacetylpiperazine derivatives possess hepatotoxicity comparable or less than that of albendazole.

These results also confirmed the hypothesis that the introduction of a piperazine ring into the structure of 5(6)-(1*H*-benzimidazol-2-ylthio)acetic acid is favourable for the interaction of these molecules with the biological targets.

7. Experimental part

Melting points (mp) were determined on an Electrothermal AZ 9000 3MK4 apparatus and were uncorrected. The thin layer chromatography (TLC, R_f values) was performed on silica gel 60 plates F_{254} (Merck, 0.2 mm thick) using mobile phase benzene:methanol − 3:1, and visualization was effected with ultraviolet light. IR spectra were recorded on a Specord 71 IR spectrophotometer as potassium bromide discs. All NMR spectra were recorded on a Bruker Avance DRX 250 spectrometer (Bruker, Faelanden, Switzerland) operating at 250.13 MHz for ^{1}H and at 62.89 MHz for ^{13}C, using a dual 5 mm ^{1}H/^{13}C probehead. The studied compounds were dissolved in $CDCl_3$ or DMSO-d_6 (see Section 5.1 for details). Chemical shifts were expressed relative to tetramethylsilane (TMS) and were reported as δ (ppm). The measurements were carried out at ambient temperature (300 K). The atom numbering used for description of the spectra is shown in Fig. 1.

Mass spectrometric measurements were performed using an LCQ DECA instrument (Thermo Finnigan, Palo Alto, CA, USA). Compounds were solubilized in CH_3OH. Solutions (10^{-6} M) of compounds were prepared and directly infused into the ESI source. The ions were produced using spray voltage, capillary voltage and capillary temperature of 4 kV, 8 kV and 220 °C, respectively. The microanalyses for C, H, N and S were performed on Perkin–Elmer elemental analyzer.

7.1. Chemistry

7.1.1. General procedure for compound *4*

4-Chloro-1,2-diamino-benzene (0.019 mol) and water (3 mL) were added to a solution of sodium hydroxide (0.022 mol) in ethanol (20 mL) and carbon disulphide (0.022 mol). The mixture was heated under reflux for 3 h. Charcoal is then added cautiously, and after the mixture has been refluxed for 10 min the charcoal was removed by filtration. The filtrate is heated to 60–70 °C and quenched with warm water (70°, 20 mL), and then 50% acetic acid (9 mL) was added by good stirring. The product was separated and after cooling in refrigerator for 3 h the crystallization was completed. Yield: 69%.

7.1.2. General procedure for compounds *5–8*

Solution of sodium hydroxide (0.012 mol) in ethanol (14 mL) and 5(6)-(un)substituted-1*H*-benzimidazole-2-thiol (0.0067 mol) was refluxed for 1 h. After cooling, chloroacetic acid (0.0067 mol) was added and the refluxing was continued for 1–5 h more. Then the reaction mixture was cooled, poured into water and acidified with diluted acetic acid. The crystallized product was filtrated, carefully washed with water and re-crystallized. Yield: 59–75%.

7.1.3. General procedures for compounds *9–12*

Method A: solution of 5(6)-(un)substituted-(1*H*-benzimidazol-2ylthio)acetic acid (0.104 mol) and acetic anhydride (19.5 mL) in dry pyridine (65 mL) was heated by refluxing

for 10 min. Products were crystallized as pale orange sediment after cooling and water (100 mL) was added in small portions by stirring. Yield: 50–71%.

Method B: DCC (0.0144 mol) in pyridine (10 mL) was added dropwise to compounds **5–8** (0.0144 mol) diluted in dry pyridine (60 mL). The solution was stirred for 10–12 h at 5–10 °C. The precipitate of dicyclohexylurea was removed and the filtrate was evaporated under reduced pressure to dryness. The residue was dissolved in chloroform and filtrated after heating with charcoal. After cooling and filtering the products were re-crystallized.

*7.1.4. General procedures for compounds **15–26***

Method A: a mixture of 3.16 mmol [1,3]thiazolo[3,2-*a*]benzimidazol-3(2*H*)-one and 3.79 mmol *N*-substituted piperazine in 12 mL ethyl alcohol was refluxed for 3 h. The solvent was distillated under reduced pressure and the obtained oil product was washed with water several times in order to remove the excess of piperazine. By addition of ethanol to the oil the compounds crystallized out.

Method B: to 3.9 mmol *N*-substituted piperazine, dissolved in 5 mL dry pyridine solution of 3.9 mmol DCC in 5 mL pyridine was added. The solution was cooled down to 5–10 °C by stirring and 3.9 mmol of (1*H*-benzimidazol-2ylthio)acetic acid was added. The stirring went on for 20–25 h at the same temperature. The obtained precipitate of dicyclohexylurea was removed through filtration and the native solution was evaporated to dryness in vacuum. The obtained oil product was washed with water several times in order to remove the excess of piperazine, after that ethanol was added and the compounds crystallized out.

*7.1.4.1. 2-[(2-Oxo-2-piperazin-1-ylethyl)thio]-1H-benzimidazole **15*** Piperazine was used in twofold excess; yield – 45% (Method A), 53% (Method B); mp. 93–96 °C; re-crystallized with ethanol; $R_f = 0.81$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR (DMSO-d_6): 2.61–2.74 (m, 4H, $^2CH_2$–NH–$^6CH_2$); 3.37–3.60 (m, 4H, $^3CH_2$–NH–$^5CH_2$); 4.39 (s, 2H, SCH_2CO); 7.08–7.15 (m, 2H–^{17}CH, ^{18}CH, Ar–bz); 7.43 (m, 2H–^{16}CH, ^{19}CH, Ar–bz); 7.45 (br s, NH); ^{13}C NMR: 165.5 (C=O), 149.9 (C=N–S), 140.8 (2C–^{13}C, ^{14}C, Ar), 121.4 (2CH–^{17}CH, ^{18}CH, Ar), 113.7 (2CH–^{16}CH, ^{19}CH, Ar), 46.93 ($^2CH_2$–NH), 45.73 ($^6CH_2$–NH), 45.32 ($^3CH_2$–N), 42.82 ($^5CH_2$–N), 35.18 ($^9CH_2$–S). Analysis: calculated for $C_{13}H_{16}N_4OS$: C – 56.50, H – 5.84, N – 20.27, S – 11.60; found: C – 56.68, H – 5.61, N – 20.39, S – 11.48.

*7.1.4.2. 1,4-Bis[(1H-benzimidazol-2-ylthio)acetyl]piperazine **16*** The ratio between **9** and piperazine was 2:1; yield – 48% (Method A); mp. 275–277 °C; re-crystallized with ethanol; $R_f = 0.61$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR ($CDCl_3$): 2.88–2.97 (m, 4H, $^2CH_2$–NH–$^6CH_2$); 3.54–3.71 (m, 4H, $^3CH_2$–NH–$^5CH_2$); 3.95 (s, 4H, $2SCH_2CO$); 7.18–7.23 (m, 8H, 2Ar–bz); 7.56 (br s, 2H, 2NH); ESI, *m/z* (%): MH^+ – 467 (100); MNa^+ – 489 (10); MS, *m/z* (%): MH^+ – 467 (100), 277, 191, 163, 118. Analysis: calculated for $C_{22}H_{22}N_6O_2S_2$: C – 56.63, H – 4.75, N – 18.01; found: C – 56.75, H – 4.98, N – 18.11.

*7.1.4.3. 1,4-Bis[(5(6)-methyl-1(H)-benzimidazol-2-ylthio)acetyl]piperazine **17*** Yield – 62% (Method A), 70% (Method B); mp. 137–139 °C; re-crystallized with ethanol; $R_f = 0.48$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR (DMSO-d_6): 2.38 (s, 6H, $2CH_3$); 3.58 (m, 8H, $2CH_2$–N–CH_2); 4.43 (s, 4H, $2SCH_2CO$); 6.93 (dd, 2H, Bzi, $J = 8.16$ Hz); 7.21–7.29 (m, 4H, Bzi); 12.46 (br s, 2H, 2NH). Analysis: calculated for $C_{24}H_{26}N_6O_2S_2$: C – 58.28, H – 5.30, N – 16.99, S – 12.97; found: C – 58.15, H – 5.42, N – 16.81, S – 13.09.

*7.1.4.4. 2-{[2-Oxo-2-(4-benzhydrylpiperazin-1-yl)ethyl]thio}-1H-benzimidazole **18*** Yield – 58% (Method A); mp. 194–196 °C; re-crystallized with ethanol; $R_f = 0.62$; ^{1}H NMR ($CDCl_3$): 2.38 (s, 4H, $^6CH_2$–N–$^2CH_2$); 3.50–3.66 (m, 4H, $^3CH_2$–N–$^5CH_2$); 4.00 (s, 2H, SCH_2CO); 4.20 (s, 1H, ^{7}CH–$(Ph)_2$); 7.18–7.55 (m, 14H, 2Ph; Bzi); ^{13}C NMR: 167.7 (C=O), 149.2 (N=C–S), 141.8 (2C–^{8}C, ^{16}C), 139.0 (2C–^{26}C, ^{27}C), 128.6 (4CH–^{9}C, ^{13}C, ^{17}C, ^{21}C), 127.7 (4CH–^{10}C, ^{12}C, ^{18}C, ^{20}C), 127.2 (2CH–^{11}C, ^{19}C), 122.2 (2CH–^{30}C, ^{31}C), 114.5 (2CH–^{29}C, ^{32}C), 75.7 (^{7}CH), 51.6 ($^2CH_2$), 51.2 ($^6CH_2$), 46.6 ($^3CH_2$), 42.6 ($^5CH_2$), 33.5 ($^{22}CH_2$); ESI mass spectral data: MH^+ – *m/z* 443 (100); MNa^+ – *m/z* 465 (12); MS, *m/z* (%): 443 (100), 357, 167. Analysis: calculated for $C_{26}H_{26}N_4OS_2$: C – 70.56, H – 5.92, N – 12.66; found: C – 70.71, H – 6.10, N – 12.83.

*7.1.4.5. 2-{[2-Oxo-2-(4-benzhydrylpiperazin-1-yl)ethyl]thio}-5(6)-methyl-1H-benzimidazole **19*** Yield – 57% (Method A), 64% (Method B); mp. 149–151 °C; re-crystallized with ethanol; $R_f = 0.60$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR ($CDCl_3$): 2.44 (m, 7H–CH_3 and $^2CH_2$–N–$^6CH_2$); 3.59–3.72 (m, 4H, $^3CH_2$–N–$^5CH_2$); 3.95 (s, 2H, SCH_2CO); 4.25 (s 1H, ^{7}CH–$(Ph)_2$); 7.01–7.42 (m, 13H, Ph and Bzi); ^{13}C NMR: 167.6 (C=O), 148.6 (N=C–S), 141.8 (2C–^{8}C, ^{16}C), 139.0 (2C–^{26}C, ^{27}C), 132.0 (^{30}C), 128.6 (4CH–^{9}C, ^{13}C, ^{17}C, ^{21}C), 127.7 (4CH–^{10}C, ^{12}C, ^{18}C, ^{20}C), 127.2 (2CH–^{11}C, ^{19}C), 123.6 (^{31}CH), 113.9 (2CH–^{29}C, ^{32}C), 75.7 (^{7}CH), 51.6 ($^2CH_2$), 51.2 ($^6CH_2$), 46.6 ($^3CH_2$), 42.5 ($^5CH_2$), 33.7 ($^{22}CH_2$), 21.5 (CH_3). Analysis: calculated for $C_{27}H_{28}N_4OS$: C – 71.02, H – 6.18, N – 12.27, S – 7.02; found: C – 70.15, H – 6.09, N – 12.19, S – 7.21.

*7.1.4.6. 2-({2-[4-(4-Methylphenyl)piperazin-1-yl]-2-oxoethyl}thio)-1H-benzimidazole **20*** Yield – 68% (Method A); mp. 183–185 °C; re-crystallized with ethanol; $R_f = 0.38$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR ($CDCl_3$): 2.28 (s, 3H, CH_3); 3.12–3.19 (m, 4H $^2CH_2$–NH–$^6CH_2$); 3.72–3.87 (m, 4H, $^3CH_2$–N–$^5CH_2$); 4.10 (s, 2H, SCH_2CO); 6.82–6.85 (m, 2H–^{8}CH, ^{12}CH); 7.08–7.11 (m, 2H–^{9}CH, ^{11}CH); 7.18–7.24 (m, 2H–^{24}CH, ^{25}CH); 7.52–7.57 (m, 2H–^{23}CH, ^{26}CH); ^{13}C NMR: 167.7 (C=O), 149.2 (N=C–S), 148.4 (^{7}C), 138.9 (^{20}C, ^{21}C), 130.4 (^{10}C), 129.8 (2CH–^{11}CH, ^{9}CH), 122.4 (2CH–^{24}CH, ^{25}CH), 117.1 (2CH–^{8}CH, ^{12}CH), 114.3 (2CH–^{23}CH, ^{26}CH), 50.1 ($^2CH_2$), 49.8 ($^6CH_2$), 46.5 ($^3CH_2$), 42.4 ($^5CH_2$), 33.8 ($^{16}CH_2$), 20.4 (CH_3); ESI, *m/z* (%): MH^+ – 367 (100); MNa^+ –

389 (28); MS, *m/z* (%): MH^+ − 367 (100), 177, 134, 119. Analysis: calculated for $C_{20}H_{22}N_4OS$: C − 65.55, H − 6.05, N − 15.29; found: C − 65.39, H − 6.20, N − 15.37.

7.1.4.7. 2-({2-[4-(4-Chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio) -1H-benzimidazole ***21*** Yield − 70% (Method A); mp. 187–188 °C; re-crystallized with ethanol; $R_f = 0.50$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR ($CDCl_3$): 3.09–3.15 (m, 4H, $^2CH_2$–N–$^6CH_2$); 3.69–3.85 (m, 4H, $^3CH_2$–N–$^5CH_2$); 4.16 (s, 2H, SCH_2CO); 6.78–6.83 (m, 2H–^{8}CH, ^{12}CH); 7.18–7.26 (m, 4H–^{9}CH, ^{11}CH, ^{24}CH and ^{25}CH); 7.49–7.54 (m, 2H–^{23}CH, ^{26}CH); ^{13}C NMR: 167.9 (C=O), 149.2 (N=C–S), 149.1 (^{7}C), 139.2 (2C–^{20}C, ^{21}C), 129.1 (2CH–^{11}C, ^{9}C), 125.8 (^{10}C), 122.4 (2CH–^{24}C, ^{25}C), 117.9 (2CH–^{8}C, ^{12}C), 114.3 (2CH–^{23}C, ^{26}C), 49.5 ($^2CH_2$), 49.2 ($^6CH_2$), 46.3 ($^3CH_2$), 42.2 ($^5CH_2$), 33.8 ($^{16}CH_2$); ESI, *m/z* (%): MH^+ − 387 (100); MNa^+ − 409 (12); MS, *m/z* (%): MH^+ − 387 (100), 197, 154, 119. Analysis: calculated for $C_{19}H_{19}ClN_4OS$: C − 58.98, H − 4.95, N − 14.48; found: C − 58.81, H − 5.07, N − 14.59.

7.1.4.8. 2-({2-[4-(4-Methylphenyl)piperazin-1-yl]-2-oxoethyl}thio) -5(6)-methyl-1(H)-benzimidazole ***22*** The ratio between **10** and piperazine was 1:2; yield − 68% (Method A), 74% (Method B); mp. 180–182 °C; re-crystallized with ethanol; $R_f = 0.68$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR ($CDCl_3$): 2.28 (s, 3H, CH_3–Ar); 2.44 (s, 3H, CH_3–Bzi); 3.09–3.15 (m, 4H, $^2CH_2$–N–$^6CH_2$); 3.60–3.79 (m, 2H, $^3CH_2$–N–$^5CH_2$); 4.08 (s, 2H, SCH_2CO); 6.79–6.85 (m, 2H, ^{8}CH и ^{12}CH); 7.00 (dd, 1H, ^{25}H, $J = 1.37$, 8.22 Hz); 7.05–7.09 (m, 2H, ^{9}H and ^{11}H); 7.27 (d, 1H, ^{23}H, $J = 1.37$ Hz); 7.39 (d, 1H, ^{26}H, $J = 8.22$ Hz); ^{13}C NMR: 167.5 (C=O), 148.6 (N=C–S), 148.4 (^{7}C), 139.0 (^{20}C), 137.7 (^{21}C), 132.1 (^{24}C), 130.3 (^{10}C), 129.7 (2CH–^{9}C, ^{11}C), 123.7 (^{25}CH), 117.0 (2CH–^{8}C, ^{12}C), 114.1 (^{26}CH), 113.9 (^{23}CH), 49.9 ($^2CH_2$), 49.7 ($^6CH_2$), 46.3 ($^3CH_2$), 42.3 ($^5CH_2$), 34.0 ($^{16}CH_2$), 21.5 ($^{27}CH_3$), 20.4 ($^{15}CH_3$); ESI, *m/z* (%): MH^+ − 381 (100), MNa^+ − 403 (31); MS, *m/z*: 205, 177, 133, 177, 134, 119. Analysis: calculated for $C_{21}H_{24}N_4OS$: C − 66.29, H − 6.36, N − 14.72; found: C − 66.19, H − 6.51, N − 14.91.

7.1.4.9. 2-({2-[4-(4-Chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio) -5(6)-methyl-1(H)-benzimidazole ***23*** Yield − 69% (Method A); mp. 172–174 °C; re-crystallized with ethanol; $R_f = 0.73$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR ($CDCl_3$): 2.39 (s, 3H, CH_3); 3.04 (m, 4H, $^2CH_2$–N–$^6CH_2$); 3.59–3.740 (m, 4H, $^3CH_2$–N–$^5CH_2$); 4.13 (s, 2H, SCH_2CO); 6.74 (m, 2H, ^{8}CH and ^{12}CH); 6.96–6.99 (d, 1H, ^{25}CH, $J = 7.99$ Hz); 7.18 (m, 2H, ^{9}CH and ^{11}CH); 7.26 (br s, 1H, ^{23}CH); 7.38 (d, 1H, ^{26}CH, $J = 7.99$ Hz); 10.78 (br s, 1H, NH); ^{13}C NMR: 167.4 (C=O), 149.1 (N=C–S), 148.5 (^{7}C), 139.0 (^{20}C), 137.5 (^{21}C), 132.2 (^{24}C), 129.0 (2CH–^{9}C, ^{11}C), 125.5 (^{10}C), 123.8 (^{25}CH), 117.8 (2CH–^{8}C, ^{12}C), 114.1 (^{26}CH), 113.8 (^{23}CH), 49.3 ($^2CH_2$), 49.0 ($^6CH_2$), 46.0 ($^3CH_2$), 42.1 ($^5CH_2$), 34.1 ($^{16}CH_2$), 21.6 (CH_3). Analysis: calculated for $C_{20}H_{21}ClN_4OS$: C − 59.91, H − 5.28, N − 13.97; found: C − 60.07, H − 5.18, N − 13.71.

7.1.4.10. 2-({2-[4-(4-Methylphenyl)piperazin-1-yl]-2-oxoethyl}thio) -5(6)-nitro-1(H)-benzimidazole ***24*** Yield − 60% (Method A), 68% (Method B); mp. 210–213 °C (decomp.); $R_f = 0.66$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR (DMSO-d_6): 2.19 (s, 3H, CH_3); 3.09 (m, 4H, $^2CH_2$–N–$^6CH_2$); 3.65 (m, 4H, $^3CH_2$–N–$^5CH_2$); 4.53 (s, 2H, SCH_2CO); 6.86 (m, 2H–^{8}CH, ^{12}CH, Ar); 7.03 (m, 2H–^{9}CH, ^{11}CH, Ar); 7.57 (d, 1H, ^{26}CH, $J = 8.16$ Hz); 8.04 (br d, 1H, ^{25}CH, $J = 8.16$ Hz); 8.28 (br s, 1H, ^{23}CH); 13.2 (br s, 1H, NH); ^{13}C NMR: 165.3 (C=O), 156.2 (N=C–S), 148.7 (^{7}C), 143.7 (^{21}C), 142.1 (^{24}C), 139.6 (^{20}C), 129.5 (2CH–^{9}C, ^{11}C), 128.3 (^{10}C), 117.5 (^{25}CH), 116.2 (2CH–^{8}C, ^{12}C), 113.4 (^{26}CH), 110.3 (^{23}CH), 49.08 ($^2CH_2$), 48.68 ($^6CH_2$), 45.37 ($^3CH_2$), 41.57 ($^5CH_2$), 35.29 ($^{16}CH_2$), 20.1 (CH_3). Analysis: calculated for $C_{20}H_{21}N_5O_3S$: C − 58.38, H − 5.14, N − 17.02.

7.1.4.11. 2-({2-[4-(4-Chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio) -5(6)-nitro-1(H)-benzimidazole ***25*** Yield − 65% (Method A), 71% (Method B); mp. 240–242 °C (decomp.); $R_f = 0.67$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR (DMSO-d_6): 3.09–3.21 (m, 4H, $^2CH_2$–N–$^6CH_2$); 3.59–3.71 (m, 4H, $^3CH_2$–N–$^5CH_2$); 4.54 (s, 2H, SCH_2CO); 6.98 (m, 2H, ^{8}CH и ^{12}CH); 7.26 (m, 2H, ^{9}CH и ^{11}CH); 7.59 (d, 1H, ^{26}CH, $J = 8.23$ Hz); 8.05 (dd, 1H, ^{25}CH, $J = 1.9$, 8.23 Hz); 8.29 (d, 1H, ^{23}CH, $J = 1.9$ Hz); ^{13}C NMR: 166.4 (C=O), 157.2 (N=C–S), 150.5 (^{7}C), 146.5 (^{21}C), 143.1 (^{24}C), 142.6 (^{20}C), 129.7 (2CH–^{9}C, ^{11}C), 123.8 (^{10}C), 118.4 (^{25}CH), 118.3 (2CH–^{8}C, ^{12}C), 114.5 (^{26}CH), 111.0 (^{23}CH), 49.20 ($^2CH_2$), 48.85 ($^6CH_2$), 46.12 ($^3CH_2$), 42.35 ($^5CH_2$), 36.18 ($^{16}CH_2$). Analysis: calculated for $C_{19}H_{18}ClN_5O_3S$: C − 52.84, H − 4.20, N − 16.22, S − 7.42; found: C − 52.99, H − 4.20, N − 16.41, S − 7.31.

7.1.4.12. 2-({2-[4-(4-Chlorophenyl)piperazin-1-yl]-2-oxoethyl}thio) -5(6)-chloro-1(H)-benzimidazole ***26*** Yield − 63% (Method A); mp. 176–179 °C, $R_f = 0.78$; mobile phase, benzene:methanol = 3:1; ^{1}H NMR (DMSO-d_6): 3.13–3.24 (m, 4H, $^2CH_2$–N–$^6CH_2$); 3.61–3.71 (m, 4H, $^3CH_2$–N–$^5CH_2$); 4.47 (s, 2H, SCH_2CO); 6.98 (m, 2H, ^{8}CH and ^{12}CH); 7.14 (dd, 1H, ^{25}CH, $J = 1.96$, 8.51 Hz); 7.26 (m, 2H, ^{9}CH and ^{11}CH); 7.41–7.49 (2H, ^{23}CH и ^{26}CH, overlapped); 12.74 (br s, 1H, NH). Analysis: calculated for $C_{19}H_{18}Cl_2N_4OS$: C − 54.16, H − 4.31, N − 13.30, S − 7.61; found: C − 54.39, H − 4.45, N − 13.12, S − 7.49.

7.2. Biological screening

7.2.1. Antitrichinellosis activity

The parasitological pharmaco-therapeutical experiments in vitro for antitrichinellosis activity of investigated compounds were carried out according to Campbell's method [11,12].

Encapsulated *T. spiralis* larvae were used in the parasitological pharmaco-therapeutic experiment in vitro, 100 specimens for 1 mL physiological solution. The tested benzimidazole derivatives were dissolved in DMSO–water. The used concentrations are given in Table 3. The samples were incubated in "humid" chamber with thermostat at 37 °C. The control for vitality of the *T. spiralis* larvae was carried out 24 h and 48 h after the treatment using stereomicroscope MBC-9.

7.2.2. Antihelminthic activity against S. obvelata

In the experiments for each concentration were used 72 white mice with 18–20 g weight, divided into groups of eight mice for every one of the tested compounds. Each of the control groups consisted of six mice. All mice were infected with the nematode *S. obvelata* by equal conditions.

The tested compounds were introduced into the oesophagus of each mouse by means of thin metallic probe in a single dose of 20 and 100 mg/kg mw, respectively, during three days. The compounds were used as water–DMSO solution in volume of 0.25 mL. The first control group was without treatment and the mice in the second control group were given only the solvent (DMSO) during three days. The other control groups were administrated albendazole in doses of 20 mg/kg mw and 100 mg/kg mw, respectively, and piperazine hexahydrate in a dose of 100 mg/kg mw as a three-day treatment course. One control group was given one dose of ivermectin of 0.200 μg/kg mw. Three days after the treatment course a partial postmortem examination of the large intestine (that is the final localization of the imaginal forms of *S. obvelata*) was carried out in order to determine the number of mature parasites microscopically [22].

7.2.3. Hepatotoxicity test

Rat hepatocytes were used to examine the hepatotoxicity of the synthesized compounds. The rat was anesthetized with sodium pentobarbital. In situ liver perfusion and cell isolation were performed (Fau et al. [24]). The cells were counted under microscope and the viability was estimated by trypan blue (0.05%) exclusion. The initial viability averaged 90%. Incubations were carried out in 25-mL Erlenmeyer flasks. The cells were diluted with Krebs–Ringer-carbonate buffer, pH = 7.4. Each flask contained 3 mL of the cell suspension (i.e., 9×10^6 hepatocytes) and the corresponding compound in concentration of 250 μM. The incubation was performed under a carbogen atmosphere.

The significance of differences between groups was determined by Student's *t*-test.

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