

Evaluation of the anxiolytic properties of benzimidazole-2-thioethanones, 2-alkylthio- and 2-alkylsulfonylbenzimidazoles

Dmitry V. Maltsev^{1,2}, Kristina I. Adzhienko^{1,2}, Maxim A. Perfiliev^{1,2}, Dmitry A. Nekrasov¹, Maria O. Maltseva², Karina R. Magomedova², Raul I. Musaev^{1,2}, Pavel M. Vasiliev^{1,2}, Olga N. Zhukovskaya³, Alexander A. Spasov^{1,2}

¹ Research Center for Innovative Medicines; 39 Novorossiyskaya str., Volgograd 400087 Russia;

² Volgograd State Medical University; 1 Pavshih Bortsov Sq., Volgograd 400131 Russia;

³ Research Institute of Physical and Organic Chemistry, Southern Federal University; 194 Stachki av., building 2, Rostov-on-Don 344090 Russia.

Corresponding author: Maria O. Maltseva (maria.maltseva.volsmu@mail.ru)

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Abstract

Introduction: Fabomotizole is an anxiolytic drug produced in Russia, a 2-mercaptobenzimidazole derivative. The pleiotropic effects of the benzimidazole scaffold, combined with proven neuropsychotropic properties, make fabomotizole a promising target for the synthesis of its analogs to obtain new derivatives with pronounced biological activity.

Materials and Methods: The study involved 125 white outbred male mice, randomly divided into 25 experimental groups (n=5). Twenty-one derivatives of benzimidazole-2-thioethanone, 2-alkylthio-, and 2-alkylsulfonylbenzimidazole were studied. The screening dose was calculated at the level of 1/100 of LD₅₀, determined *in silico* by the consensus ADMET prediction. The behavioral patterns of the animals were assessed *in vivo* in Light/Dark Box and Open Field in comparison with etifoxine (50 mg/kg), fabomotizole (20 mg/kg), and phenazepam (0.01 mg/kg). In the final stage, the studied structures were optimized using quantum chemistry methods for subsequent analysis of the structure-activity relationship.

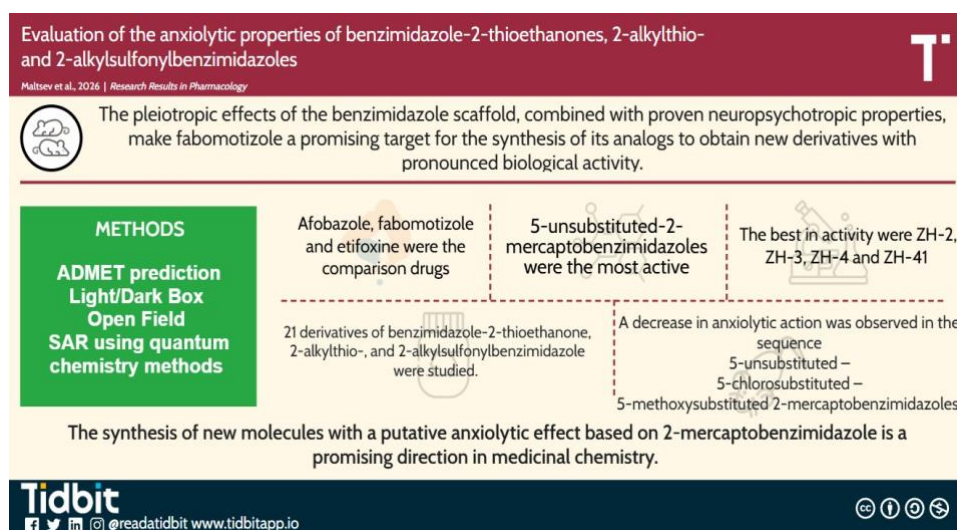
Results and Discussion: The most active derivatives of 2-mercaptobenzimidazole were derivatives of 5-unsubstituted-2-mercaptobenzimidazole: ZH-2, ZH-3, ZH-4, and ZH-41. Among all the studied compounds, a decrease in anxiolytic effect was observed in sequence: 5-unsubstituted – 5-chloro-substituted – 5-methoxy-substituted 2-mercaptobenzimidazoles. A study of the mercaptobenzimidazole fragment using molecular mechanics and quantum chemistry methods revealed that the addition of a substituent to the benzene ring leads to a redistribution of the electron density of cyclic structures.

Conclusion: The synthesis of new molecules with a putative anxiolytic effect based on 2-mercaptobenzimidazole is a promising direction in medicinal chemistry. The most active compounds are those without a substituent at position 5 of the benzimidazole ring.



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Graphical Abstract



Keywords

open Field; Light/Dark Box; ADMET; 2-mercaptobenzimidazole; quantum chemistry; fabomotizol; phenazepam; etifoxine

Introduction

Benzimidazole is a widely studied nitrogen-containing fused heterocycle in medicinal chemistry, serving as the basis for the creation of a plenty of drugs (Alzhrani et al. 2022). Benzimidazole derivatives have been shown to exhibit a wide range of pharmacological activities, including antihistamine, antimicrobial, antidiabetic, antitumor, anti-inflammatory, analgesic, and other activities (Vasava et al. 2020). In the early 2000s, Russian scientists at the Zakusov Institute of Pharmacology developed **fabomotizole**, a drug based on a benzimidazole scaffold containing a 2-mercaptobenzimidazole core. The compound was shown to have a pronounced anxiolytic effect, mediated by interaction with sigma-1 receptors and combined with the absence of muscle relaxation and sedation inherent in classical benzodiazepine drugs.

The spectrum of pharmacological effects of **fabomotizole** has now been expanded to include a neuroprotective effect in the 6-hydroxydopamine model of Parkinson's disease (Voronin et al. 2019), antidepressant properties in the tail suspension test (Kapitsa et al. 2023), cardioprotective effects in an isolated culture of human endothelial cells HUVEC (Kryzhanovskii et al. 2021), as well as hypoglycemic, antioxidant, anti-inflammatory, and antiapoptotic effects in a model of streptozotocin-induced diabetes (Wahba et al. 2025). A study by Dabburu et al. (2025) also reported the potential of **fabomotizole** as a mimic of the human respiratory pacemaker protein, which may find application in the treatment of SARS-CoV-2. Thus, the pleiotropic effects of the benzimidazole scaffold, combined with proven neuropsychotropic properties, make **fabomotizole** a promising target for chemical optimization of its structure and the synthesis of analogues in order to obtain new derivatives with pronounced biological activity.

Previous studies have demonstrated the anxiolytic properties of some 2-mercaptobenzimidazole derivatives of the AZH series (Spasov et al. 2020). The most active substance was identified as AZH-57, which is 4-(2-((5-methyl-1H-benzo[d]imidazol-2-yl)thio)ethyl)morpholine dihydrochloride. This compound differs from **fabomotizole** by the substitution of the ethoxy group with a methyl group at position 5 of the benzimidazole base. In this study, new analogs of the AZH series substances, coded ZH, were synthesized. These derivatives are 5-unsubstituted-2-mercaptobenzimidazole, as well as 5-chloro-2-mercaptobenzimidazole, 5-methoxy-2-mercaptobenzimidazole, and 2-sulfonyl-1H-benzo[d]imidazole.

The aim of the study was to evaluate the anxiolytic potential of new 2-mercaptobenzimidazole derivatives, namely benzimidazole-2-thioethanone, 2-alkylthio- and 2-alkylsulfonylbenzimidazole, using *in vivo* methods, followed by the establishment of structure-activity relationships.

Materials and Methods

Experimental drugs

Twenty-one 2-mercaptobenzimidazole derivatives were studied, namely benzimidazole-2-thioethanone, 2-alkylthio-, and 2-alkylsulfonylbenzimidazole. Of these, eight were derivatives of 5-methoxy-2-mercaptobenzimidazole, six were derivatives of 5-chloro-2-mercaptobenzimidazole, and seven were derivatives of 5-unsubstituted-2-mercaptobenzimidazole, including two 2-sulfonyl-1*H*-benzo[d]imidazoles. The compounds were synthesized by O.N. Zhukovskaya, PhD, at the Institute of Physical Chemistry, Southern Federal University, Rostov-on-Don (Russia). The study doses were 1/100 of the pre-calculated LD₅₀ (Table 1). All compounds were administered orally to animals via an atraumatic metal probe at a dose of 0.1 mL of solution per 10 g of body weight 30 minutes before the start of the experiment. Control group received distilled water (vehicle) in equivalent volume. Fabomotizole (20 mg/kg), etifoxine (50 mg/kg), and phenazepam (0.01 mg/kg) were selected as reference drugs.

Table 1. Calculated screening doses of the studied 2-mercaptobenzimidazole derivatives of the ZH series

№	Code	LD ₅₀ , mg/kg	Calculated dose, mg/kg
1	ZH-2	2735	27.35
2	ZH-3	551	5.51
3	ZH-4	1161	11.61
4	ZH-5	503	5.03
5	ZH-6	2735	27.35
6	ZH-7	734	7.34
7	ZH-8	1039	10.39
8	ZH-9	2058	20.58
9	ZH-10	2577	25.77
10	ZH-11	764	7.64
11	ZH-12	2735	27.35
12	ZH-13	201	2.01
13	ZH-14	1317	13.17
14	ZH-15	3501	35.01
15	ZH-16	3744	37.44
16	ZH-17	411	4.11
17	ZH-18	3193	31.93
18	ZH-40	3000	30.0
19	ZH-41	2000	20.0
20	ZH-42	700	7.0
21	ZH-43	450	4.5

Experimental animals

The study involved 125 white outbred male mice weighing 20±2 g, obtained from the Rappolovo nursery, Leningrad Region (Russia). The animals were randomly divided into 25 experimental groups (n=5). The animals were kept in the vivarium of the Research Center for Innovative Medicines, Department of Pharmacology and Bioinformatics, Volgograd State Medical University, with natural light conditions, a relative air humidity of 40-50%, a temperature of 22-24°C, and a standard complete diet for laboratory animals (GOST R 50258-92). All studies were approved by the Biomedical Ethics Committee of Volgograd State Medical University IRB 00005839 IORG 0004900 (OHRP) No. 2023/191 dated 2 June 2023.

Experimental models

In silico determination of acute toxicity

In the first stage of the study, a consensus prediction of acute oral toxicity was performed for 21 studied chemical compounds using several programs and web resources. The tools used included programs and services employing various machine learning models. The prediction was performed using ProTox-III (Alwaili et al. 2024), SwissADME (Daina et al. 2017), admetSAR (Yang et al. 2019), pkCSM (Pires et al. 2015) and ADMETlab 3.0 (Dong et al. 2018).

Light/dark box

The study involved 25 groups of outbred white male mice (n=5). The anxiolytic activity of the compounds was studied in the light/dark box (Campos-Cardoso et al. 2023). The experiment was conducted in a setup consisting of a dark and a light compartment separated by a partition with a door. At the beginning of testing, the animal was placed in the dark compartment to freely

explore the space. The door was then opened, and the time (s) spent in the light compartment during a 5-minute observation period was recorded.

Open Field test

The assay involved 25 groups of outbred white male mice ($n=5$). To determine the influence of 2-mercaptobenzimidazole derivatives on the behavioral patterns of mice, the Open Field test (da Silva et al. 2018) was used. The setup consisted of a circular arena with high walls, the floor of which was divided into quadrants with a circular center. Blind holes were located at the intersections of the quadrants. During the 5-minute observation period, the number of quadrants crossed and rearings score were recorded, as well as the number of holes examined (search activity).

Structure-activity relationship

At the final stage of the work, the studied structures of 2-mercaptobenzimidazoles were sequentially optimized using molecular mechanics and quantum chemistry methods for the subsequent analysis of the structure-activity relationship between two groups with the absence or presence of a substituent in the benzimidazole benzene ring. For each structure, 10 energetically most favorable conformers were generated in the MarvinSketch program (ChemAxon Ltd., Hungary). All conformers were then processed in the MOPAC2012 program using the semi-empirical quantum chemical method PM7 (Stewart et al. 2013), and one best conformer with the minimum total energy was selected for each compound. Based on the optimization results, such molecular parameters as the energy gap (HOMO-LUMO gap - HLG) were analyzed, calculated as the difference in the energy of the lowest unoccupied orbital (LUMO) and the highest occupied orbital (HOMO), used as an approximate indicator of the reactivity of the structure. The sums of partial charges of non-hydrogen atoms were also calculated for the benzene ring, the imidazole ring, separately for the sulfur atom and for the adjacent elements of the conjugated system: the benzene and imidazole rings in total, the imidazole ring and sulfur in total, and also for the entire mercaptobenzimidazole fragment in total.

Statistical analysis

The obtained data were processed in GraphPad Prism 8.0 using the Kolmogorov-Smirnov test to determine the normality of the data distribution. For parametric distributions in multiple comparisons, one-way ANOVA with Dunnett's posttest was used. Comparison of groups of studied structures based on quantum chemical parameters was performed using the Mann-Whitney test. A p value of <0.05 was considered the criterion for statistical significance.

Results

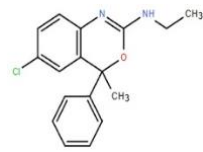
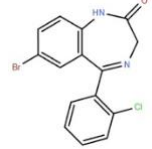
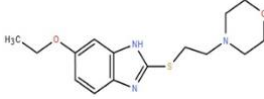
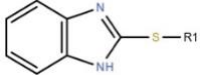
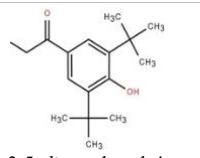
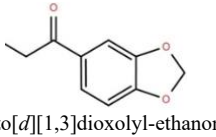
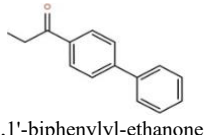
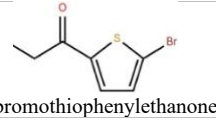
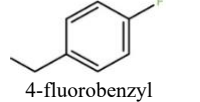
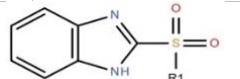
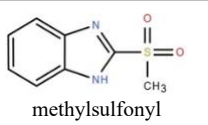
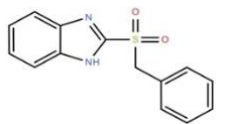
In the first stage, the predicted median lethal dose (LD_{50}) for the studied compounds was estimated, using network pharmacology methods. Consensus prediction using the ProTox-III, admetSAR, ADMETlab 3.0, pkCSM, and SwissADME software suite yielded LD_{50} values for each substance. Based on this value, a screening dose was calculated for each compound individually, amounting to 1/100 of the LD_{50} . The resulting values are presented in Table 1.

In the second stage, the anti-anxiety properties of the studied compounds were assessed in the Light/Dark Box. For all comparison drugs studied (etifoxine, phenazepam, fabomotizole), a significant difference with the control group was demonstrated with a confidence level of at least 95%, allowing the adjusted method to be considered valid for assessing the pharmacological effects of the new compounds.

Among the 5-unsubstituted-2-mercaptobenzimidazole derivatives, including two 2-sulfonyl-1*H*-benzo[*d*]imidazole derivatives, 6 of the 7 studied substances exhibited anxiolytic activity comparable to that of the reference drugs (Table 2). The most pronounced effect was demonstrated by the derivatives containing an aromatic radical with a relatively large molecular weight (ZH-2, ZH-3, and ZH-4), as well as by ZH-41, a substance containing 4-fluorobenzyl. It should be noted that the absence of a substituent at position 5 of the benzimidazole ring is the most favorable option for the manifestation of anxiolytic activity by the studied substances.

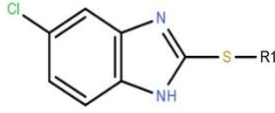
According to the results of the testing, two out of six 5-chloro-substituted mercaptobenzimidazole compounds were found to be active: ZH-9, containing a thiophenyl-ethanone radical, and ZH-6, a 3,5-*di-tert*-butyl-4-hydroxyphenyl-ethanone derivative. The anxiolytic effect of these compounds corresponded to that of fabomotizole and etifoxine. Among 5-methoxy-substituted mercaptobenzimidazoles, one substance out of eight stood out: ZH-40, which contains 4-bromobenzyl. The effect of this compound exceeded that of the reference drugs fabomotizole and etifoxine, without, however, reaching the level of phenazepam. Thus, the presence of chlorine or a methoxy group at position 5 of the core structure of these groups of compounds generally had a negative impact on the level of the desired effect (Tables 3, 4).

Table 2. Anxiolytic effect of 5-unsubstituted-2-mercaptobenzimidazole derivatives, including 2-sulfonyl-1*H*-benzo[*d*]imidazoles, in the Light/Dark Box, mice, M±SEM

No	Comparison drugs	Structure	Time spent by mice in the light compartment of the Light/Dark Box, s
1	Vehicle	—	30.6±5.49
2	Etifoxine 6-chloro-2-ethylamino-4-methyl-4-phenyl-4 <i>H</i> -3,1-benzoxazine hydrochloride		75.0±5.77*
3	Phenazepam 7-bromo-5-(<i>ortho</i> -chlorophenyl)-2,3-dihydro-1 <i>H</i> -1,4-benzodiazepin-2-one		102.8±4.79****
4	Fabomotizole 5-ethoxy-2-[2-(morpholino)-ethylthio]benzimidazole dihydrochloride		78.0±13.66**
 derivatives of 5-unsubstituted-2-mercaptobenzimidazole			
1	ZH-2 2-((1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(3,5- <i>di-tert</i> -butyl-4-hydroxyphenyl)ethan-1-one hydrobromide	 3,5- <i>di-tert</i> -butyl-4-hydroxyphenyl-ethanone	91.4±9.46***
2	ZH-3 2-((1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-benzo[<i>d</i>][1,3]dioxol-5-yl)ethan-1-one hydrobromide	 benzo[<i>d</i>][1,3]dioxolyl-ethanone	93.8±12.49****
3	ZH-4 2-((1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-([1,1'-biphenyl]-4-yl)ethan-1-one hydrobromide	 1,1'-biphenyl-yl-ethanone	92.00±5.50***
4	ZH-5 2-((1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(5-bromothiophen-2-yl)ethan-1-one hydrobromide	 5-bromothiophenylethanone	72.2±9.63*
5	ZH-41 2-((4-fluorobenzyl)thio)-1 <i>H</i> -benzo[<i>d</i>]imidazole hydrochloride	 4-fluorobenzyl	106.8±6.25****
 2-sulfonyl-1 <i>H</i> -benzo[<i>d</i>]imidazole derivatives			
6	ZH-42 2-(methylsulfonyl)-1 <i>H</i> -benzo[<i>d</i>]imidazole hydrochloride	 methylsulfonyl	73.4±7.58*
7	ZH-43 2-(benzylsulfonyl)-1 <i>H</i> -benzo[<i>d</i>]imidazole hydrochloride	 benzylsulfonyl	49.4±14.61

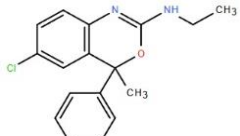
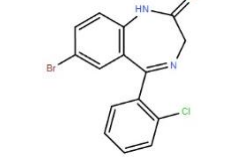
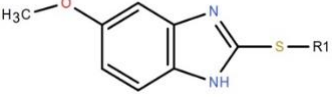
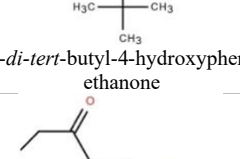
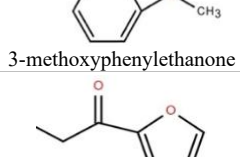
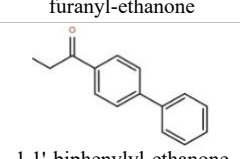
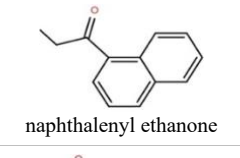
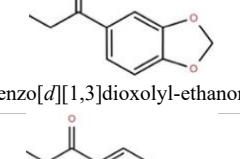
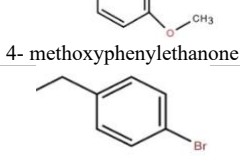
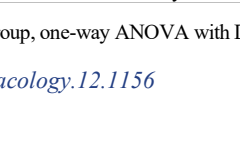
Note: data are significant in comparison with the control group, one-way ANOVA with Dunnett's posttest: * – $p < 0.05$, *** – $p < 0.001$, **** – $p < 0.0001$.

Table 3. Anxiolytic effect of 5-chloro-2-mercaptobenzimidazole derivatives in the Light/Dark Box, mice, M±SEM

№	Comparison drugs	Structure	Time spent by mice in the light compartment of the Light/Dark Box, s
1	Vehicle	—	30.6±5.49
2	Etifoxine 6-chloro-2-ethylamino-4-methyl-4-phenyl-4 <i>H</i> -3,1-benzoxazine hydrochloride		75.0±5.77*
3	Phenazepam 7-bromo-5-(<i>ortho</i> -chlorophenyl)-2,3-dihydro-1 <i>H</i> -1,4-benzodiazepin-2-one		102.8±4.79****
4	Fabomotizole 5-ethoxy-2-[2-(morpholino)-ethylthio]benzimidazole dihydrochloride		78.0±13.66**
 5-chloro-2-mercaptobenzimidazole derivatives			
1	ZH-6 2-((5-chloro-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(3,5- <i>di-tert</i> -butyl-4-hydroxyphenyl)ethan-1-one hydrobromide	 3,5- <i>di-tert</i> -butyl-4-hydroxyphenyl-ethanone	78.6±9.23**
2	ZH-7 2-((5-chloro-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(3-methoxyphenyl)ethan-1-one hydrobromide	 3-methoxyphenylethanone	36.4±10.43
3	ZH-8 1-(benzo[<i>d</i>]dioxol-5-yl) 2-((5-chloro-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)ethan-1-one hydrobromide	 benzo[<i>d</i>][1,3]dioxolyl-ethanone	21.4±11.55
4	ZH-9 2-((5-chloro-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(thiophen-2-yl)ethan-1-one hydrobromide	 thiophenylethanone	74.4±11.72*
5	ZH-10 2-((5-chloro-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(furan-2-yl)ethan-1-one hydrobromide	 furanylethanone	57.0±6.07
6	ZH-11 2-((5-chloro-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(3,4-dimethoxyphenyl)ethan-1-one hydrobromide	 3,4-dimethoxyphenylethanone	16.6±5.05

Note: data are significant in comparison with the control group, one-way ANOVA with Dunnett's posttest: * – $p < 0.05$.

Table 4. Anxiolytic effect of 5-methoxy-2-mercaptobenzimidazole derivatives in the Light/Dark Box, mice, M±SEM

№	Comparison drugs	Structure	Time spent by mice in the light compartment of the Light/Dark Box, s
1	Vehicle		30.6±5.49
2	Etifoxine 6-chloro-2-ethylamino-4-methyl-4-phenyl-4 <i>H</i> -3,1-benzoxazine hydrochloride		75.0±5.77*
3	Phenazepam 7-bromo-5-(<i>ortho</i> -chlorophenyl)-2,3-dihydro-1 <i>H</i> -1,4-benzodiazepin-2-one		102.8±4.79****
4	Fabomotizole 5-ethoxy-2-[2-(morpholino)-ethylthio]benzimidazole dihydrochloride		78.0±13.66**
 5-methoxy-2-mercaptobenzimidazole derivatives			
1	ZH-12 1-(3,5- <i>di-tert</i> -butyl-4-hydroxyphenyl)-2-((5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)ethan-1-one hydrobromide		27.6±8.52
2	ZH-13 2-((5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(3-methoxyphenyl)ethan-1-one hydrobromide		21.8±2.88
3	ZH-14 1-(furan-2-yl)-2-(5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)ethan-1-one hydrobromide		27.0±1.94
4	ZH-15 1-([1,1'-biphenyl]-4-yl)-((5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)ethan-1-one hydrobromide		20.2±7.37
5	ZH-16 2-((5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-naphthalen-1-yl)ethan-1-one hydrobromide		12.2±3.47
6	ZH-17 1-(benzo[<i>d</i>][1,3]dioxol-5-yl)-2-((5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)ethan-1-one hydrobromide		12.6±2.24
7	ZH-18 2-((5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazol-2-yl)thio)-1-(4-methoxyphenyl)ethan-1-one hydrobromide		34.4±11.89
8	ZH-40 2-((4-bromobenzyl)thio)-5-methoxy-1 <i>H</i> -benzo[<i>d</i>]imidazole hydrobromide		84.8±8.61**

Note: data are significant in comparison with the control group, one-way ANOVA with Dunnett's posttest: ** – p<0.01.

Under the conditions of the Open Field test, a statistically significant difference with the control group in the parameter of the number of quadrants crossed was shown for the groups of compounds coded ZH-4 ($p<0.05$), ZH-5 ($p<0.05$) and ZH-9 ($p<0.05$), as well as the reference drug **phenazepam** ($p<0.001$). The rearings score of mice was significantly increased under the influence of substances ZH-3 ($p<0.05$) and ZH-42 ($p<0.05$). The search activity of animals was significantly more intense under the influence of ZH-4 ($p<0.01$), ZH-41 ($p<0.01$) and ZH-42 ($p<0.05$), as well as **etifoxine** ($p<0.01$) and **phenazepam** ($p<0.0001$). The results of the Open Field test are presented in Figure 1 (A, B, C).

At the final stage of the study, SMILES codes were generated for all the studied compounds based on their 2D structures. These codes were used to automatically construct 3D molecular models, which were then sequentially optimized using molecular mechanics and quantum chemistry methods using MarvinSketch and MOPAC2012. A total of 270 conformers were processed. The calculations resulted in *.out files containing the calculated thermodynamic and electronic parameters characterizing the stability and reactivity of the studied compounds. The mean and median HLG values in the group of substituted 2-mercaptobenzimidazoles were 7.839 ± 0.118 and 7.794 , respectively (range: 7.614 to 8.000). For unsubstituted structures, the mean and median HLG values were 8.181 ± 0.362 and 8.018 , respectively (range: 7.826 to 8.773). The total charges of the labile electron density on the 2-mercaptobenzimidazole fragments were calculated for the two groups of compounds studied (Table 5).

Table 5. Total partial charges on individual fragments of mercaptobenzimidazole

Code	Group	Ch_B	Ch_Im	Ch_S	Ch_BIm	Ch_ImS	Ch_BImS
ZH-2	1	-0.492	-0.521	0.156	-1.013	-0.365	-0.857
ZH-3	1	-0.483	-0.536	0.157	-1.020	-0.380	-0.863
ZH-4	1	-0.478	-0.534	0.157	-1.012	-0.377	-0.855
ZH-5	1	-0.477	-0.545	0.166	-1.022	-0.379	-0.856
ZH-41	1	-0.476	-0.551	0.111	-1.026	-0.439	-0.915
ZH-42	1	-0.486	-0.721	2.096	-1.207	1.375	0.889
ZH-43	1	-0.490	-0.698	2.239	-1.188	1.542	1.052
ZH-6	2	-0.333	-0.493	0.159	-0.826	-0.334	-0.667
ZH-7	2	-0.323	-0.512	0.164	-0.834	-0.347	-0.670
ZH-8	2	-0.330	-0.510	0.161	-0.840	-0.349	-0.678
ZH-9	2	-0.328	-0.514	0.170	-0.841	-0.343	-0.671
ZH-10	2	-0.336	-0.509	0.178	-0.845	-0.331	-0.667
ZH-11	2	-0.331	-0.504	0.162	-0.835	-0.343	-0.673
ZH-12	2	-0.265	-0.513	0.144	-0.779	-0.369	-0.634
ZH-13	2	-0.270	-0.532	0.154	-0.802	-0.378	-0.648
ZH-14	2	-0.280	-0.529	0.167	-0.810	-0.363	-0.643
ZH-15	2	-0.269	-0.528	0.151	-0.797	-0.377	-0.646
ZH-16	2	-0.264	-0.527	0.157	-0.791	-0.370	-0.633
ZH-17	2	-0.273	-0.530	0.151	-0.803	-0.379	-0.652
ZH-18	2	-0.274	-0.515	0.153	-0.788	-0.362	-0.636
ZH-40	2	-0.269	-0.544	0.113	-0.813	-0.431	-0.700
U		0	15	65	0	46	34
p		$7.45\cdot10^{-5}$	0.00202	0.861	$7.45\cdot10^{-5}$	0.200	0.0476
Med(1)		-0.481	-0.548	0.159	-1.024	-0.378	-0.857
Med(2)		-0.280	-0.520	0.161	-0.813	-0.349	-0.652
Med(1)-Med(2)		-0.200	-0.028	-0.003	-0.212	-0.030	-0.205

Note: Code – compound code, Group – label indicating that the compound belongs to the group of unsubstituted mercaptobenzimidazole derivatives (1) and the structure of substituted mercaptobenzimidazole derivatives (2); Ch_B – the total charge over the benzene ring; Ch_Im – the total charge on the imidazole ring; Ch_S – the charge on the sulfur atom; Ch_BIm – the total charge on benzimidazole; Ch_ImS – the total charge on the imidazole ring with sulfur; Ch_BImS – the total charge on the entire mercaptobenzimidazole system; U – the numerical value of the Mann-Whitney test; p – the level of statistical significance; Med – the median value for the first and second groups.

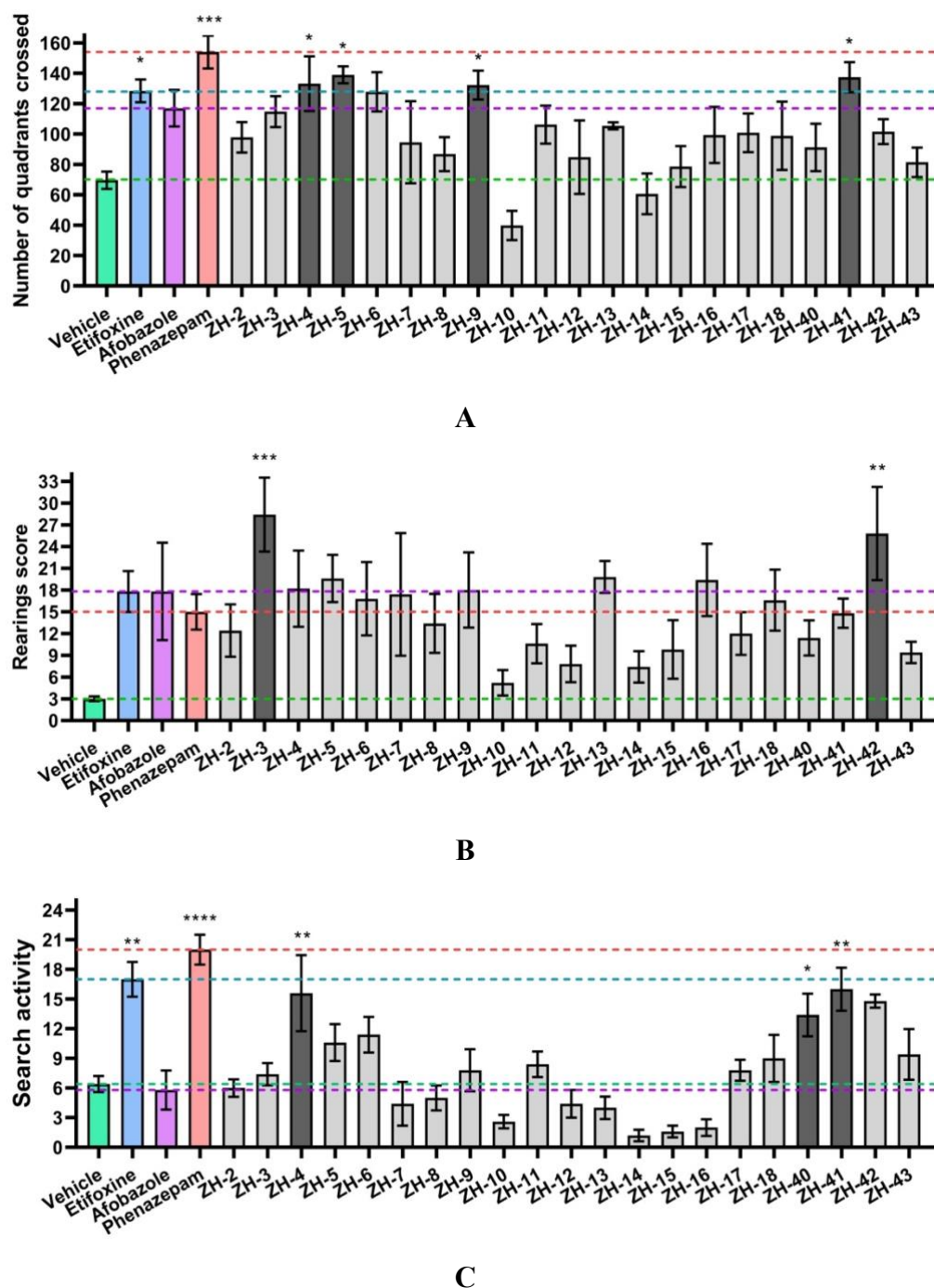


Figure 1. Behavioral patterns of animals under the influence of 2-mercaptobenzimidazole derivatives in the Open Field test in comparison with *etifoxine* (50 mg/kg), *phenazepam* (0.01 mg/kg) and *fabomotizole* (20 mg/kg), mice, M±SEM. A – number of quadrants crossed, B – rearing score, C – search activity. **Note:** data are significant in comparison with the control group, one-way ANOVA with Dunnett's posttest: * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$, **** – $p < 0.0001$.

Figure 2 shows the general structures of the studied 2-mercaptobenzimidazole groups, optimized by quantum chemistry methods, with the calculated labile electron density on the benzene ring as the most statistically significant difference between the compounds.

According to data obtained using quantum chemistry methods, the structures of substituted mercaptobenzimidazole are generally more reactive. However, a study of the mercaptobenzimidazole fragment revealed that the appearance of a substituent on the benzene ring leads to a redistribution of electron density across the rings that make up mercaptobenzimidazole. The median total charge of the system in the first group is significantly greater than that in the second ($p < 0.05$). The redistribution has a particularly strong effect on the benzene ring ($p = 7.45 \times 10^{-5}$), which leads to a significant decrease in the electron charge and will likely lead to a decrease in the pharmacological activity of compounds from the substituted mercaptobenzimidazole group. It is notable that the charge on the sulfur atom has virtually no effect on the charge of the entire mercaptobenzimidazole fragment.

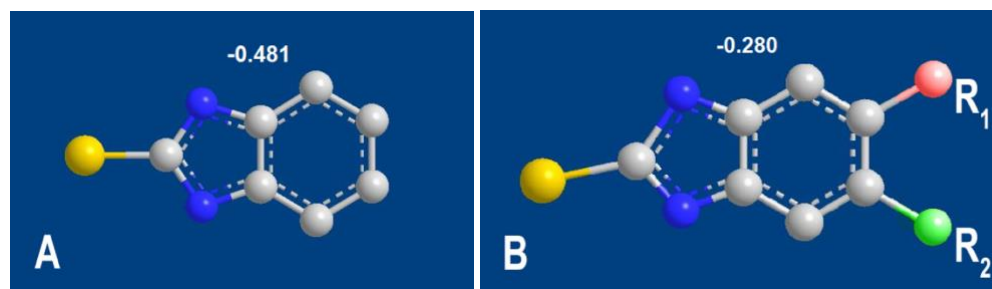


Figure 2. General structure of unsubstituted (A) and substituted (B) 2-mercaptobenzimidazole with median values of total labile electron density on the benzene ring.

Discussion

As noted previously, the most favorable structure for the anxiolytic effect of the new compounds was the benzimidazole core, which contains no substituents at position 5. The structure of the radical adjacent to the mercapto group in the structure of these derivatives had virtually no effect on the anti-anxiety effect of compounds ZH-2, ZH-3, ZH-4, ZH-5, and ZH-41. At the same time, the radicals of these substances are similar in having aromatic properties, being quite massive, as well as in containing strong electronegative atoms (oxygen, sulfur, bromine, fluorine) both as a side substituent and a heteroatom. It is possible that the rigidity of the structure during interaction with the target determines the higher anxiolytic effect of new 2-mercaptobenzimidazoles.

When examining 3,5-*di-tert*-butyl-4-hydroxyphenylethanone derivatives ZH-2, ZH-6 and ZH-12, which differ in the substituent at the 5-position of the benzimidazole ring, a decrease in the anti-anxiety effect can be noted from unsubstituted ZH-2 (91.4 ± 9.46) to chloro-substituted ZH-6 (78.6 ± 9.23) and methoxy-substituted ZH-12 (27.6 ± 8.52). A similar pattern was noted when examining the benzo[*d*][1,3]dioxolylethanone compounds ZH-3, ZH-8, and ZH-17. The outstanding effect of unsubstituted ZH-3 (93.8 ± 12.49) is reduced several times in the chlorine-substituted version ZH-8 (21.4 ± 11.55) and is leveled out in the methoxy-substituted ZH-17 (12.6 ± 2.24), decreasing below the control values. The indicated effect was maintained when comparing 1,1'-biphenyl-ethanone-containing substances – unsubstituted ZH-4 (92.00 ± 5.50) and methoxy derivative ZH-15 (20.2 ± 7.37), as well as comparing 3-methoxyphenyl-ethanone molecules – unsubstituted ZH-7 (36.4 ± 10.43) and methoxy-substituted ZH-13 (21.8 ± 2.88), and also furanyl-ethanone compounds – chlorine-containing ZH-10 (57.0 ± 6.07) and methoxy-containing ZH-14 (27.0 ± 1.94). Thus, the length and heterogeneity of the radical in position 5 of the benzimidazole ring are inversely related to the level of anxiolytic effect of the new compounds.

A compound coded ZH-40 is of interest from this perspective. Its anti-anxiety activity is comparable to such in *etifoxine* and *fabomotizole*, despite belonging to the methoxy-substituted 2-mercaptobenzimidazole group. The absence of a keto group and the presence of a heavy bromine, which, as fluorine in ZH-41, promotes optimal lipophilicity and π -stacking with aromatic residues of the receptor (presumably sigma-1) in the structure of such compounds may be a positive factor in the development of new anxiolytics.

The 2-sulfonyl-1*H*-benzo[*d*]imidazole derivatives ZH-42 and ZH-43 are particularly noteworthy. Methylsulfonyl ZH-42 demonstrated a pronounced anti-anxiety effect comparable to such of reference drugs, while benzylsulfonyl ZH-43 demonstrated no difference in activity compared to control values. This finding may be due to the steric hindrance created by benzylsulfonyl, as well as its ability to participate in hydrogen bonding with the receptor, which may interfere with more effective allosteric interactions. Methylsulfonyl, on the other hand, exhibits a positive inductive effect and can fill a small hydrophobic cavity within the receptor pocket, ensuring tight contact and high affinity.

Summarizing the obtained results, it can be noted that from the point of view of searching for anxiolytic agents, the most active compounds are ZH-2 (91.4 ± 9.46), ZH-3 (93.8 ± 12.49), ZH-4 (92.00 ± 5.50), and ZH-41 (106.8 ± 6.25). When ZH-3 was administered to animals, an increase in the number of rearings performed by mice during the experiment was noted compared to sucu in the control ($p < 0.001$). ZH-4 and ZH-41 are characterized by an increase in the number of quadrants crossed in the Open Field, significantly different from such in the control group ($p < 0.05$), as well as a statistical increase in search activity ($p < 0.01$). These data may indicate some stimulating action of ZH-3, ZH-4 and ZH-41, characteristic of non-sedative anxiolytics such as tofisopam. The parameters of ZH-2 under the conditions of the specified method did not

differ from the control values. It is worth noting that when interpreting Open Field data, it is important to consider the conflict between animals' exploratory interest in new spaces (rearing score, holes explored, total distance traveled, taking into account interest in central and open areas) and their natural preference for safe, enclosed burrows. In this case, the Open Field test can help evaluate the effects of previously unstudied anxiolytic substances, so the results of this testing are best considered in conjunction with basic anti-anxiety techniques.

Studies using molecular mechanics and quantum chemistry methods revealed that the addition of a substituent to the benzene ring of the mercaptobenzimidazole system leads to a redistribution of electron density with a significant reduction in the charge on the benzene ring itself, which likely leads to a weakening of the anxiolytic effects in the group of substituted 2-mercaptobenzimidazoles. Overall, the data obtained through *in silico* analysis are consistent with those obtained experimentally.

Conclusion

In this study, the anxiolytic potential of new 2-mercaptobenzimidazole derivatives was assessed using the Light/Dark Box and Open Field tests in mice in comparison with *etifoxine*, *fabomotizole*, and *phenazepam* after single administration. The most active compounds were derivatives of 5-unsubstituted-2-mercaptobenzimidazole containing 3,5-*di-tert*-butyl-4-hydroxyphenylethanone (ZH-2, 27.35 mg/kg), benzo[d][1,3]dioxolylethanone (ZH-3, 5.51 mg/kg), 1,1'-biphenylethanone (ZH-4, 11.61 mg/kg), and 4-fluorobenzyl (ZH-41, 20.0 mg/kg) fragments. Among all the compounds studied, a decrease in anxiolytic effect was observed in the series 5-unsubstituted – 5-chlorosubstituted – 5-methoxysubstituted 2-mercaptobenzimidazoles. An exception is the highly active methoxy-containing substance ZH-40, which lacks a keto group conjugated to the mercapto group. Using molecular mechanics and quantum chemistry methods, it was shown that the introduction of a substituent into the benzene ring of the core nucleus leads to a significant reduction in the system's charge, which weakens the anxiolytic effects of substituted 2-mercaptobenzimidazoles compared to the unsubstituted ones. Therefore, the design of new molecules with a putative anxiolytic effect based on 2-mercaptobenzimidazole is a promising direction in medicinal chemistry.

Additional Information

Conflict of interest

The authors declare that they have no conflicts of interest.

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Ethics statement

All studies were approved by the Biomedical Ethics Committee of Volgograd State Medical University IRB 00005839 IORG 0004900 (OHRP) No. 2023/191 dated 2 June 2023.

Data availability

All relevant data generated and analyzed during this study are included in this article.

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Author Contribution

- **Dmitriy V. Maltsev**, Doctor of Biological Sciences, Associate Professor, Professor in the Department of Pharmacology and Bioinformatics, Volgograd State Medical University; Head of the Laboratory of Anxiolytic and Antidepressant Drugs, Research Center for Innovative Medicines, Volgograd, Russia; e-mail: maltsevdmitriy@rambler.ru; **ORCID ID**: <https://orcid.org/0000-0002-2005-6621>. Author defined the research idea, developed the study design, and was the project administrator.
- **Kristina I. Adzhienko**, Assistant Professor in the Department of Pharmacology and Bioinformatics, Volgograd State Medical University; Junior Researcher in the Laboratory of Anxiolytic and Antidepressant Drugs, Research Center for Innovative Medicines, Volgograd, Russia; e-mail: kris959688@yandex.ru; **ORCID ID**: <https://orcid.org/0009-0003-2860-8456>. Author took part in experimental work *in vivo*.
- **Maxim A. Perfiliev**, PhD in Medicine, Assistant Professor, Department of Pharmacology and Bioinformatics; Junior Researcher, Laboratory of Anxiolytic and Antidepressant Drugs, Volgograd State Medical University, Volgograd, Russia; e-mail: maxim.firu@yandex.com; **ORCID ID**: <https://orcid.org/0000-0002-5326-3299>. Author performed *in silico* estimation of acute toxicity.
- **Dmitriy A. Nekrasov**, MD, Junior Researcher, Laboratory of Anxiolytic and Antidepressant Drugs, Volgograd State Medical University, Volgograd, Russia; e-mail: dmitrynekrasov@mail.ru; **ORCID ID**: <https://orcid.org/0000-0002-1722-011X>. Author took part in experimental work *in vivo*.
- **Maria O. Maltseva**, PhD in Medicine, Associate Professor, Department of Surgical Dentistry and Maxillofacial Surgery, Volgograd State Medical University, Volgograd, Russia; e-mail: maria.maltseva.volsmu@mail.ru; **ORCID ID**: <https://orcid.org/0000-0002-4173-7143>. Author performed statistical analysis, wrote and revised the manuscript.
- **Karina R. Magomedova**, Assistant Lecturer, Department of Pharmacology and Bioinformatics, Volgograd State Medical University; Junior Researcher, Laboratory of Anxiolytic and Antidepressant Drugs, Research Center for Innovative Medicines, Volgograd, Russia; e-mail: kerryreich666@gmail.com. Author took part in experimental work *in vivo*.

- **Raul I. Musaev**, Assistant Lecturer, Department of Pharmacology and Bioinformatics, Volgograd State Medical University; Junior Researcher, Laboratory of Anxiolytic and Antidepressant Drugs, Research Center for Innovative Medicines, Volgograd, Russia; e-mail: raulraulraul76@gmail.com; **ORCID ID:** <https://orcid.org/0009-0006-3973-0184>. Author took part in experimental work *in vivo*.
- **Pavel M. Vasiliev**, Doctor of Biological Sciences, Full Professor, Department of Pharmacology and Bioinformatics, Volgograd State Medical University; Head of the Laboratory of Information Technologies in Pharmacology and Computer Modeling of Drugs, Research Center for Innovative Medicines, Volgograd, Russia; e-mail: pvassilev@mail.ru; **ORCID ID:** <https://orcid.org/0000-0002-8188-5052>. Author provided *in silico* structure-activity relationships.
- **Olga N. Zhukovskaya**, PhD in Chemistry, Senior Researcher, Department of Chemistry of Heterocyclic Compounds, Institute of Physical and Organic Chemistry, Southern Federal University, Rostov-on-Don, Russia; e-mail: zhukowskaia.ol@yandex.ru; **ORCID ID:** <https://orcid.org/0000-0003-2485-2139>. Author designed and synthesized the substances.
- **Alexander A. Spasov**, Honored Scientist of the Russian Federation, Full member of the Russian Academy of Sciences, Doctor Habil. of Medical Sciences, Full Professor, Head of the Department of Pharmacology and Bioinformatics, Volgograd State Medical University, Volgograd, Russia; e-mail: aspasov@mail.ru; **ORCID ID:** <https://orcid.org/0000-0002-7185-4826>. Author was the principal investigator of the study.