ISSN1027-5495. Functional Materials, 32, No.3 (2025), p. 471-486
doi:http://dx.doi.org/10.15407/fm32.03.471

New pharmaceutical materials with analgesic activity based on 1,2,3-triazolo-1,4-benzodiazepine

I. Botsula[1], I. Kireyev[1], L. Perekhoda[1], M. Suleiman[1], R. Figat[2], Grzegorz Nalecz-Jawecki[2], O. Koshovyi[1], I. Vladymyrova[1], M. Mazur[3,4], V. Chebanov[3,4]

[1] National University of Pharmacy, Hryhoriia Skovorody street 53, 61002, Kharkiv (Ukraine)
[2] Medical University of Warsaw, Banacha str., 1, 02-097, Warsaw, Poland
[3] Institute of Functional Materials Chemistry, State Scientific Institution "Institute for Single Crystals" of National Academy of Sciences of Ukraine, Nauky Avenue 60, 61072, Kharkiv, Ukraine
[4] Faculty of Chemistry, V. N. Karazin Kharkiv National University, Svobody Square 4, 61022, Kharkiv, Ukraine

Received May 5, 2025

Medicines and their components are essential molecular functional materials while developing and transferring them into production is a priority for many interdisciplinary research teams. Hybrid compounds that integrate multiple potent pharmacophoric fragments are regarded as highly promising candidates in the development of such materials. Hence, the incorporation of 1,2,3-triazole moieties into conventional 1,4-benzodiazepine frameworks presents a promising strategy. This structural modification is anticipated to enhance drug-receptor interactions and improve pharmacological profiles by increasing selectivity for specific GABA-A receptor subtypes.

The objective of this study was to assess new functional materials for pharmaceutical use derived from 1,2,3-triazolo-1,4-benzodiazepine compounds. The research specifically focused on conducting molecular docking studies at the benzodiazepine site of the GABA receptor, evaluating their safety profile in vitro, and determining their analgesic potential in vivo.

During the molecular docking study of five compounds for their binding affinity towards the benzodiazepine site of the GABA receptor, a detailed analysis of the formed complexes indicated that all S-configurations of the new compounds exhibit a binding mode in the benzodiazepine site comparable to classical benzodiazepines. The findings demonstrated that the investigated derivatives do not negatively impact cell proliferation in the MTT and NRU tests. No genotoxicity of the investigated derivatives was observed under the conditions of the umu-test. Additionally, it has been established that the compounds exhibit moderate antinociceptive activity in experimental models involving both peripheral and central mechanisms of pain reaction formation.

Keywords: molecular functional materials, pharmaceutical materials, benzodiazepines; molecular docking; cytotoxicity; genotoxicity; umu-test; analgesic; hot plate; writhing.

Нові фармацевтичні матеріали з аналгетичною активністю на основі 1,2,3-триазоло-1,4-бензодіазепіну. *І. Боцула, І. Кірєєв, Л. Перехода, М. Сулейман, Р. Фігат, Г. Налеч-Явецький, О. Кошовий, І. Володимирова, М. Мазур, В. Чебанов*

Лікарські засоби та їх компоненти є важливими молекулярними функціональними матеріалами, а їх розробка та впровадження у виробництво є пріоритетним завданням для багатьох міждисциплінарних дослідницьких груп. Гібридні сполуки, які інтегрують декілька потужних фармакофорних фрагментів, розглядаються як дуже перспективні кандидати в розробці таких матеріалів. Таким чином, включення 1,2,3-тріазольних фрагментів у традиційні 1,4-бензодіазепінові каркаси є багатообіцяючою стратегією. Очікується, що ця структурна модифікація посилить взаємодію лікарського засобу з рецептором та покращить фармакологічні профілі за рахунок підвищення селективності до певних підтипів ГАМК-рецепторів.

Метою цього дослідження було вивчити нові функціональні матеріали фармацевтичного призначення, отримані на основі 1,2,3-триазоло-1,4-бензодіазепінових сполук. Дослідження було зосереджено на проведенні молекулярних докінгових досліджень бензодіазепінової ділянки ГАМК-рецептора, оцінці їх профілю безпеки in vitro та визначенні їх анальгетичного потенціалу in vivo.

Під час молекулярного докінгу п'яти сполук на афінність зв'язування з бензодіазепіновим сайтом ГАМК-рецептора детальний аналіз утворених комплексів показав, що всі S-конфігурації нових сполук демонструють характер зв'язування з бензодіазепіновим сайтом, порівнянний з класичними бензодіазепінами. Отримані результати показали, що досліджувані похідні не чинять негативного впливу на проліферацію клітин в тестах MTT та NRU. Генотоксичності досліджуваних похідних в умовах umu-тесту не виявлено. Додатково встановлено, що сполуки проявляють помірну протибольову активність в експериментальних моделях із залученням як периферичних, так і центральних механізмів формування больової реакції.

1. Introduction

Benzodiazepines represent one of the most extensively prescribed classes of psychotropic medications, playing a crucial role in treating anxiety, insomnia, and seizure disorders [1]. However, their long-term use is associated with significant drawbacks, including dependence, cognitive impairment, and adverse effects on psychomotor function [2]. These limitations have spurred intensive research into new benzodiazepine derivatives that maintain therapeutic efficacy while minimizing undesirable effects.

The integration of 1,2,3-triazole moieties into traditional 1,4-benzodiazepine scaffolds has emerged as a promising approach in medicinal chemistry and pharmacology [3]. This structural modification can potentially enhance drug-receptor interactions and improve pharmacological profiles through increased selectivity for specific GABA-A receptor subtypes [4]. These molecules exhibit a broad spectrum of pharmacological activities, including antiviral, anti-inflammatory, antitumor, analgesic, anxiolytic, sedative, anticonvulsant, and muscle relaxant effects [5, 6]. Their primary action on the central nervous system is mediated through interactions with GABA-A receptors, functioning as modulators of neural signaling [7]. Due to their unique structural features, triazolobenzodiazepines offer the potential for enhanced selectivity, reducing the risk of side effects such as sedation and dependence.

International drug development efforts have yielded varying approaches to addressing benzodiazepine-associated concerns. In Japan, researchers have focused on developing partial agonists with reduced sedative effects [8, 9]. Preclinical and clinical investigations in the United States have significantly advanced our understanding of benzodiazepines, particularly regarding their mechanisms of action, tolerance development, and potential for dependence [10, 11]. Our research has shown promising anxiolytic, antidepressive effects, absence of negative impact on muscle tone and coordination of rodent movements [12–14].

Despite these advances, there remains a critical gap in understanding how triazole incorporation affects analgesic properties and long-term safety profiles. Previous studies have primarily focused on anxiolytic effects, leaving the potential analgesic benefits largely unexplored [15, 16]. Additionally, while preliminary docking studies suggest promising receptor interactions, comprehensive toxicological assessments of these new derivatives are lacking.

This research aims to evaluate novel 1,2,3-triazolo-1,4-benzodiazepine derivatives, specifically focusing on conducting molecular docking studies at the benzodiazepine site of GABA receptor, determining their safety profile *in vitro* and analgesic potential *in vivo*. By

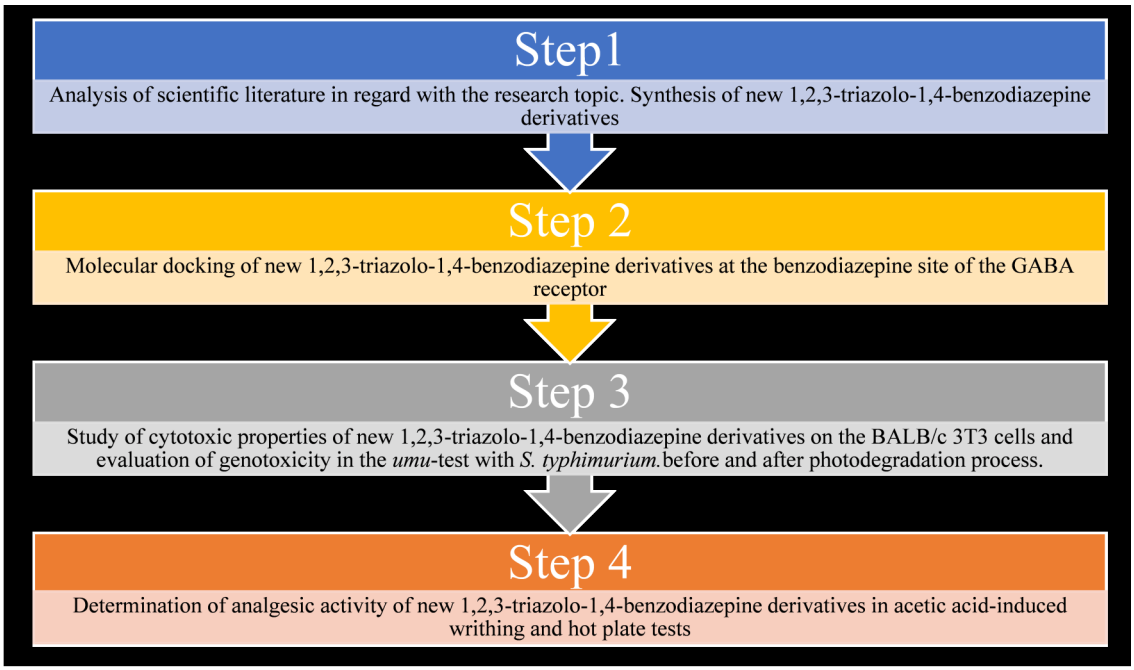

Fig. 1. Study protocol and milestones

combining molecular docking studies with toxicological assessments and analgesic activity evaluations, we seek to develop safer alternatives to traditional benzodiazepines with enhanced therapeutic properties.

2. Planning of the research

The study protocol and main steps are presented in Fig. 1.

3. Materials and methods

3.1 Object of research

Five new 1,2,3-triazolo-1,4-benzodiazepine derivatives were synthesized using the methods described earlier by Mazur et al. (2021) and used as APIs in the present study [17]. Structures of tested compounds are shown in Figure 2 where:

MA-252 - 5-(4-bromophenyl)-N-(*tret*-butyl)-4-oxo-3-phenyl-5,6-dihydro-4H-benzo[f][1,2,3]triazolo[1,5-a][1,4]diazepine-6-carboxamide

MA-253 - N-(*tret*-butyl)-4-oxo-3,5-diphenyl-5,6-dihydro-4H-benzo[f][1,2,3]triazolo[1,5-a][1,4]diazepine-6-carboxamide

MA-254 - N-(*tret*-butyl)-5-(5-methylisoxazol-3-yl)-4-oxo-5,6-dihydro-4H-benzo[f][1,2,3]triazolo[1,5-a][1,4]diazepine-6-carboxamide

MA-255 - N-(*tret*-butyl)-4-oxo-5-phenyl-5,6-dihydro-4H-benzo[f][1,2,3]triazolo[1,5-a][1,4]diazepine-6-carboxamide

MA-261 - N-(*tret*-butyl)-5-butyl-4-oxo-3-phenyl-5,6-dihydro-4H-benzo[f][1,2,3]triazolo[1,5-a][1,4]diazepine-6-carboxamide

3.2 Molecular Docking study

The Autodock 4.2 software package was used for receptor-based flexible docking. Ligands were prepared using MGL Tools 1.5.6. Ligand optimization was performed using the

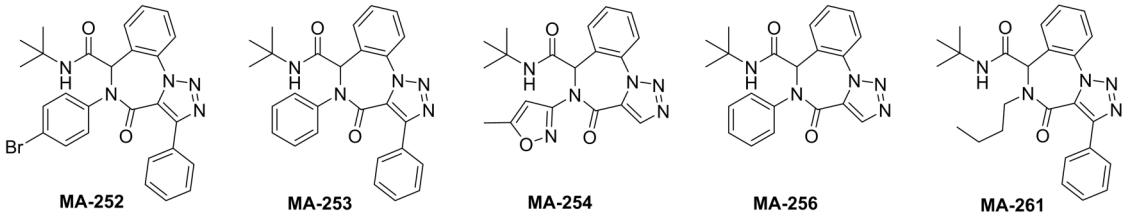

Fig. 2 Structures of new 1,2,3-triazolo-1,4-benzodiazepine derivatives.

Avogadro program. The original data formats of the receptor and ligands were converted to a special PDBQT format using Autodock 4.2. The active site (αγ benzodiazepine binding site) of the GABA receptor (PDB ID: 6HUP) from the Protein Data Bank (PDB) was used as a biological target for docking. Receptor maps were created in MGL Tools and AutoGrid programs. Water molecules, ions, and ligands were removed from the PDB file. All compounds used in the docking study contained a 1,2,3-triazole ring as a core structural motif. No compounds with alternative heterocyclic fragments were included in the dataset.

The following docking parameters were set, which were described in previous studies [18]: the translational motion step was 2 Å, the coefficient of torsional freedom was 0.2983. The cluster tolerance was 2 Å. The external energy of the lattice was 1000, the maximum initial energy was 0, and the maximum number of attempts was 10,000. The number of structures in the population is 150, the maximum number of energy estimation steps is 2500000, the maximum number of generations is 27 000, the number of structures that pass to the next generation is 1, the gene mutation rate is 0.02, the crossover rate is 0.8, and the crossover method is arithmetic. The α-parameter of the Gaussian distribution is 0, the ß-parameter of the Gaussian distribution is 1.

The visualization of the obtained complexes of the studied molecules in the active site of the receptor was performed using the Discovery Studio Visualizer program.

In addition to the reference drug diazepam (PDB ID: 6HUP), we performed the molecular docking procedure for gidazepam, given that it was selected as a comparison drug for pharmacological studies.

- The scoring function that indicates the enthalpy component of the free binding energy (Affinity DG) for the best conformational positions of the tested compounds in the benzodiazepine site of the GABA receptor;

- Binding free energy values and binding constants (EDoc kcal/mol and Ki uM (micromolar) for certain conformational positions of the tested compounds.

The benzodiazepine site (between subunits α and γ) is a well-known and well-documented binding site for known benzodiazepines, so the docking procedure was performed in this particular region of the GABA receptor and the crystallographic model which has PDB ID: 6HUP in the Protein Data Bank database was chosen as a biological target.

3.3 Toxicological assessment in vitro

Dulbecco's modified Eagle's medium (DMEM) and bovine calf serum (BCS) were obtained from Thermo Fisher Scientific, Inc. Ethanol, ethyl acetate, methanol, and dimethylsulfoxide (DMSO), streptomycin, penicillin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (for trypsinization), Trypsin, Triton, neutral red (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride), Glacial acetic acid solution were purchased from Sigma Aldrich.

Preparation and irradiation of tested solutions

Investigated new 1,2,3-triazolo-1,4-benzodiazepine derivatives are practically insoluble in water [12, 19], so they were dissolved in 30% DMSO to prepare stock solutions. Then the working solutions were prepared by the dilution of the stock solutions with PBS because of the relatively high sensitivity of mammalian cells to the changes in the medium osmolarity [20].

Cytotoxicity tests

MTT and NRU tests were performed on the BALB/c 3T3 cells, clone A31 (e.g., CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA), for new 1,2,3-triazolo-1,4-benzodiazepine derivatives [21]. The cells were seeded in 96-well microplates in the DMEM culture medium (supplemented with 10% BCS, 100 IU/mL penicillin and 0.1 mg/mL streptomycin) and incubated for 24 h (5% CO_2, 37 °C, > 90% humidity). Then, each plate was examined under a microscope to ensure the growth of cells they have to form a half-confluent monolayer. Then cells were treated by investigated derivatives in different concentrations and incubated for 24 hours.

MTT assay: After exposure, cells were washed with PBS and treated with medium containing MTT. Measurement of cell viability in this assay was based on the metabolic activity of the cells. Mitochondrial succinate dehydrogenase reduced the water-soluble MTT salt in viable cells to a purple insoluble formazan that could be quantified colorimetrically after dissolution in alcohol. The dye-containing medium was removed after 2 hours and replaced with isopropanol, which lyses the cells and releases formazan from the cells. Color intensity, which correlates with cell culture viability, was assessed at 570 nm using a microplate spectrophotometer. PBS and Triton X-100 were used as negative and positive controls, respectively.

NRU assay: The treatment medium was removed from the bottle; the cells were washed with PBS and treated with the neutral red medium for 3 h. Then, the medium was discarded; the cells were washed with PBS again and treated with desorb solution which is consist of an ethanol, acetic acid solution and water in proportion 49:1:50. The quantitative estimation of viable cells was evaluated based on the neutral red uptake in comparison to the negative control. The amount of neutral red released from the cells was evaluated colorimetrically at 540 nm. As negative and positive controls, PBS and Triton X-100 were used.

The cytotoxicity of all the samples was tested in a dilution series of 7 concentrations. The range of tested concentrations was from 0.78 μM/L to 50 μM/L for investigated derivatives, respectively.

Samples were considered cytotoxic if they reduced cell survival or mitochondrial metabolic activity below 50%, compared to the untreated cells. When the BALB/c 3T3 cell viability was not decreased below 50% across the whole range of tested dilutions of the samples, it was considered non-cytotoxic in this range of concentrations.

Umu-test

Salmonella typhimurium (S. *typhimurium*) TA1535/pSK1002 was purchased from Deutsche Sammlung von Mikroorganismen Und Zellkulturen GmbH in Germany. *Salmonella typhimurium* TA1535 is modified to contain plasmid pSK1002. This plasmid contains the umuC gene fused to a lacZ reporter gene. The umuC gene is induced as part of the bacterial SOS response when genetic damage occurs following exposure to potentially genotoxic compounds. The fusion of the SOS-Umu response gene with the lacZ gene facilitates Salmonella to generate ß-galactosidase, which has the capability to convert ortho-nitrophenyl-ß-galactoside (ONPG) into ortho-nitrophenol that can be detected by a colorimetric change from the ONPG substrate (colorless) to 2-nitrophenol (yellow) [22, 23]. Growth factor, ß-galactosidase activity and induction ratio were registered during the test for investigation the influence of new derivatives on these indexes [24]. The relative units (RU) were obtained at OD600 for the growth factor (G), OD420 for ß-galactosidase activity (UT), and for the induction ratio (IR). The quality criteria to classify a sample as genotoxic according to blank and negative controls is IR ≥ 1.5.

ß-galactosidase activity of the tested compound was calculated according to the formula:

$$\text{I-galactosidase units} = \frac{\text{OD420}_{\text{SAMPLE/CONTROL}}}{\text{OD600}_{\text{SAMPLE/ CONTROL}}}$$

where

OD420 - the absorption at 420 nm indicated the intensity of enzymatic reaction;

OD600 – the absorption at 600 nm indicated the bacteria growth.

The measurements were performed with Asys UVM340 Hightech microplate spectrophotometer. The induction ratio was calculated as the ß-galactosidase activity of the tested compound relatively to the negative control:

$$\text{IR} = \frac{\text{I-galactosidase units}_{\text{SAMPLE}}}{\text{I-galactosidase units}_{\text{CONTROL}}}$$

The bacteria growth (G) is evaluated by measurement of an optical density to determine the genotoxicity of tested samples.

The antigenotoxic potential was evaluated against the genotoxic action of two known genotoxic agents 4-nitroquinoline oxide (4NQO) and 2-aminoanthracene (2-AA) in the absence and presence of metabolic activation with rat's liver s9 fraction respectively.

The genotoxicity of all the samples was tested in a dilution series of 4 concentrations. The range of tested concentrations was from 6.25 μM/L to 50 μM/L for investigated derivatives, respectively.

3.4 Pharmacological study

The study of analgesic activity was carried out *in vivo* [25].

Hot plate test is usually used for assessing the central component of analgesia. As part of the method, a behavioral model of nociception was used, where animal actions (jumping and licking hind paws) occur after an irritating thermal stimulus [26]. The model of thermal irritation of the limbs was reproduced by placing rats on a metal plate heated to 55 °C (this the temperature was maintained using a thermostat). The reaction time was considered the interval between the moment the animal was placed on the plate and the time it started licking its claws or jumping [27]. The corresponding time was calculated reactions in seconds (licking paws, jumping out, squeaking).

The calculation was carried out according to the following formula:

$$\text{AA}=\frac{\Delta\text{Te}-\Delta\text{Tc}}{\Delta\text{Tc}}\times 100\%,$$

where

AA – analgesic activity, %;

ΔTe – difference in latency of the corresponding response in an experimental group before and after the administration of the potential analgesic agent;

ΔTc – difference in latency of the corresponding response in a control group before and after the administration of the solvent.

The acetic acid-induced writhing test is aimed at the study of acute visceral and somatic deep pain. The specific pain reaction of "cramps" by the method of chemical irritation of the peritoneum is induced by intraperitoneal injection of 0.6% acetic acid (0.1 ml / 10 g of body weight). After the injection, the animal was placed in the test chamber. Writhing responses, characterized by contraction of the abdominal wall, pelvic rotation, and subsequent hind limb extension, were recorded throughout the 15-minute duration of the test [28]. The analgesic activity of the new derivatives in doses of 0.75 mg/kg and 1 mg/kg was assessed for their ability to decrease the number of pain reactions in mice with administered acetic acid intraperitoneally. Comparison drugs used in this test were metamizole sodium in the dose of 50 mg/kg and diclofenac sodium in the dose of 8 mg/kg.

The calculation was carried out according to the following formula:

$$AA = \frac{Wc - We}{Wc} \times 100\%,$$

where

AA – analgesic activity, %;

Wc – average number of writhes in control group;

We – average number of writhes in experimental group.

The experiments were carried out in accordance with the "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes" (Council Directive 2010/63/EU, 2010), and the study was approved by the Bioethics Commission of the National University of Pharmacy (protocol No. 4 from 02.10.2020) [29, 30].

Statistical analysis

Results are shown in tables or figures where each value is expressed as mean ± standard deviation of 3-6 independent biological replicates. All data were analyzed using MS Excel (Microsoft Excel 2016, version 16.0, Microsoft Corporation, USA). Student's t-test was used to determine intergroup differences in the case of normal distribution of data, and Mann-Whitney U-test was used in the absence of normal distribution. Changes at $p<0.05$ were considered statistically significant.

4. Results

Molecular docking study

The investigated molecules 1-5 contain an asymmetric carbon atom with a substitution in the 6-position of the 1,4-benzodiazepine framework, therefore, they can exist in the form of two enantiomers, R and S (Fig. 3). At the stage of 3D optimization of the tested structures, the corresponding conformations of the 1-5R and 1-5S enantiomers were created.

According to the estimated docking values (Table 1), among the tested R and S enantiomers of new benzodiazepines 1-5, the best (low-

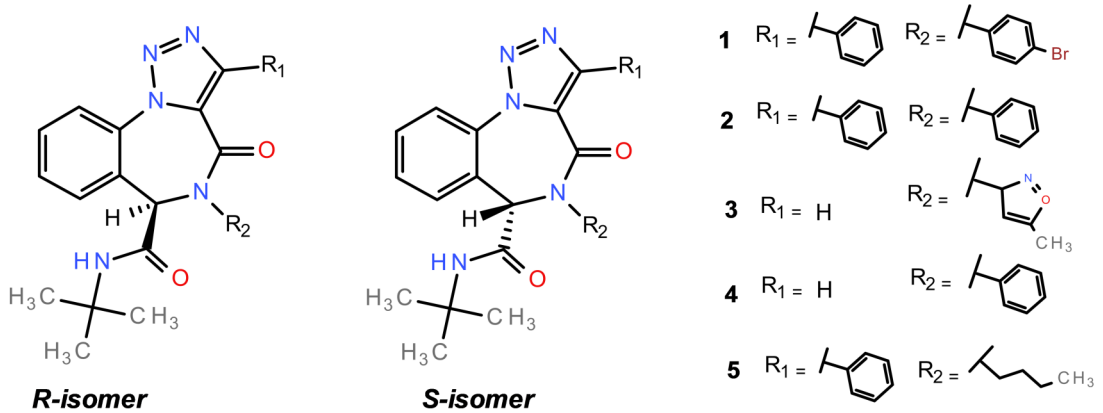

Fig. 3. R and S structures of the studied derivatives

Table 1. Estimated docking values of the studied derivatives at the benzodiazepine site of the GABA receptor

Molecule	Scoring function, kcal/mol	Free binding energy, kcal/mol	Binding constant, μM
1-R	-8.0	-6.32	23.33
1-S	-9.0	-6.94	8.17
2-R	-7.6	-5.52	90.44
2- S	-10.1	-6.59	14.82
3-R	-7.4	-5.02	208.92
3- S	-8.6	-7.46	3.39
4-R	-7.7	-5.11	178.73
4- S	-7.6	-6.98	7.59
5-R	-6.8	-3.97	1220
5- S	-8.9	-6.40	20.49
Diazepam	-9.9	-7.00	7.40
Gidazepam	-8.9	-6.72	11.84

est) values were obtained for the S enantiomers. Among them, the 2-S molecule exceeded the absolute value of the scoring function of diazepam and gidazepam, although it was slightly inferior to them in terms of free energy and binding constants. However, it was found that the 3-S molecule with a moderate affinity had binding constant values 2 and 3 times better than diazepam and gidazepam, respectively. The value of the binding constant of the 4-S compound is 1.5 times better than that of gidazepam. The 1-S and 5-S molecules were at the level of gidazepam according to the scoring function, although they had non-correlated values of EDoc and Ki in comparison with the reference values.

As a result of flexible docking of reference drugs against the selected target and visualization of the formed complexes, we found that they occupy the benzodiazepine binding site at the α1/γ2 interface, where they form a whole complex of interactions with the amino acid residues of the site (Fig. 4).

To assess the binding modes of the new molecules at the benzodiazepine site, we conducted a thorough analysis of the geometric configuration of energetically favorable positions and examined all the complexes formed with the target under investigation.

The visualization results reveal that all R-enantiomers of the tested molecules 1-5 exhibit a slightly distinct binding mode compared to classical benzodiazepines. They occupy the region beneath the bottom of the pocket, which is constrained by the amino acid residue Asn60 of the γ2 site, while interactions occur with the amino acid residue Ser195γ2.

According to the results of docking, the most promising molecule is 2-S, which had higher absolute values of the scoring function compared to diazepam and gidazepam, although it had free energy and binding constant at the level of gidazepam (Affinity DG = -10.1 kcal/mol, EDoc = -6.59 kcal/mol, Ki = 14.82 μM). In Fig. 5 shows the superposition and diagram of intermolecular interactions of the most promising 2-S hit molecule in the allosteric binding site benzodiazepines of the GABA receptor. It should also be noted that the hit molecule has a similar type of binding to the classical benzodiazepines in the allosteric site of the GABA receptor. It forms a complex due to two π-π stacking interactions between 5,6-dihydro-4H-benzo[f][1,2,3]triazolo[1,5-a][1,4]diazepine fragment and Tyr58 residue. The formation of a stable complex between the compound and the receptor was also facilitated by two π-σ bonds between the N-tert-butyl substituent and the Tyr210 residue, π-Alk interaction between the phenyl substituent substituted at position 5 substituent of the benzodiazepine backbone and the amino acid Val203. The formation of such interactions contributes to the formation of a hydrophobic pocket, which is stabilized by two hydrogen bonds between the oxygen atom of the 4-oxo group of the benzodiazepine backbone with the Tyr58 residue and the oxygen atom of the carboxamide group substituted in the 6th position with amino acid residue His102 (Fig. 5).

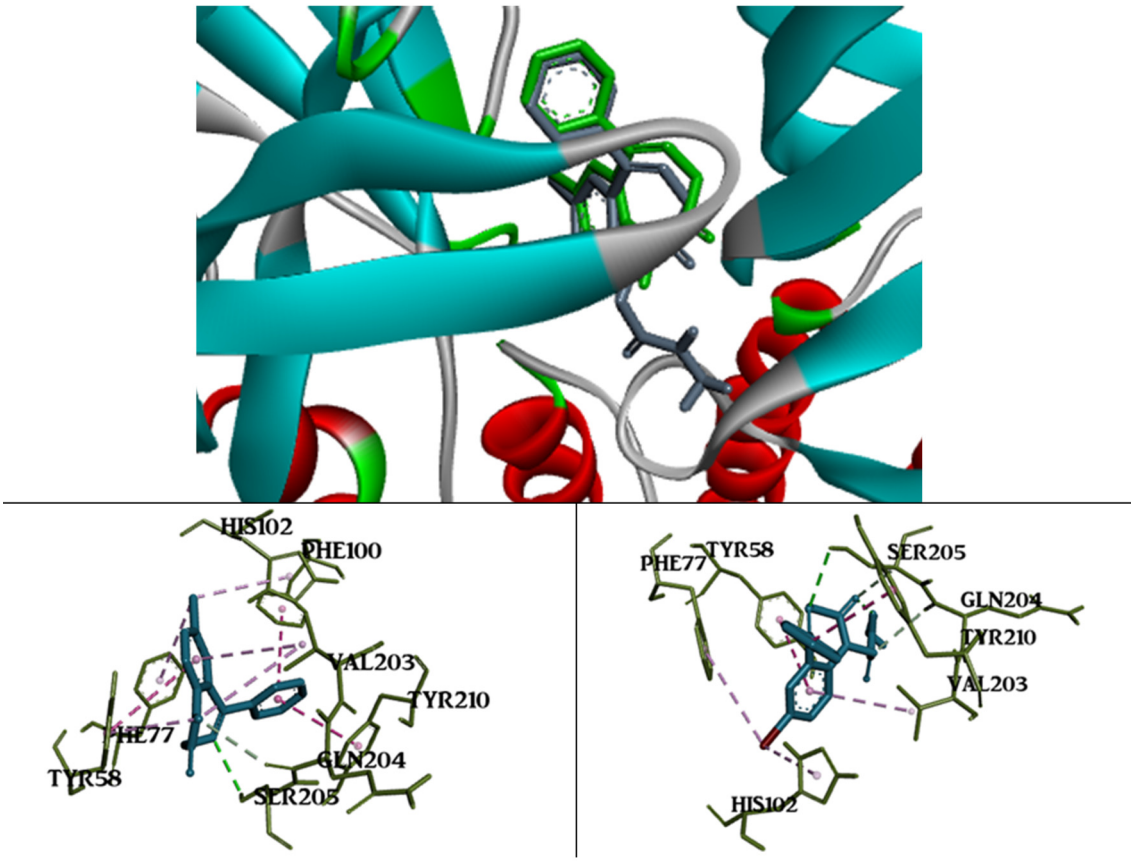

Fig. 4 Superposition of reference drugs (top panel) in the canonical benzodiazepine binding site at the α1/γ2 interface: diazepam - green, gidazepam - blue. Binding modes are diazepam on the left and gidazepam on the right.

In vitro study of toxicity of new 1,2,3-triazolo-1,4-benzodiazepine derivatives

Measurement of cell viability in this assay was based on the metabolic activity of the cells. Mitochondrial succinate dehydrogenase reduced the water-soluble MTT salt in viable cells to a purple insoluble formazan that could be quantified colorimetrically after dissolution in alcohol.

As investigated, the cytotoxicity test showed that new 1,2,3-triazolo-1,4-benzodiazepine derivatives did not affect the mitochondrial metabolic activity of the BALB/c 3T3 cells, did not cause damage effect to the cell viability and demonstrated cell growth more than 50% that allows to consider derivatives as non-toxic under the conditions used in the MTT test (Fig. 6).

The BALB/c 3T3 cells viability under the condition of the NRU test did not fall below 50% in comparison to the control (Fig. 7). The results showed that investigated samples were non-toxic and did not cause harmful effects under the conditions used in this test.

Genotoxicity of new 1,2,3-triazolo-1,4-benzodiazepine derivatives by the umu-test

In the *umu*-test with *S. typhimurium*, none of the tested concentrations exhibited the genotoxic effect with (+S9) or without (-S9) metabolic activation (IR < 1.5) (Table 2). According to results new 1,2,3-triazolo-1,4-benzodiazepine derivatives didn't affect on the growth factor of bacteria in this model since the value was not lower than in the negative control group.

The study of analgesic activity of new 1,2,3-triazolo-1,4-benzodiazepine derivatives

The obtained results of molecular docking demonstrated a high affinity of the 1,2,3-triazolo-1,4-benzodiazepine derivatives to the GABA receptor, which indicates their potential as anti-anxiety agents. To verify the properties predicted by docking, a series of *in vivo* pharmacological tests were performed to assess in particular anxiolytic activity. The screening studies demonstrated the presence of anxiolytic activity of five new 1,2,3-triazolo-1,4-benzodiazepine derivatives in the elevated plus maze and dark-light chamber tests, which was manifested

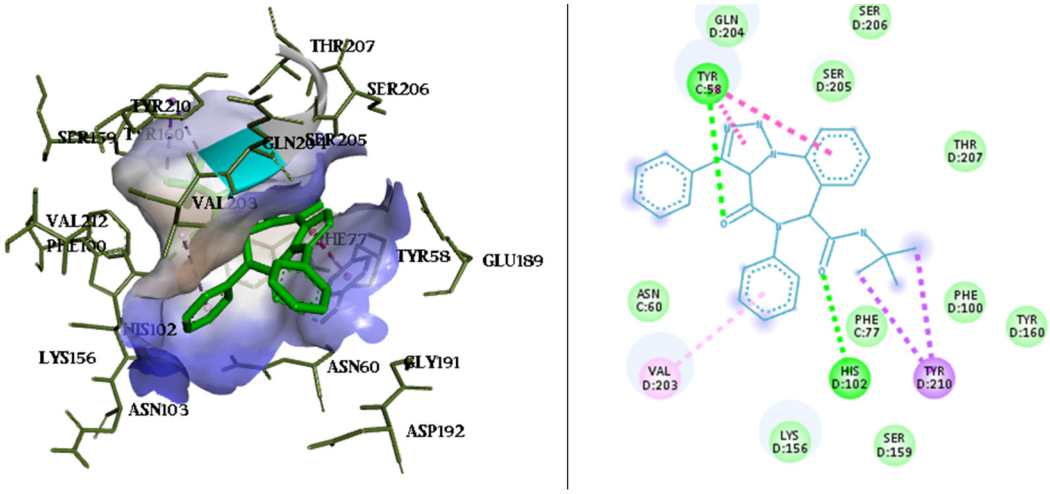

Fig. 5 Superposition (left panel) of the 2-S molecule; diagram of intermolecular interactions (right panel) in the allosteric benzodiazepine binding site of the GABA receptor. The visualization of the bonds is shown by dashed lines of the corresponding color: *Vander Waals forces - light green; hydrogen bonds - green; π-σ bond - lilac; π-π interaction - purple; π-Alk interaction - pink.*

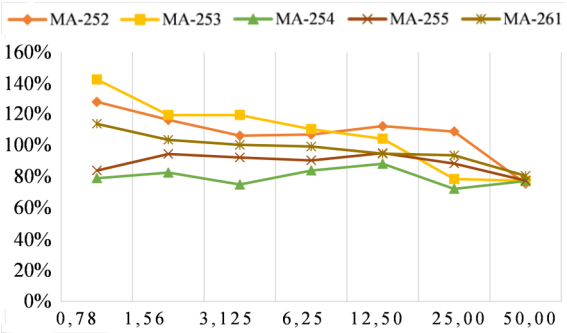

Fig. 6. The results of MTT assay. The y-axis shows the cell viability, while the x-axis represents the concentration (μM/L) of investigated derivatives.

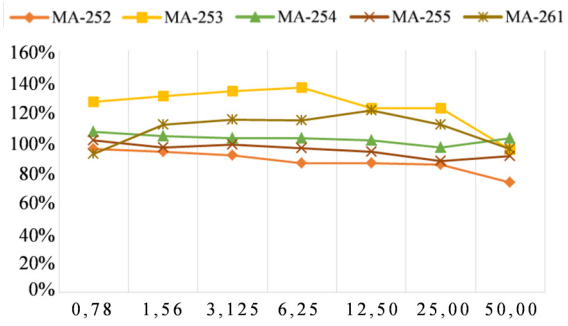

Fig. 7. The results of NRU assay. The y-axis shows the cell viability, while the x-axis represents the concentration (μM/L) of investigated derivatives.

in an increase in the duration of mice staying in the open space of the devices, the main indicator of anxiety behavior of animals [12].

Methods of thermal nociceptive stimulation occupy one of the leading places for the study of analgesic activity in experiments. The hot plate test is commonly used to assess the central component of analgesia and is a basic method for measuring the threshold of pain sensitivity and the potential analgesic effect of pharmacological agents in response to thermal stimuli. Paw licking is considered a rapid response to nociceptive thermal stimuli and a characterization of pain threshold. Jumping characterizes a more complex delayed response and includes an emotional component of escape. The results of the study of the analgesic activity of the investigated derivatives obtained during research are presented in Table 3.

As a result of pharmacological studies, it was found that among the new 1,2,3-triazolo-1,4-benzodiazepine derivatives MA-253 at a dose of 1 mg/kg exhibits the most pronounced analgesic activity in the hot plate test. The latency period of the reaction to the thermal stimulus in the group receiving this derivative was 43.5% higher in 30 min after its administration, and 49.1% higher in 60 min compared to the control group. The response time of the rodents peaked 120 min after administration of MA-253 and reached 17.2 s on average in the group, which is 55.7% higher than the corresponding response in the control group. The analgesic activity of the new derivative increased by 45.4% after 180 minutes and by 36.7% after 240 minutes compared to the control group. Moreover, it was interesting to observe that the latency period at these time points was higher than in the

Table 2 . Evaluation of genotoxic activity of new 1,2,3-triazolo-1,4-benzodiazepine derivatives based on growth factor (G) and induction ratio (IR) values

Compound	Dose, μM/L	-S9		+S9	
		G	IR	G	IR
MA-252	6.25	1.07±0.15	1.05±0.15	1.03±0.07	1.04±0.11
	12.5	1.14±0.21	0.97±0.15	1.07±0.08	1.15±0.11
	25	1.07±0.12	0.99±0.14	1.03±0.02	1.12±0.04
	50	1.13±0.12	1.03±0.15	1.10±0.08	1.34±0.17
MA-253	6.25	1.08±0.11	1.13±0.12	1.01±0.07	1.04±0.14
	12.5	1.26±0.07	0.89±0.07	1.10±0.06	1.03±0.10
	25	1.25±0.08	0.80±0.03	1.10±0.04	1.04±0.11
	50	1.04±0.12	1.00±0.03	1.11±0.04	1.22±0.06
MA-254	6.25	1.06±0.11	1.02±0.10	1.01±0.06	0.95±0.08
	12.5	1.15±0.09	0.99±0.07	1.06±0.04	1.12±0.26
	25	1.17±0.04	0.73±0.07	1.08±0.04	0.96±0.06
	50	1.15±0.05	0.78±0.07	1.15±0.05	1.05±0.08
MA-255	6.25	1.04±0.13	0.98±0.15	0.83±0.05	1.23±0.23
	12.5	0.99±0.07	1.00±0.16	0.89±0.04	1.27±0.18
	25	0.96±0.05	0.96±0.06	0.94±0.08	1.07±0.09
	50	0.92±0.05	1.19±0.13	1.01±0.04	1.16±0.20
MA-261	6.25	0.98±0.06	1.10±0.09	0.90±0.11	1.04±0.09
	12.5	0.98±0.05	1.15±0.17	1.10±0.03	1.07±0.09
	25	0.99±0.05	0.96±0.10	1.11±0.02	0.88±0.07
	50	0.95±0.04	1.09±0.20	1.03±0.07	0.99±0.10
NG	-	1.00±0.07	1.00±0.20	1.00±0.07	1.00±0.20
PC	-	0.91±0.08	3.47±0.30	0.97±0.14	3.13±0.68
SC	-	0.96±0.04	1.09±0.06	0.99±0.09	1.21±0.17

Note: NG – negative control (water); PC – positive control (-S9 – NQO; +S9 – 2-AA); SC – solvent control (3% DMSO)

groups of rodents treated with diclofenac sodium or metamizole, although the difference was not statistically significant. The maximum analgesic effect of diclofenac sodium and metamizole was observed 2 hours after their administration to experimental animals, where it averaged 18 and 16.9 s, respectively, which is 62.7% and 53.1% higher than in the control group.

Only 120 minutes after the administration of 1,2,3-triazolo-1,4-benzodiazepine derivatives MA-252 and MA-254 at doses of 1 mg/kg, a significant increase in the period from the moment of placing the experimental animal on the hot surface of the device to the appearance of a behavioral response to the thermal stimulus was noted, where it reached 14.0 s and 15.0 s, respectively ($p<0.05$ vs. control). The response time of the animal after administration of MA-255 did not differ from that of the control group.

The results of the experiment indicate an increase in the latency period of the behavioral response to painful stimulation of thermoreceptors in the limbs of mice under the influence of MA-252, MA-253 and MA-254 derivatives at a dose of 1 mg/kg and the presence of a pronounced analgesic effect in the background of MA-253 administration.

In the “acetic acid induced writhing” test, the analgesic effect is expressed as a significant decrease in the manifestations of a specific reaction to chemical irritation - the number of “writhings” compared to the pain sensitivity of animals from the control pathology group. According to the results obtained in this study (Table 4), it should be noted that derivatives under code MA-252 in a dose of 1 mg/kg, MA-253 and MA-254 in doses of 0.75 and 1 mg/kg showed the potential in reducing the development of writhing compared to the control group.

Table 3. Analgesic activity of 1,2,3-triazolo-1,4-benzodiazepine derivatives in the hot plate test (M±m, n=6)

Group	Dose, mg/kg	The time of discomfort occurrence (seconds) / Analgesic activity (%) in relation to [control] and (reference drug) after administration in				
		30 min	60 min	120 min	180 min	240 min
Control	-	10.6 ± 0.5	10.6 ± 0.8	10.8 ± 0.6	10.4 ± 0.7	9.8 ± 0.5
MA-252	0.75	11.3 ± 0.5 [7.1] (-25.8)#	12.3 ± 0.6 [15.7] (-23.5) #	11.8 ± 1.2 [6.6] (-29.1) #	10.7 ± 0.9 [2.6] (-23.3) #	11.2 ± 0.8 [14.3] (-5.0)
	1	11.9 ± 1.0 [12.1] (-22.3) #	13.1 ± 0.8 [23.3] (-18.5) #	14.0 ± 0.8 [27.0]* (-15.5) #	11.8 ± 0.6 [13.1] (-15.3)	10.3 ± 0.7 [5.5] (-12.3)
MA-253	0.75	12.2 ± 1.2 [15.4] (-20.0)	13.2 ± 1.4 [24.7] (-17.6)	13.6 ± 0.8 [22.8]* (-18.4) #	12.2 ± 0.7 [17.1] (-12.4)	12.5 ± 0.8 [27.6]* (6.1)
	1	15.2 ± 0.2 [43.5]* (-0.5)	15.8 ± 0.3 [49.1]* (-1.5)	17.2 ± 0.5 [55.7]* (3.5)	15.1 ± 0.7 [45.4]* (8.8)	13.3 ± 0.7 [36.7]* (13.6)
MA-254	0.75	12.9 ± 0.5 [22.0]* (-15.4) #	11.8 ± 0.5 [11.3] (-26.4) #	11.6 ± 0.5 [4.7] (-30.4) #	11.0 ± 0.5 [5.4] (-21.1) #	10.0 ± 0.4 [2.9] (-14.5) #
	1	12.2 ± 0.7 [15.6] (-19.9) #	13.3 ± 1.3 [25.8] (-16.8)	15.0 ± 1.5 [35.7]* (-9.7)	12.9 ± 1.1 [23.9] (-7.3)	11.7 ± 1.0 [19.8] (-0.4)
MA-255	0.75	11.7 ± 0.8 [10.1]* (-23.7) #	11.7 ± 0.8 [12.1] (-27.1) #	10.8 ± 0.7 [0.2] (-33.4) #	9.5 ± 0.5 [0.8] (-24.6) #	9.2 ± 0.5 [1.0] (-16.0) #
	1	11.7 ± 0.7 [10.6] (-23,4) #	11.3 ± 0.3 [6.6] (-29.5) #	10.5 ± 0.4 [-0.5] (-33.8) #	9.7 ± 0.4 [0.3] (-24.9) #	9.2 ± 0.4 [2.2] (-15.0) #
Diclofenac sodium	8	16.3 ± 0.5 [53.9]* (6.7)	16.7 ± 0.4 [57.4]* (4.1)	18.0 ± 0.6 [62.7]* (8.2)	14.5 ± 0.5 [39.7]* (4.6)	12.7 ± 0.2 [30.2]* (8.2) #
Metamizole sodium	50	15.3 ± 0.2 [44.3]*	16.0 ± 0.3 [51.3]*	16.9 ± 0.2 [53.1]*	13.9 ± 0.1 [33.7]*	11.8 ± 0.3 [20.3]*

Note: * Significant at $p < 0.05$ compared to the control group; # Significant at $p < 0.05$ compared to the group treated with metamizole sodium.

Moreover, the derivative MA-253 in the dose of 1 mg/kg, like the reference drugs metamizole and diclofenac sodium, exhibited a significant analgesic effect and was 74.2%. The analgesic activity of diclofenac sodium in this test was 62.6% and of metamizole was 87.9%.

5. Discussion

Benzodiazepine derivatives are important drugs that enhance GABA-induced chloride ion flow by binding to the GABAA receptor at the α+/γ2– interface, causing neuronal hyperpolarization [31]. However, their exact binding mode remains unclear, with conflicting hypotheses reported in the literature and *in silico* studies [32]. Elgarf and authors predicted the binding site for benzodiazepines in the pocket between α2 and γ2 subunits. Then they performed radioligand displacement studies and investigated that triazolam-like compound

Table 4. Effect of test samples in acetic acid induced writhing test (M±m, n=6)

Group	Dose (mg/kg)	Writhing count	Analgesic activity to control group, %
Control		70.5±1.6	
MA-252	0.75	59.8±4.8 #,&	15.1
	1	39.3±2.6 *,#,&	44.2
MA-253	0.75	57.0±1.8 *,#,&	19.1
	1	18.2±1.2 *,#	74.2
MA-254	0.75	53.3±3.9 *,#,&	24.3
	1	51.5±3.9 *,#,&	27.0
MA-255	0.75	70.0±3.0 # ,&	0.7
	1	69.7±2.3 # ,&	1.2
Metamizole	50	8.5±1.5 *	87.9
Diclofenac sodium	8	26.3±7.3 *	62.6

Note: * Significant at $p < 0.05$ compared to the control group; # Significant at $p < 0.05$ compared to the group treated with metamizole; & Significant at $p < 0.05$ compared to the group treated with diclofenac sodium.

2-S demonstrated significant higher binding affinities in α2 and α5 GABAAR subtypes than in α1 and α3 subtypes while 2-R failed to displace [3H]flunitrazepam binding at all GABAA receptor subtypes. Meanwhile diazepam-like compound 3-S showed low subtype selectivity [33]. According to the results of the estimated values of molecular docking of new 1,2,3-triazolo-1,4-benzodiazepine derivatives relative to the benzodiazepine site of the GABA receptor in the terms of our research, it was found that the S-enantiomers had the best energy-favorable positions. Due to the calculated estimated values and detailed analysis of the location, a 2-S hit molecule (MA-253) was found to be promising for experimental studies, which had higher absolute values of the scoring function compared to diazepam and gidazepam. Based on flexible receptor-oriented molecular docking, the prospects of 1,2,3-triazolo-1,4-benzodiazepine derivatives as compounds with potential antianxiety activity were shown.

The cytotoxicity of benzodiazepines and triazolobenzodiazepine derivatives can depend on a variety of factors, including the specific compound being studied, the cell type being tested, and the concentration of the compound. Some benzodiazepines and its derivatives have been shown to induce apoptosis in certain types of cancer cells, suggesting that they may have potential as anticancer agents [34–36]. Almeida and authors [37] have investigated the cytotoxicity of a sedative benzodiazepine flunitrazepam (FNZ) used as a hypnotic and preanesthetic agent, and the results showed no cytotoxic action in hepatoma cells from Chinese hamsters and rats (Rattus norvegicus). The addition of FNZ at various concentrations (0.2, 1.0, 5.0 and 10.0 μG/mL) did not cause the inhibition of cell proliferation, furthermore stimulated cell proliferation. Cytotoxicity of 14 benzodiazepines incubated with CYP2Cs in HepG2 cells were investigated by to determine drug-induced hepatotoxicity [38]. Flunitrazepam, nimetazepam, nitrazepam and clonazepam that belong to the group of nitrobenzodiazepines and have a nitro group in the side chain were more cytotoxic than other 10 investigated benzodiazepine derivatives.

According to received results during the research the investigated derivatives don't affect negatively on cell proliferation which could be considered as non-totoxic. The BALB/c 3T3 cells viability and mitochondrial metabolic activity did not fall below 50% in comparison to the control. No genotoxicity of investigated 1,2,3-triazolo-1,4-benzodiazepine derivatives has been demonstrated under the conditions in the *umu*-test.

GABA-A receptors and glycine receptors play an important role in the spinal control of nociception and pain. Their transmission dysfunction contributes to the emergence of chronic pain conditions [39]. Restoring their proper function with positive allosteric modulators should be a rational approach to the treatment of acute and chronic pain syndromes, including the reduction of inhibitory control of spinal pain. Acute pain can be easily modeled in experimental conditions as a physiological or behavioral response to harmful stimuli or tissue damage.

During the hot plate test, an increase in the latency period of the behavioral response to painful stimulation of thermoreceptors of the extremities of mice under the influence of

MA-252, MA-253 and MA-254 derivatives at a dose of 1 mg/kg was observed and the presence of a pronounced analgesic effect was determined against the background of MA-253 administration 2 hours after its administration. These results differ from the previous opinion on the reorganization of rodent behavior under test conditions due to the manifestation of the drug's sedative properties and indicate that the identified derivatives block nociceptive reactions at the supraspinal level [40].

In the acetic acid-induced writhing model, derivatives MA-252 at a dose of 1 mg/kg, MA-253 and MA-254 at doses of 0.75 and 1 mg/kg demonstrated a potential reduction in the number of writhing compared to the control group, which indicate an analgesic effect. Studies on 3-substituted derivatives of 1,4-benzodiazepines demonstrated notable analgesic effects in acetic acid-induced writhing test, suggesting efficacy in mitigating visceral pain. Among them 3-propoxy-7-bromo-5-(2'-chloro)phenyl-1,2-dihydro-3H-1,4-benzodiazepin-2-one showed potential as a lead compound for developing bradykinin receptor antagonists with analgesic and anti-inflammatory effects [41]. Experimental studies have shown that some benzodiazepine derivatives, such as diazepam, clonazepam, lorazepam, and midazolam, have pronounced antihyperalgesic activity in inflammatory and neuropathic pain. Their mechanism of action is associated with interaction with α2/α3-subunits of benzodiazepine receptor, and they do not cause sedation, impaired motor activity, or tolerance development [42, 43]. Therefore, the results obtained in this study demonstrate the possibility of realization of analgesic effect through direct or indirect interaction of studied compounds with GABA receptor.

Study limitations. During the study of toxicological properties of new 1,2,3-triazolo-1,4-benzodiazepine derivatives, just BALB/c 3T3 cell line were used in the research. However, it would have been intriguing to assess its activity using other cell lines, such as neuronal cell lines, to provide a more comprehensive understanding of its effects across different cell types. The second limitation is that we used only one species of mice in *in vivo* research. Other species may give different results.

Prospects for further research. More research about the pharmacological activity of new 1,2,3-triazolo-1,4-benzodiazepine derivatives need to be done to discover the prospects of using them in the treatment of anxiety disorder especially in the context of cancer in the pre- and postoperative periods, as well as in patients with war trauma or traumatic experience. The potential side effects should also be considered and studied in the further study.

6. Conclusions

This study aims to develop new pharmaceutical materials with analgesic activity based on the 1,2,3-triazolo-1,4-benzodiazepine framework. We conducted molecular docking of five synthesized compounds, comparing their binding affinity to the benzodiazepine site of the GABA receptor with diazepam and gidazepam. Analysis revealed that all S-configurations of the new compounds have a similar binding mode to classical benzodiazepines. The molecule MA-253 showed promise for experimental studies with higher binding scores than diazepam and gidazepam. These findings suggest potential antianxiety activity for 1,2,3-triazolo-1,4-benzodiazepine derivatives.

Based on the results of this study, new 1,2,3-triazolo-1,4-benzodiazepine derivatives can be regarded as safe materials, as they exhibited no cytotoxic or genotoxic activities at the investigated doses. Although these substances demonstrated promising anticytotoxic and antigenotoxic potential, further research is required to evaluate their pharmacological activity and safety in mammalian cells *in vivo*.

Furthermore, it has been determined that the derivatives demonstrate moderate antinociceptive activity in experimental models that involve both peripheral and central mechanisms of pain response formation. Regarding the potency of the analgesic effect, MA-253 is slightly less effective compared to the reference drugs: the nonsteroidal anti-inflammatory drug diclofenac sodium and the analgesic-antipyretic metamizole sodium.

Funding

This research was partially supported by National Academy of Sciences of Ukraine, project "New compounds with antibacterial properties based on nitrogen-containing heterocycles", 0125U000603.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

Acknowledgement

The authors sincerely thank all the defenders of Ukraine who made the performance of this study possible. The authors sincerely appreciate the support of Prof. Piotr Luliński (Medical University of Warsaw) and Partners who stand with Ukraine.

References

1. Coteur, K., Mamouris, P., Vaes, B., Van Nuland, M., Matheï, C., Schoenmakers, B. (2022). Evolution of benzodiazepine receptor agonist prescriptions in general practice: A registry-based study. Frontiers in Public Health, 10, 1014734. doi: https://doi.org/10.3389/fpubh.2022.1014734
2. Ritvo, A. D., Foster, D. E., Huff, C., Finlayson, A. J. R., Silvernail, B., Martin, P. R. (2023). Long-term consequences of benzodiazepine-induced neurological dysfunction: A survey. PLOS ONE, 18(6), e0285584. doi: https://doi.org/10.1371/journal.pone.0285584
3. Aziz Ali, A. (2021). 1,2,3-Triazoles: Synthesis and Biological Application. In A. Kuznetsov (Ed.), Azoles - Synthesis, Properties, Applications and Perspectives. IntechOpen. doi: https://doi.org/10.5772/intechopen.92692
4. Maramai, S., Benchekroun, M., Ward, S. E., Atack, J. R. (2020). Subtype Selective γ-Aminobutyric Acid Type A Receptor (GABAAR) Modulators Acting at the Benzodiazepine Binding Site: An Update. Journal of Medicinal Chemistry, 63(7), 3425–3446. doi: https://doi.org/10.1021/acs.jmedchem.9b01312
5. Padhyar, K. T., Patil, R. S., Wadwale, N. B. (2022). A brief review on Triazole and its Pharmacological Application. International Journal of Current Science Research and Review, 05(01). doi: https://doi.org/10.47191/ijcsrr/V5-i1-25
6. Kharb, R., Sharma, P. C., Yar, M. S. (2011). Pharmacological significance of triazole scaffold. Journal of Enzyme Inhibition and Medicinal Chemistry, 26(1), 1–21. doi: https://doi.org/10.3109/14756360903524304
7. Ankali, K. N., Rangaswamy, J., Shalavadi, M., Naik, N. (2022). Synthesis, Molecular Docking and In Vivo Biological Evaluation of Iminostilbene Linked 1,2,3-Triazole Pharmacophores as Promising Anti- Anxiety and Anti-Inflammatory Agents. Medicinal Chemistry (Shariqah (United Arab Emirates)), 18(2), 260–272. doi: https://doi.org/10.2174/1573406417666210608141746
8. Deng, X., Sun, H., Youssif, B. G. M. (2023). Editorial: Pharmaceutical insights into the triazoles: Recent advances. Frontiers in Chemistry, 11, 1149133. doi: https://doi.org/10.3389/fchem.2023.1149133
9. Dai, J., Tian, S., Yang, X., Liu, Z. (2022). Synthesis methods of 1,2,3-/1,2,4-triazoles: A review. Frontiers in Chemistry, 10. doi: https://doi.org/10.3389/fchem.2022.891484
10. Guina, J., Merrill, B. (2018). Benzodiazepines I: Upping the Care on Downers: The Evidence of Risks, Benefits and Alternatives. Journal of Clinical Medicine, 7(2), 17. doi: https://doi.org/10.3390/jcm7020017
11. Duke, A. N., Meng, Z., Platt, D. M., Atack, J. R., Dawson, G. R., Reynolds, D. S., Tiruveedhula, V. V. N. P. B., Li, G., Stephen, M. R., Sieghart, W., Cook, J. M., Rowlett, J. K. (2018). Evidence That Sedative Effects of Benzodiazepines Involve Unexpected GABAA Receptor Subtypes: Quantitative Observation Studies in Rhesus Monkeys. The Journal of Pharmacology and Experimental Therapeutics, 366(1), 145–157. doi: https://doi.org/10.1124/jpet.118.249250
12. Botsula, I., Schavikin, J., Heinämäki, J., Laidmäe, I., Mazur, M., Raal, A., Koshovyi, O., Kireyev, I., Chebanov, V. (2024). Application of nanofiber-based drug delivery systems in improving anxiolytic effect of new 1,2,3-triazolo-1,4-benzodiazepine derivatives. European Journal of Pharmaceutical Sciences, 106712. doi: https://doi.org/10.1016/j.ejps.2024.106712
13. Botsula, I. V., Kireyev, I. V., Mazur, M. O., Chebanov, V. A. (2024). The study of antidepressant properties of new 1,2,3-triazolo-1,4-benzodiazepine derivatives. News of Pharmacy, 107(1), 136–143. doi: https://doi.org/10.24959/nphj.24.144
14. Botsula, I. V., Kireyev, I. V., Koshovyi, O. M., Mazur, M. O., Chebanov, V. A. (2023). Behavioral reactions of rodents after administration of the 1,2,3-triazolo-1,4-benzodiazepine derivatives in the open field test. Pharmaceutical review, (4), 70–77. doi: https://doi.org/10.11603/2312-0967.2023.4.14297
15. Griessner, J., Pasieka, M., Böhm, V., Grössl, F., Kaczanowska, J., Pliota, P., Kargl, D., Werner, B., Kaouane, N., Strobelt, S., Kreitz, S., Hess, A., Haubensak, W. (2021). Central amygdala circuit dynamics underlying the benzodiazepine anxiolytic effect. Molecular Psychiatry, 26(2), 534–544. doi: https://doi.org/10.1038/s41380-018-0310-3
16. Rosso, M., Wirz, R., Loretan, A. V., Sutter, N. A., Pereira da Cunha, C. T., Jaric, I., Würbel, H., Voelkl, B. (2022). Reliability of common mouse behavioural tests of anxiety: A systematic review and meta-analysis on the effects of anxiolytics. Neuroscience & Biobehavioral Re-

views, 143, 104928. doi: https://doi.org/10.1016/j.neubiorev.2022.104928

17. Mazur, M. O., Zhelavskyi, O. S., Zviagin, E. M., Shishkina, S. V., Musatov, V. I., Kolosov, M. A., Shvets, E. H., Andryushchenko, A. Y., Chebanov, V. A. (2021). Effective microwave-assisted approach to 1,2,3-triazolobenzodiazepinones via tandem Ugi reaction/catalyst-free intramolecular azide-alkyne cycloaddition. Beilstein Journal of Organic Chemistry, 17, 678–687. doi: https://doi.org/10.3762/bjoc.17.57
18. Mohapatra, R. K., Perekhoda, L., Azam, M., Suleiman, M., Sarangi, A. K., Semenets, A., Pintilie, L., & Al-Resayes, S. I. (2021). Computational investigations of three main drugs and their comparison with synthesized compounds as potent inhibitors of SARS-CoV-2 main protease (Mpro): DFT, QSAR, molecular docking, and in silico toxicity analysis. Journal of King Saud University. Science, 33(2), 101315. https://doi.org/10.1016/j.jksus.2020.101315
19. Botsula, I., Kireyev, I., Koshovyi, O., Heinämäki, J., Ain, R., Mazur, M., Chebanov, V. (2024). Semi-solid extrusion 3D printing of functionalized polyethylene oxide gels loaded with 1,2,3-triazolo-1,4-benzodiazepine nanofibers and valine-modified motherwort (Leonurus cardiaca L.) dry extract. ScienceRise: Pharmaceutical Science, (1(47)), 40–48. doi: https://doi.org/10.15587/2519-4852.2024.299205
20. Zgadzaj, A., Kornacka, J., Jastrzębska, A., Parzonko, A., Sommer, S., Nałęcz-Jawecki, G. (2018). Development of photoprotective, antiphototoxic, and antiphotogenotoxic formulations of ocular drugs with fluoroquinolones. Journal of Photochemistry and Photobiology B: Biology, 178, 201–210. doi: https://doi.org/10.1016/j.jphotobiol.2017.11.011
21. OECD (2010). Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systematic Toxicity Tests, OECD Series on Testing and Assessment, No. 129, OECD Publishing, Paris, doi: https://doi.org/10.1787/d77a7e39-en.
22. Meyer, D., Marin-Kuan, M., Debon, E., Serrant, P., Cottet-Fontannaz, C., Schilter, B., Morlock, G. E. (2021). Detection of low levels of genotoxic compounds in food contact materials using an alternative HPTLC-SOS-Umu-C assay. ALTEX - Alternatives to animal experimentation, 38(3), 387–397. doi: https://doi.org/10.14573/altex.2006201
23. Molecular Toxicology Inc. - MOLTOX. Available at: https://moltox.com/categories.php?parent=54&name=UMU%20Genotoxicity%20Test%20Kit&cat=54
24. Skrzypczak, A., Przystupa, N., Zgadzaj, A., Parzonko, A., Sykłowska-Baranek, K., Paradowska, K., Nałęcz-Jawecki, G. (2015). Antigenotoxic, anti-photogenotoxic and antioxidant activities of natural naphthoquinone shikonin and acetylshikonin and Arnebia euchroma callus extracts evaluated by the umu-test and EPR method. Toxicology in Vitro, 30(1, Part B), 364–372. doi: https://doi.org/10.1016/j.tiv.2015.09.029
25. Bannon, A. W., Malmberg, A. B. (2007). Models of Nociception: Hot-Plate, Tail-Flick, and Formalin Tests in Rodents. Current Protocols in Neuroscience, 41(1). doi: https://doi.org/10.1002/0471142301.ns0809s41
26. Deuis, J. R., Dvorakova, L. S., Vetter, I. (2017). Methods Used to Evaluate Pain Behaviors in Rodents. Frontiers in Molecular Neuroscience, 10, 284. doi: https://doi.org/10.3389/fnmol.2017.00284
27. Nunes, E. A., Medeiros, L. F., de Freitas, J. S., Macedo, I. C., Kuo, J., de Souza, A., Rozisky, J. R., Caumo, W., Torres, I. L. S. (2017). Morphine exposure during early life alters thermal and mechanical thresholds in rats. International Journal of Developmental Neuroscience, 60, 78–85. doi: https://doi.org/10.1016/j.ijdevneu.2016.12.008
28. Drapak, I., Zimenkovsky, B., Perekhoda, L., Yeromina, H., Ieromina, Z., Paykush, M., Logoyda, L., Lubenets, V., Holubieva, T., Yaremkevych, R., Shchur, O., Seredynska, N. (2022). Synthesis of New 3-Morpholyl-Substituted 4-Aryl-2-Arylimino-2,3-Dihydrothiazole Derivatives and Their Anti-Inflammatory and Analgesic Activity. Chemistry & Chemical Technology, 16(4), 532–542. doi: https://doi.org/10.23939/chcht16.04.532
29. Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. (2010). Official Journal of the European Communities, L 256, 33–79.
30. Guide for the Care and Use of Laboratory Animals: Eighth Edition. (2011). Washington, D.C.: National Academies Press. doi: https://doi.org/10.17226/12910
31. Richter, L., de Graaf, C., Sieghart, W., Varagic, Z., Mörzinger, M., de Esch, I. J., Ecker, G. F., Ernst, M. (2012). Diazepam-bound GABAA receptor models identify new benzodiazepine binding-site ligands. Nature chemical biology, 8(5), 455–464. doi: https://doi.org/10.1038/nchembio.917

32. Sieghart, W. (2015). Allosteric modulation of GABAA receptors via multiple drug-binding sites. Advances in Pharmacology (San Diego, Calif.), 72, 53–96. doi: https://doi.org/10.1016/bs.apha.2014.10.002
33. Elgarf, A. A., Siebert, D. C. B., Steudle, F., Draxler, A., Li, G., Huang, S., Cook, J. M., Ernst, M., Scholze, P. (2018). Different Benzodiazepines Bind with Distinct Binding Modes to GABAA Receptors. ACS Chemical Biology, 13(8), 2033–2039. doi: https://doi.org/10.1021/acschembio.8b00144
34. Singh, R. K., Prasad, D. N., Bhardwaj, T. R. (2017). Design, synthesis and in vitro cytotoxicity study of benzodiazepine-mustard conjugates as potential brain anticancer agents. Journal of Saudi Chemical Society, 21, S86–S93. doi: https://doi.org/10.1016/j.jscs.2013.10.004
35. Dourlat, J., Liu, W.-Q., Gresh, N., Garbay, C. (2007). Novel 1,4-benzodiazepine derivatives with antiproliferative properties on tumor cell lines. Bioorganic & Medicinal Chemistry Letters, 17(9), 2527–2530. doi: https://doi.org/10.1016/j.bmcl.2007.02.016
36. Francis, T. M., Sundberg, T. B., Cleary, J., Groendyke, T., Opipari, A. W., Glick, G. D. (2006). Identification of cytotoxic, T-cell-selective 1,4-benzodiazepine-2,5-diones. Bioorganic & Medicinal Chemistry Letters, 16(9), 2423–2427. doi: https://doi.org/10.1016/j.bmcl.2006.01.113
37. Almeida, I. V. de, Domingues, G., Soares, L. C., Düsman, E., Vicentini, V. E. P. (2014). Evaluation of cytotoxicity and mutagenicity of the benzodiazepine flunitrazepam in vitro and in vivo. Brazilian Journal of Pharmaceutical Sciences, 50(2), 251–256. doi: https://doi.org/10.1590/S1984-82502014000200003
38. Mizuno, K., Katoh, M., Okumura, H., Nakagawa, N., Negishi, T., Hashizume, T., Nakajima, M., Yokoi, T. (2009). Metabolic activation of benzodiazepines by CYP3A4. Drug Metabolism and Disposition: The Biological Fate of Chemicals, 37(2), 345–351. doi: https://doi.org/10.1124/dmd.108.024521
39. Zeilhofer, H. U., Neumann, E., Munro, G. (2019). Spinal GABAA receptors for pain control: back to the future? BJA: British Journal of Anaesthesia, 123(2), e176–e179. doi: https://doi.org/10.1016/j.bja.2019.01.030
40. Casarrubea, M., Sorbera, F., Santangelo, A., Crescimanno, G. (2012). The effects of diazepam on the behavioral structure of the rat's response to pain in the hot-plate test: anxiolysis vs. pain modulation. Neuropharmacology, 63(2), 310–321. doi: https://doi.org/10.1016/j.neuropharm.2012.03.026
41. Pavlovsky, V. I., Tsymbalyuk, O. V., Martynyuk, V. S., Kabanova, T. A., Semenishyna, E. A., Khalimova, E. I., Andronati, S. A. (2013). Analgesic Effects of 3-Substituted Derivatives of 1,4-Benzodiazepines and their Possible Mechanisms. Neurophysiology, 45(5), 427–432. doi: https://doi.org/10.1007/s11062-013-9389-y
42. Wright, S. L. (2020). Limited Utility for Benzodiazepines in Chronic Pain Management: A Narrative Review. Advances in Therapy, 37(6), 2604–2619. doi: https://doi.org/10.1007/s12325-020-01354-6
43. Zeilhofer, H. U., Möhler, H., Di Lio, A. (2009). GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. Trends in Pharmacological Sciences, 30(8), 397–402. doi: https://doi.org/10.1016/j.tips.2009.05.007