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Research Article

Molecular docking, ADMET study and in vivo pharmacological research of N-(3,4-dimetoxyphenyl)-2-{[2-methyl-6-(pyridine-2-yl)-pyrimidin-4-yl]thio} acetamide as a promising anticonvulsant

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Abstract

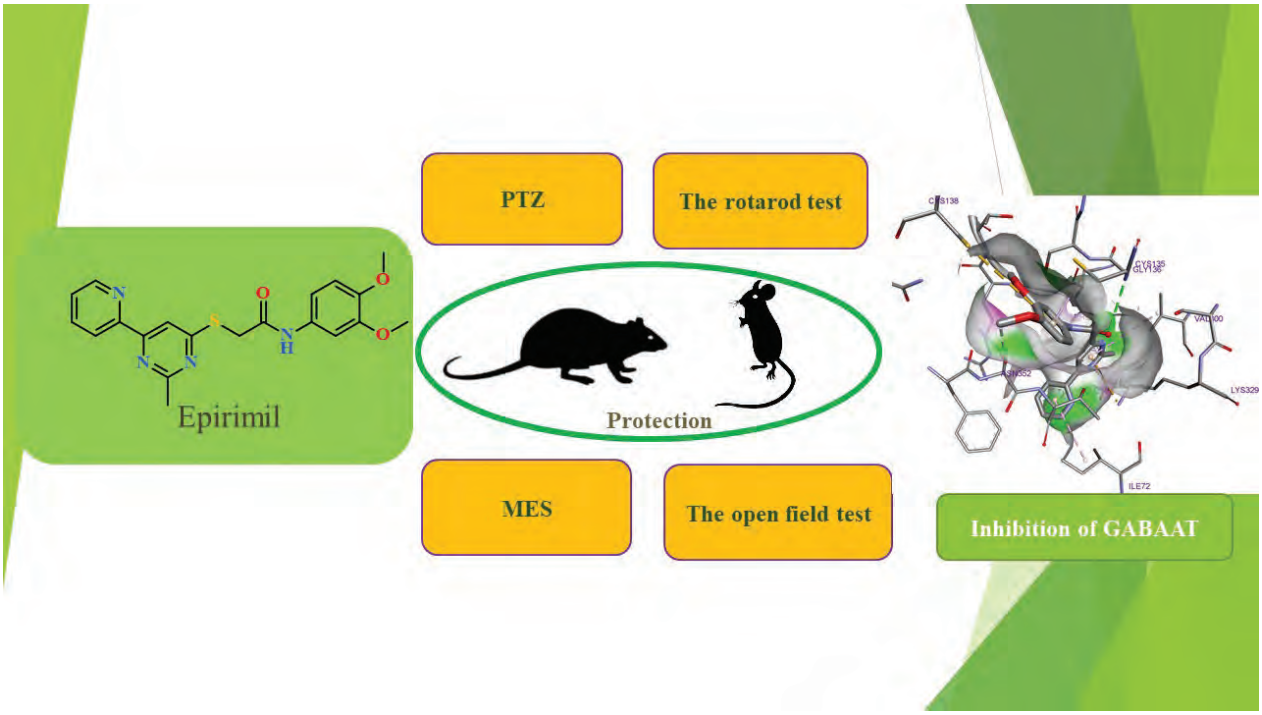
Introduction: The search for new anticonvulsants for epilepsy treatment with higher efficacy and better tolerability remains important. The aim of the present research was an *in silico* and *in vivo* pharmacological study of N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)pyrimidin-4-yl)thio)acetamide (Epirimil) as a promising anticonvulsant.

Materials and methods: A ^{1}H and ^{13}C NMR spectroscopy, LS/MS, and an elemental analysis were used to determine Epirimil structure. An ADMET analysis, as well as a docking study using anticonvulsant biotargets, e.g.: GABAAR, GABAAT, CA II, NMDAR, and AMPAR, was carried out. Anticonvulsant activity was proved, using PTZ- and MES-induced seizures in rats and mice, and neurotoxicity was determined using a rotarod test. Influence of Epirimil on the psycho-emotional state of the laboratory animals was determined by an open field test.

Results and discussion: A synthesis method of a promising anticonvulsant Epirimil was modified. The calculated ADMET parameters and a molecular docking into the active sites of anticonvulsant biotargets allowed evaluating the research prospects and predicting possible mechanisms for implementing anticonvulsant activity. A prominent anticonvulsant activity of Epirimil was established using *in vivo* studies on the model of PTZ-induced seizures in rats and MES-induced seizures in mice. In terms of toxicity, Epirimil belongs to class IV – low toxic substances. The open field test showed that Epirimil had almost no effect on the animals' behavioral responses: it neither changed their psycho-emotional activity, nor increased their anxiety level. ED_{50}, TD_{50} and protective index of Epirimil according to its anticonvulsant activity were calculated.

Conclusion: The obtained experimental results substantiate the prospects of N-(3,4-Dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)pyrimidin-4-yl)thio)acetamide as a promising active pharmaceutical ingredient having a multifactor mechanism of anticonvulsant activity.

Graphical abstract



Keywords

anticonvulsant, docking, pyrimidine, rotarod test.

Introduction

Nowadays, epilepsy is considered to be one of the most widespread chronic neurological diseases (Yuen et al. 2018; Gesche et al. 2019). Despite the successful development of various new anti-epileptic drugs (AEDs) over the recent decades (Vossler et al. 2018), all of them are characterized by a wide range of side effects, most of which are CNS disorders, i.e. depression, anxiety states, and cognitive disorders (Piedad et al. 2012; Perucca 2014). The spread of refractory epilepsy is also an important problem for today (Laxer et al. 2014; Janmohamed et al. 2020). It is worth mentioning that almost one-third of all epilepsy cases are drug-resistant and thus require new therapy approaches (Steinhoff 2015). Therefore, the search for new innovative drugs with better efficacy and tolerability remains an important goal for scientists. Finding and developing a new AED depend to a great extent on the rational, reasonable and comprehensive preclinical use of screening animal models in combination with *in silico* methods (Löscher 2017).

Our previous research (Severina et al. 2012) resulted in a number of new compounds, e.g.: 2,5,6-substantiated derivatives of pyrimidine-4(3*H*)-one (thione) and carrying out their pharmacological screening on the basic model of pentylenetetrazole-induced seizures in rats (Severina et al. 2013). This pharmacological model is recognized as the gold standard for screening tests and allows predicting anticonvulsant activity in relation to generalized myoclonic seizures (Krall et al. 1978, Bialer and White 2010). According to the experimental results, the most active compound – N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)pyrimidin-4-yl)thio)acetamide, or Epirimil, was found (Fig. 1), which completely prevented seizure development in 100% of the experimental animals and did not lead to any changes in their behavior compared to the control group.

According to the international approaches to searching for new AEDs – Anticonvulsant Drug Development (ADD) Program of the National Institutes of Health (Stables and Kupferberg 1997) and Epilepsy therapy screening program (ETSP) (Kehne et al. 2017) – to determine an anticonvulsant activity spectrum along with antagonism to pentylenetetrazole, the study of the effect of the compound on the model of primary-generalized clonic-tonic seizures induced by electrocution (maximal electroshock seizure test, MES) is also necessary (Castel-Branco et al. 2009). The mechanism of convulsion development in the MES model is caused by depolarization of the nerve cell membranes due sodium ions influx. This particular

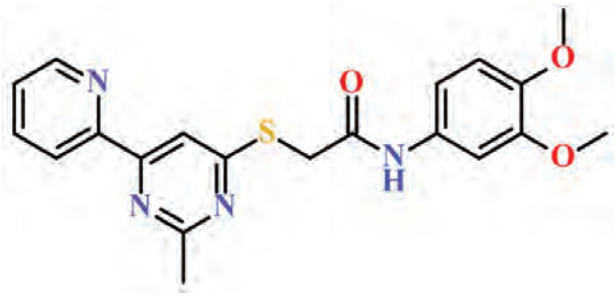


Figure 1. N-(3,4-dimethoxyphenyl)-2((2-methyl-6-(pyridin-2-yl)-pyrimidin-4-yl)thioacetamide (Epirimil).

model is recommended for the primary pharmacological screening to search for AEDs efficient for drug resistant epilepsy (Löscher 2017). Determination of the effective dose range, the main parameters of acute toxicity, including neurotoxicity, therapeutic and protective index evaluation, is a necessary stage of the search for new AEDs. As seizures are often accompanied by emotional state disorders in patients, it is vital to determine how the test compound will influence them (Brooks-Kayal et al. 2013).

Scientific achievements of the last decades concerning determination of anticonvulsant activity mechanisms, the crystal structure of target proteins, and an amino acid composition of active receptor sites, as well as a range of *in silico* methods developed to analyze and evaluate the ligand affinity to the receptor and ADMET parameters, make it possible to rationalize the search for new AEDs (Danielson et al. 2017). Relevance and future outlook of the further research of Epirimil was evaluated at the first stage, using virtual screening tools, namely molecular docking into active sites of the known anticonvulsant biotargets, and calculating ADMET (absorption, distribution, metabolism, excretion, and toxicity) – physico-chemical, pharmacokinetic, drug-like and related – parameters.

Therefore, the aim of the present research was an *in silico* and *in vivo* pharmacological study of N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)pyrimidin-4-yl) thio)acetamide (Epirimil) as a promising anticonvulsant.

Materials and methods

Molecular docking study

Flexible molecular docking as a main approach of the search for molecules having affinity to specific biological targets was used for the given study. Protein Data Bank was used to select specific macromolecules, i.e.: GABAAR (PDB ID 4COF), GABAAT (PDB ID 10HW), hBCATc (PDB ID 2COI), CA II (PDB ID 3IEO), NMDAR (PDB ID 5TP9), and AMPAR (PDB ID 5L1F) (PDB). The selection of the mentioned biological targets was substantiated according to the literature data concerning the modern anticonvulsants' mechanism of action (Bialer and White 2010). IsisDraw 2.4 software was used for depicting the ligand structures, which were saved as .mol files. At the next stage, Chem3D software was used to optimize the given molecules by MM2 molecular mechanical algorithm, with the results saved in .pdb format. Using AutoDockTools-1.5.6, the latter were converted into PDBQT, and the number of active torsions was set as default (Trott and Olson 2010). PDB-files of macromolecules were downloaded from the Protein Data Bank. Water and ligand were removed from the crystal by means of Discovery Studio Visualizer2017/R2 software tool. The structures of the obtained proteins were saved in .pdb format. Then, in AutoDockTools-1.5.6, polar hydrogen atoms were added and saved as PDBQT. Molecular docking was carried out using AutoDock Vina, and Discovery Studio Visualizer2017/R2 was used to visualize the obtained results.

ADMET

To calculate physico-chemical properties, lipophilicity, water solubility, pharmacokinetics, druglikeness and other medicinal chemistry parameters of the studied substances, SwissADME software was used (Daina et al. 2017). Table 1 demonstrates the obtained results of the calculations.

Chemistry

General methods, reagents, devices and equipment

For the present study, Sigma-Aldrich (USA) reagents were used. Their purification was carried out in accordance with the standard techniques. The thin layer chromatography (TLC) method using aluminum silica gel plates was used to control the reaction progress. The melting points of the substances (°C) were determined by the capillary method, using an electrothermal IA9100X1 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK). A Varian Mercury-400 (Varian Inc., Palo Alto, CA, USA) spectrometer (300 MHz) was used for determination of ^{1}H NMR spectra in hexadeuterodimethyl sulfoxide (DMSO-*d6*). Tetramethylsilane (TMS) was used as an internal standard. Chemical shifts were described in parts per million (ppm). ^{13}C NMR spectra were recorded on a Bruker Avance 400 (100.6 MHz). Chemical shifts were recorded in s ppm relative to TMS as an internal standard. A EuroVector EA-3000 (Eurovector SPA, Redavalle, Italy) elemental analyzer was used for the elemental analysis. The elemental composition was within ±0.4% of the theoretical values. A PE SCIEX API 150EX chromatograph was used for LC/MS spectroscopy.

Synthesis of N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)pyrimidin-4-yl)thio)acetamide (5)

Synthesis of 2-methyl-6-(pyridine-2-yl)pyrimidine-4(3*H*)-one (3). 1 mole of ethyl 3-oxo-3-(2-pyridyl) propanoate (2) was dissolved in 100 ml anhydrous methanol, then 3 mole of sodium methylate (freshly prepared solution in 300 ml of methanol) was added, and the

Table 1. ADMET Properties for the Test N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)-pyrimidin-4-yl)thio)acetamide.

Physico-chemical Properties		Lipophilicity Log P		Druglikeness violations		Medicinal Chemistry	
Molecular weight	382.44	iLOGP	3.36	Lipinski	0	PAINS[5] alerts	0
Rotatable bonds	7	XLOGP3	3.19	Ghose	0	Brenk alerts	1
H-bond acceptors	6	WLOGP	4.00	Veber	0	Leadlikeness	1
H-bond donors	1	MLOGP	2.05	Egan	0	Synthetic accessibility	3.32
Molar Refractivity	104.35	SILICOS-IT	3.21	Muegge	0		
TPSA[1] (Å)	111.53	Consensus	2.96	Bioavailability Score	0.55		
Pharmacokinetics							
GI[2] absorption	High	P-gp[4] substrate	No	CYP2C19 inhibitor	Yes	CYP2D6 inhibitor	Yes
BBB[3] permeant	Yes	CYP1A2 inhibitor	No	CYP2C9 inhibitor	No	CYP3A4 inhibitor	No

Note: [1] – Total polar surface area; [2] – Gastrointestinal; [3] – Blood-brain barrier; [4] – P-glycoprotein; [5] – Pan Assay Interference Compounds.

solutions were stirred for 30 minutes at a temperature of 25 °C, and then 1.5 mole of amidine hydrochloride (1) was added in portions for 30 minutes. Then, the mixture was heated at a temperature of 80 °C for 8 hours, cooled to 25 °C, and 3 mole of acetic acid was added. Methanol was removed using a rotary evaporator; 200 ml of water was added, and the obtained solution was stirred for 30 minutes. The formed precipitate was filtered, washed three times with 100 ml of water each time, and dried. The actual yield was 95%.

Synthesis of 2-methyl-6-(pyridin-2-yl)pyrimidine-4(3*H*)-thione (4). 1 mole of 2-methyl-6-(pyridin-2-yl)pyrimidine-4(3*H*)one (3) was mixed with 300 ml of toluene, and 1.1 mole of Lawesson reagent (LR) was added. Then the reaction mixture was kept boiling and stirred intensively for 5 hours, and then it was cooled to 25 °C. The formed precipitate was filtered, washed with toluene and crystallized from isopropanol, and then dried. The actual yield was 84%.

Synthesis of N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)-pyrimidin-4-yl)thio)acetamide (5). 1 mole of 2-methyl-6-(pyridin-2-yl)pyrimidine-4(3H)thione 4 was dissolved in 100 ml of DMF; then 1.1 mole of triethylamine and 1.1 mole of 2-chloro-N-(3,4- dimethoxyphenyl)acetamide was added at a temperature of 25 °C. The mixture was kept at a temperature of 60 °C, being and intensively stirred for 5 hours; then it was cooled, and 500 ml of water was added. The formed precipitate was filtered, washed with water and crystallized from isopropanol. The actual yield was 92%. The melting temperature 220–222 °C, molecular mass 396.47. Molecular formula: $C_{20}H_{20}N_4O_3S$. Calculated: C, % 60.59; H, 5.08; N, % 14.13; S, % 8.09. Found: C, % 60.38; H, 5.05; N, % 14.16; S, % 8.06. LC-MS: m/z = 397.1 [M+1].^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm)): 10.17 (c, 1H, NH), 8.69 (d, *J*=7.5 Hz, 1H, Ar), 8.37 (d, *J*=8 Hz, 1H, Ar), 8.08 (s, 1H, Ar), 7.90 (t, *J*=8.2 Hz, 1H, Ar), 7.59 (t, *J*=8.2 Hz, 1H, Ar), 7.20 (s, 1H, CH-5), 7.05 (d, *J*=8 Hz, 1H, Ar), 6.85 (d, *J*=8 Hz, 1H, Ar), 4.14 (s, 2H, SCH_2), 3.85 (s, 6H, $2OCH_3$), 2.60 (s, 2H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6,): 171.8, 150.0, 168.2, 166.1, 164.2, 155.3, 149.2, 145.4, 137.2, 131.1, 123.6, 121.4, 115.1, 114.9, 112.2, 105.7, 38.8, 56.1 (2C), 24.4.

Pharmacological studies

Animals

Adult male rats weighing 130–150 g each, as well as adult random-bred albino mice of both sexes weighing 20–30 g each, were used for the research. The animals were housed under vivarium conditions at the National Pirogov Memorial Medical University (Vinnytsya, Ukraine) at a temperature of 19–24 °C, 60% humidity, the natural day/night cycle in polypropylene cages, with a standard diet and free access to food and water. The pharmacological studies were conducted in compliance with *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*, the procedures and requirements of The State Expert Center of the Ministry of Health of the Ukraine , the rules of The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the resolution of the First National Congress on Bioethics (Kyiv, 2001), and the Law of Ukraine №3447-IV «On Protection of Animals from Cruel Treatment» dated 02.21.2006.

Pentylenetetrazole-Induced Seizures (Vogel 2008)

The rats were divided into 4 groups of 7 animals each. Epirimil at a dose of 50 mg/kg, as well as the reference drugs lamotrigine (Lamictal by GlaxoSmithKline, Poland) at a dose of 20 mg/kg and phenobarbital (Phenobarbital IC by Interchem, Ukraine) at the same dose were administered intragastrically to the animals. One hour before the induced seizure, Epirimil and the reference drugs were dissolved in twin-80 and given to the animals using oral gavage cannula at a volume of 0.5 ml/100 g body weight. The control group received the solvent at the equivalent amount. To simulate seizures, Pentylenetetrazole (Corazolum by Sigma, USA) as an aqueous solution was administered subcutaneously at a dose of 80 mg/kg. After its administration, every mouse was placed into a separate plastic cylindrical container (20 cm diameter and 35 cm high). The animals were continuously monitored for 60 min. If seizures did not occur within 1 hour, the la-

tency period was considered to be 60 min. The evaluation of the anticonvulsant activity was estimated according to the following indicators: latent period of clonic or tonic seizures, score of severity of paroxysms, seizure duration, and lethality. A 5-point scale was used to estimate the intensity of seizures (Gerald and Riffee 1973).

Maximal electroshockseizures (MES) model (Vogel 2008)

The study was carried out using 40 nonlinear mice of both sexes weighing 25–28 g each. The animals were randomly divided into 4 groups of 10 animals each. Group 1 was considered to be control. Epirimil (50 mg/kg), lamotrigine (20 mg/kg) (Lamictalby GlaxoSmithKline, Poland) and carbamazepine (15 mg/kg) (Tegretol by Novartis Pharma, Italy) were administered to the animals of the 2nd, the 3rd, and the 4th groups, respectively. A study of the anticonvulsant activity was carried out 1 hour after administering the studied compounds. A 57800 ECT Unit (Ugo-Basile, Italy) with corneal electrodes for mice was used to induce electrical seizures. An electric current of 50 mA, 50 Hz, and 0.2 s duration with a sine wave stimulus was used to reproduce the MES model. The electrodes were treated with a 0.9% sodium chloride solution (ARTERIUM, Ukraine). A 2% lidocaine hydrochloride solution (EGIS, Hungary) was instilled into the conjunctival sac. The number of mice with tonic seizures, total seizure duration, and lethality were recorded.

Determination of the average effective dose

The anticonvulsant activity of Epirimil was estimated according to the number of the animals that had survived after the MES procedure. 50 nonlinear mice weighing 22–26 g each were used for the study. Epirimil was given once intragastrically at doses of 10, 20, 30, 40, 50 mg/kg after dissolution in twin-80. The average effective dose of the drug (ED_{50}) was calculated on the basis of the correlation between the drug activity and its dose by probit analysis.

Acute toxicity study

Acute toxicity (LD_{50} and its confidence interval) was determined by V.B. Prozorovsky method modified by T.V. Pastushenko (Pastushenko et al. 1985). In the experiment, 34 white nonlinear mice standardized by body weight (24±3) g were used. The animals were divided into 5 groups. Epirimil in the dose range from 100 mg/kg to 700 mg/kg was given to the animals once by intragastric administration after its dissolution in twin-80 ("quick-and-dirty" experiment). As soon as the starting dose of 300 mg/kg was determined, Epirimil was administered in step-by-step according to the schemes in (Pastushenko et al. 1985). The animals were watched for 14 days. On the 1st day of the study, the animals were continuously monitored. Their behavior and weight were registered, then clinical symptoms of intoxication were determined: general condition, motor activity, breathing features, hair and skin conditions, presence of seizures, and food and water consumption; besides the number of dead animals in each group was registered.

Neurotoxicity study (Vogel 2008)

The program of studying Epirimil neurotoxicity included several consistent steps. The rotarod performance test was the main method. The additional information was received in the open-field test, which allowed studying the spectrum of Epirimil neuropsychotropic activity in detail (the presence or absence of sedative or activating, or anxiolytic effect in the studied substance, its possible effect on the emotional sphere and memory).

Rotarod test

100 mice of both sexes (weighing 24–28 g each) were used for the research. The animals were randomly divided into 10 groups. Various doses of Epirimil were administered intragastrically to the studied groups, and then neurotoxicological effects (sedation and ataxia) were observed and recorded using the rotarod test (Cortez and Snead 2006; Gaitatzis and Sander 2013). Only the animals that demonstrated their ability to remain on the rotating rod for at least 1 minute were used for the further test. Epirimil was given once by intragastric administration at the consistently increasing doses ranging from 10 to 200 mg/kg (10, 30, 50, 70, 90, 130, 140, 180, 200 mg/kg, respectively) as a tween-80 suspension 30 minutes before the test. The animals of the control group received only solvent – tween-80. The inability of the animals to remain on the rod (3 cm in diameter), rotating at a speed of 15 rpm for 60 seconds, at least in one of three attempts was considered a negative symptom.

Open-field test

The influence of Epirimil on orientational-investigative activity and psycho-emotional state in rats was determined using the open-field test (Greenshaw et al. 1988). Modification of the hole-board test as a relatively simple procedure was used to estimate simultaneously locomotion and curiosity of the animals (Adams and Geyer 1982; Geyer et al. 1986). Epirimil was administered once intragastrically to the rats at an average effective anticonvulsant dose of 12.5 mg/kg. The control group of animals received the equivalent dose of the solvent. Each group consisted of 7 animals. The studies were performed 1 hour after the drug administration. The rat was placed in the center of the field, and its investigative activity was evaluated for 3 minutes according to the number of lines and vertical racks crossed, and holes explored. The emotional condition was evaluated according to the number of washings. The anxiety level was determined in accordance with the number of defecations and urinations.

Statistical analysis

All results were expressed as the average values ± S.E.M. One-way ANOVA (analysis of variance) followed by Dunnett's test (Statistical package for social sciences, SPSS 16.0, USA) was used to analyze the obtained data. To compare the neurotoxicity differences, an X2 analysis (number of failures per test) was used. $P \leq 0.05$ was considered as significant.

Results and discussion

Docking study

Selecting biotargets

The Pro-convulsant activity of PTZ is caused by inhibiting the benzodiazepine receptor complex $GABA_A$-site and decreasing the intensity of GABA-ergic inhibitory processes in the CNS. GABA concentration in the human's brain is controlled by two pyridoxal-5'-phosphate-dependent enzymes – glutamate decarboxylase, which catalyzes glutamate transformation to GABA, and GABA-aminotransferase, which is responsible for the inhibitory neurotransmitter degradation. The previously established efficacy of N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl) pyrimidin-4-yl)thio)acetamide on the PTZ-induced seizure model(Severina et al. 2013 suggests that the mentioned compound may act as a GABA agonist or inhibit GABA aminotransferase. At the same time, such drugs as phenobarbital, benzodiazepine, gabapentin, vigabatrin, felbamate, etc. show their efficacy on the PTZ-induced seizure model, as most of these drugs have a multifactor mechanism of anticonvulsant activity.

To predict the GABA-ergic mechanism of anticonvulsant activity, the Epirimil interaction was studied with the active sites of the type-A γ-aminobutyric acid receptor ($GABA_AR$) (Miller and Aricescu 2014), γ-aminobutyrate aminotransferase ($GABA_{AT}$) enzyme (Storici et al. 1999), and cytosolic human branched-chain aminotransferase (hBCATc) (Goto et al. 2005). So the reference drugs were those phenobarbital which enhances the inhibitory neurotransmission by allosterically modulating GABAA receptor-mediated Cl-currents; vigabatrin, which increases GABA intracellular concentration in the human's brain by irreversible inhibition of GABA aminotransferase; gabapentin, which by influencing the leucine transport and increasing glutamate decarboxylase activity increases the GABA concentration (Bialer and White 2010).

The prospects of using the anticonvulsant activity of Epirimil on the MES-induced seizure model and the probability of its multifactor anticonvulsant mechanism were evaluated by molecular docking into the active sites of ionotropic glutamate receptors N-methyl-D-aspartate (NMDAR) (Villemure et al. 2017) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) (Yelshanskaya et al. 2016), as well as Carbonic anhydrase II (CA II) (Temperini et al. 2010). Native ligands of these biotargets, respectively, are modern anticonvulsant drugs, such as felbamate – NMDAR antagonist, perampanel – AMPAR antagonist, and lacosamide, the mechanism of anticonvulsant activity of which is not fully determined yet, but one of the options is its ability to enhance selectively slow inactivation of voltage-gated sodium chennels (Bialer and White 2010). All the drugs show their activity exactly on the MES model and on the 6-Hz test for partial seizures, as well as on some kindling models.

Docking results

Epirimil conformations in the active sites of anticonvulsant biotargets are shown in Figs 2–7 (hydrogen bonds are indicated with green dotted lines, hydrophobic interactions – purple dotted lines, electrostatic – orange lines, unfavorable – red lines) and the affinity quantitative features – binding energy and types of interaction with amino acid residues – are shown in Table 2. The results of docking into the active site of $GABA_A$ receptor show (Fig. 2), though not deep, but dipping into the active site, but the interaction occurs with amino acids of both chains of the protein (A and B) and with all the fragments of the ligand molecule.

The following interactions are detected: six hydrophobic bonds between an Epirimil pyrimidine cycle and methyl groups of two alanine residues (Ala 135, 245); a pyridine cycle and methyl groups of leucine (Leu99) and alanine (Ala45); a methyl group in the 2nd position of the pyridine cycle and alanine (Ala135); an aryl ring and CH_2-group of methionine (Met137). Aconventional hydrogen bond is formed between a carbonyl group of the acetamide fragment and a methionine amino group (Met137) (distance 3.16 Å), and one carbon hydrogen bond with asparagine (Asp48). The scoring function value – -7.3 kcal/mol – was close to that of the reference drug – phenobarbital (-7.6 kcal/mol), which points to the possibility Epirimil exerting an anticonvulsant activity as $GABA_A$ receptor agonist.

Molecular docking of Epirimil into the active site $GABA_{AT}$ (1OHW) showed a better affinity and a lower scoring function value (-7.8 kcal/mol) comparing to that of the native ligand – vigabatrine (-6.7 kcal/mol). Figure 3 shows that the studied ligand completely fills the active site in the target protein and engages in the hydrophobic interaction with chain A amino acids: between the methyl group in the 2nd position and phenylalanine phenyl radical (Phe189), the pyridine ring and the alkyl fragment of isoleucine (Ile72) and the methyl group of valine (Val300), as well as the phenyl ring and sulfur of cysteine (Cys135). The conformation is additionally stabilized by electrostatic bonds with amino acids residues of lysine and glutamine (Lys 329, Glu270), and it forms a bond between the phenyl ring of the acetamide residue and sulfur of cysteine (Cys138). Besides, conventional hydrogen bonds are formed between the carbon group of the acetamide fragment and the glycine amino group (Gly136), as well as a carbon hydrogen bond with asparagine (Asn 352).

Table 2. Docking Results of Epirimil and Native Ligands into Active Sites of Anticonvulsant Biotargets.

Receptor	Binding energy kcal/mol	Hydrophobic interaction	Hydrogen interaction	Other interactions	Native ligand binding energy
Epirimil Ligand					
$GABA_AR$	-7.3	Leu99(B), Ala135(B), Ala245(A), Ala135(B), Met137(B)	Met137(B), Asp48(B)		phenobarbital -7.6
$GABA_{AT}$	-7.8	Phe189(A), Ile72(A), Val300(A), Cys135 (A)	Gly136(A)	Lys 329(A),Glu270(A), Cys 138(B) (Pi-Sulfur) Asn 352(B)	vigabatrine -6.7
hBCATc	-4.2	Val175, Tyr90, Phe95, Tyr227	Arg119, Tyr193, Ala334, Thr333, Thr260, Ser331	Lys 222 (Pi-Cation)	gabapentine -7.6
CA II	-4.2	Ile91, His94, His 64	Asn62, Gln92,Phe		lacosamide -6.8
NMDAR	-8.9	Tyr144(B), Pro129(A), Val266(A)	Tyr144(B)		felbamate -7.7
AMPAR	-9.6	Ala622, Phe623, Pro520, Leu620, Arg628	Asn619	Asp519	parampanel -10.6

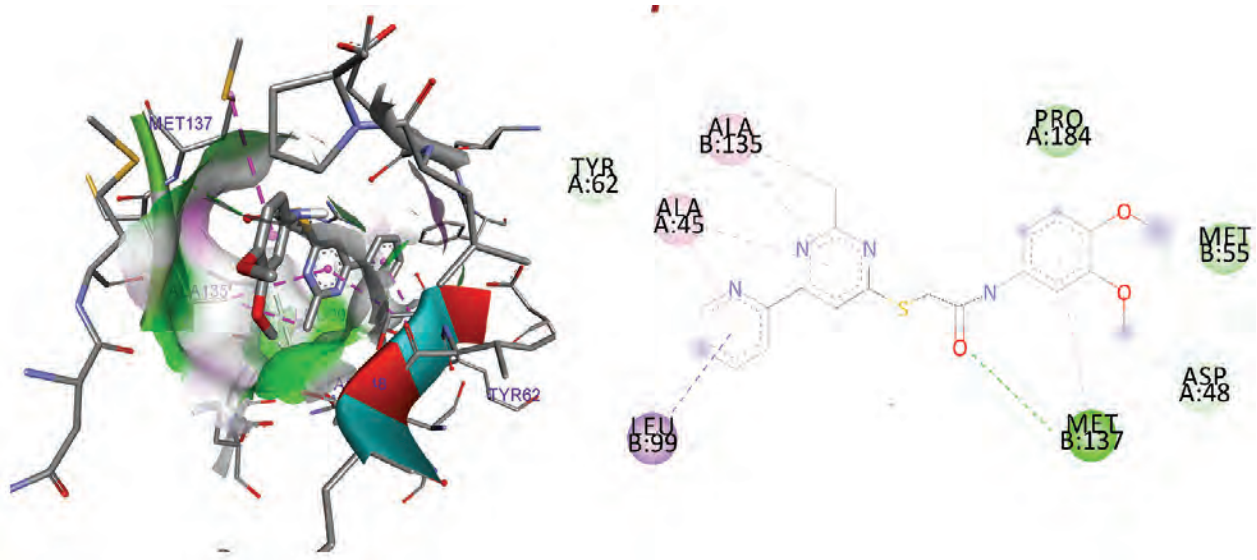


Figure 2. 3D and 2D interaction between $GABA_AR$ and Epirimil ligand.

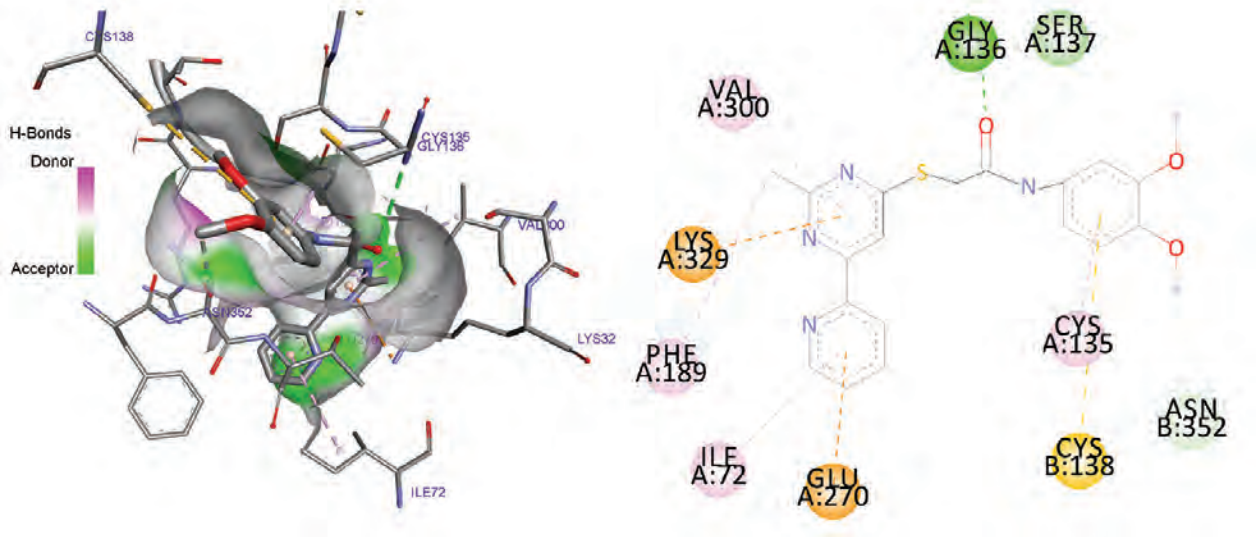


Figure 3. 3D and 2D interaction between $GABA_{AT}$ and Epirimil ligand.

When Epirimil interacted with the hBCATc active site, the binding energy was very high: -4.2 kcal/mol, while for the native ligand – gabapentin – it was -7.6 kcal/mol. This can be explained by the fact that the binding site is actually a small hydrophobic pocket where a hydrophobic fragment of the ligand – 2-methyl-6-(pyridin-2-yl)-pyrimidine – immersed (Fig. 4), forming hydrophobic interactions with valine, tyrosine, and phenylalanine (Val175, Tyr90, Phe95, Tyr227), but the acetamide fragment remains outside, forming only hydrogen bonds with the hydrophilic environment (Arg119, Tyr193, Ala334, Thr333, Thr260, Ser331). Besides, one unfavorable bump with tyrosine is predicted (Tyr193).

The interaction between Epirimil and the active site of carbonic anhydrase II (CA II) is rather superficial and one-sided (Fig. 5): only 4 hydrophobic bonds are formed with three amino acid residues – isoleucine (Ile91), histidine (His94 and 64), and hydrogen bonds – with asparagine (Asn62), glutamine (Gln92), and phenylalanine (Phe70). Superficial dipping into the active site is characterized by a high scoring function value -4.2 kcal/mol, comparing to -6.8 kcal/mol in the native lacosamide ligand, which makes such a conformation unlikely.

Epirimil had a high affinity to active sites of ionotropic glutamate receptors – NMDA and AMPA: the binding energy was -8.9 comparing to -7.7 kcal/mol for felbamate, and -9.6 comparing to -10.6 kcal/mol for perampanel. Epirimil is fully dipped into a rather narrow hydrophobic pocket of the NMDA receptor,forming strong hydrophobic bonds between the pyrimidine ring, the methyl group in the 2nd position and the aromatic ring of tyrosine (Tyr144), two bonds with the proline pyrrolidine ring (Pro129), and between valine pyridine residue and methyl group (Val266) (Fig. 6). One conventional hydrogen bond is formed between the carbonyl acetamide fragment and an OH-group of the tyrosine p-hydroxyphenyl residue (Tyr144).

The scoring function of Epirimil interacting with the AMPA-receptor was a little worse comparing to that of native ligand perampanel, but the value of -9.6 kcal/mol and a large number of hydrophobic bonds with all the molecule fragments indicates the formation of a stable and energy-efficient conformation between the ligand and the receptor: between the methyl group in the 2nd position and the phenyl radical of phenylalanine (Phe623), pyrimidine and pyridine rings with pyrrolidine fragment of proline (Pro520), the pyridine fragment and methyl group of leucine (Leu620), the aryl fragment and methyl groups of alanine (Ala622) and arginine (Arg628). The conformation is stabilized by a hydrogen bond between asparagine carbonyl (Asn619) and an NH-group of Epirimil (the bond length was 2.56 Å).

To sum up the obtained docking results, a multifactor mechanism of the anticonvulsant effect of Epirimil can be predicted, due to $GABA_{AT}$ inhibition and antagonism to glutamate ionotropic receptors – NMDA and AMPA, which demonstrates the perspectives of the further advanced pharmacological study of Epirimil.

ADMET analysis

When searching for new innovative molecules promising as future APIs, their pharmacokinetic properties are important. *In silico* calculations of ADMET parameters for N-(3,4-dimethoxyphenyl)-2-((2-methyl-6-(pyridin-2-yl)pyrimidin-4-yl)thio)acetamide (Table 1) were carried out for a preliminary prediction. According to the obtained results, physicochemical and pharmacokinetic properties, drug-likeness and medicinal chemistry friendliness were determined for Epirimil, and potentially high oral bioavailability of Epirimil was predicted. The physical and chemical parameters, including the number of H-bond donors and acceptors (1:6), molecular weight (382.44), the total polar surface area (TPSA) – 111.53 (the desired range was 20–130Å^2), turned out to be optimal. The mentioned indicators directly affect the permeability through the blood-brain barrier, as well as an ability to form stable conformations between the ligand and the receptor (Lipinski 2016). Lipophilicity as a classic descriptor also influences this ability (Lipinski 2001), and it is satisfactory for Epirimil. Epirimil will not become a substrate for glycoprotein-P, the activity of which is linked to the development of refractory epilepsy (Wang et al. 2016). The absolute druglikeness by the all program filters – Lipinski,Ghose, Veber, Egan, Muegge – demonstrates the prospect of its further *in vivo* research.

Chemistry

In order to ensure the high quality of the biologically active substance, of key importance is to select a synthesis method, which would allow the final product to be obtained with a minimal amount of concomitant impurities and, at the same time, provide a high API yield and acceptable purity. The decision to carry out an advanced pharmacological study of N-(3,4-dimethoxyphenyl)-2-[2-methyl-6-(pyridine-2-yl)pyrimidine-4-yl]thioacetamide prompted us to somewhat improve the method of its synthesis in order to increase the target product yield and to use less toxic reagents. The synthesis phasing was not changed (Scheme 1), but the reaction conditions were changed.

At the stage obtaining 2-methyl-6-(pyridine-2-yl)pyrimidine-4(3*H*)one (**3**) from amidine hydrochloride (**1**) and ethyl 3-oxo-3-(2-pyridyl)propanoate (**2**), a dioxane solvent was replaced by less toxic methanol (limit concentration 380ppm/3000ppm, respectively) (European Pharmacopoeia) with sodium methylate present as a catalyst. The given modification resulted in an increased product yield from 84% to 95%, respectively, and made it possible to further use pyrimidine-4-one without additional purification. At the stage of transformation of the carbonyl group into a thio group (B), toxic phosphorus pentasulfide was replaced by a softer Lawesson's Reagent, which is widely used in organic chemistry, and a pyridine solvent was replaced by toluene (Ozturk et al. 2007). Though both of the solvents belong to Toxicity Category 2, the limit concentrations in APIs are significantly different, e.g. 200 ppm in pyridine and 890 ppm in toluene. The

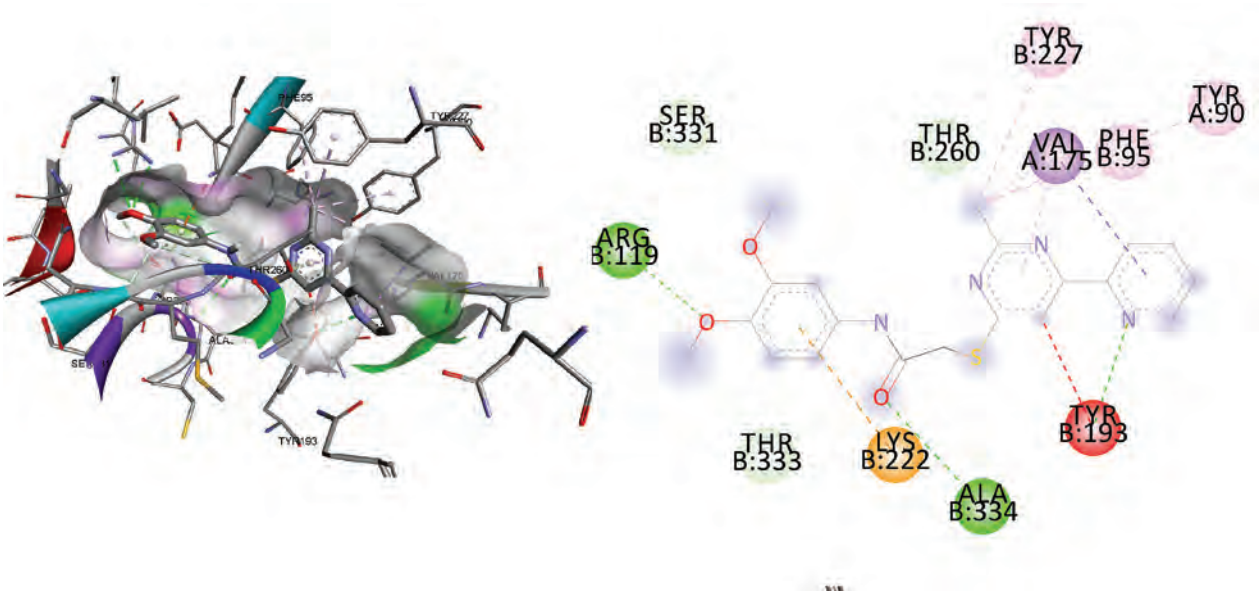


Figure 4. 3D and 2D interaction between hBCATc and Epirimil ligand.

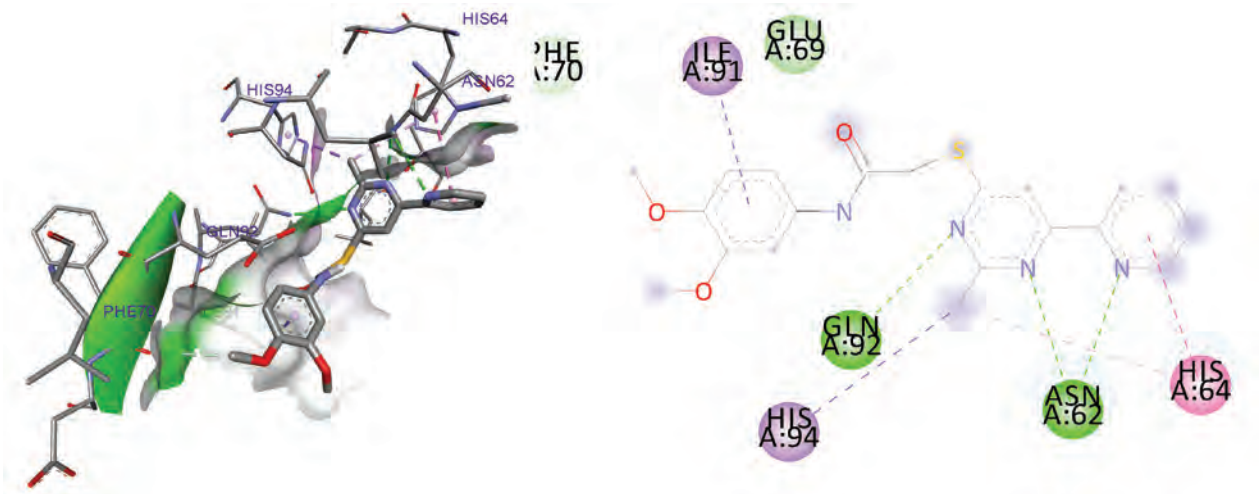


Figure 5. 3D and 2D interaction between CAII and Epirimil ligand.

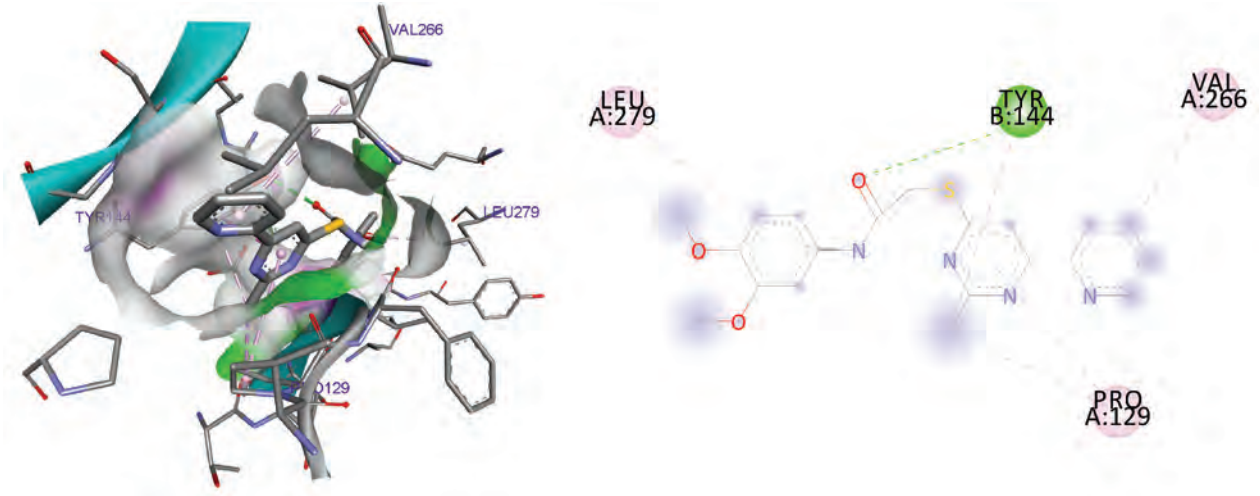


Figure 6. 3D and 2D interaction between NMDAR and Epirimil ligand.

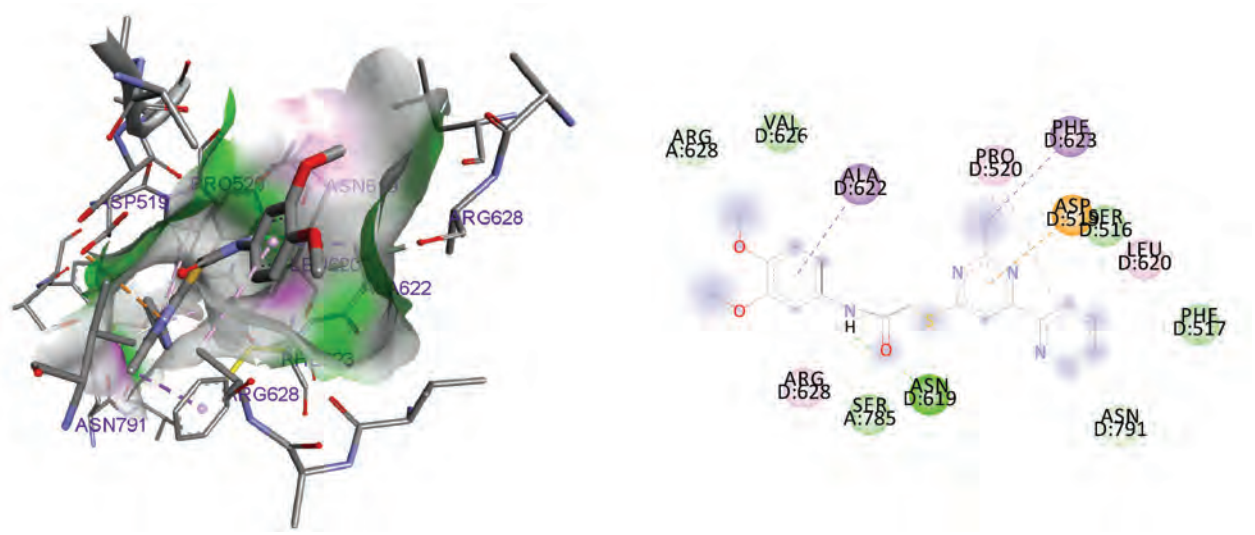
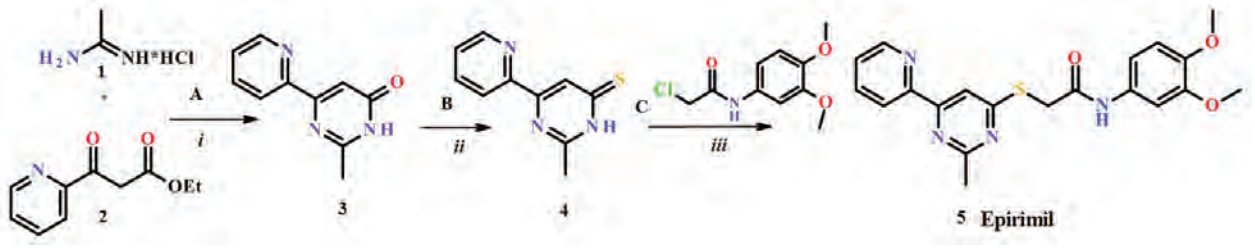


Figure 7. 3D and 2D interaction between AMPAR and Epirimil ligand.



Scheme 1. Reagents and conditions: (i) MeONa/ dry MeOH, 80 °C; (ii) Toluen, RL, t°; (iii) Et_3N, dry DMF, 60 °C.

conditions of C-alkylation of pyrimidine-4-thione (**4**) by 2-chloro-N-(3,4-dimethoxyphenyl)acetamide remained without changes, because these conditions were optimal for the alkylation of the thiogroup (Ivachtchenko et al. 2004). Only the reaction time was increased to 5 hours, which made possible to increase the alkylation product yield to 92%. The obtained N-(3,4-dimethoxyphenyl)-2-[2-methyl-6-(pyridine-2-yl)pyrimidine-4-yl]thioacetamide (**5**) by the melting point and spectral data corresponds to such described earlier (Severina et al. 2012). The alterations made allowed reducing the use of toxic reagents and increasing the total yield of Epirimil by 14%.

Pharmacological study

Anticonvulsant activity on the model of PTZ-induced seizures

It is known that various biopharmaceutical aspects, including purity, concomitant impurities, crystal form, etc., influence the manifestation of the pharmacological activity (Severina et al. 2017). As the synthesis conditions of the test substance Epirimil were changed, its anticonvulsant activity was examined, using the PTZ-induced seizures in rats to obtain more reliable results. The administration of pentylenetetrazole led to convulsions development in all the animals of the control group: the latent period lasted on average 4.7 minutes, and the convulsion period – 9.7 minutes (Table 3). Besides, the convulsive syndrome was accompanied by a significant tonic-clonic seizures, a definite tonic extension and 100% lethality were registered.

Phenobarbital significantly prevented seizures development in all the animals. After administering lamotrigine to the rats, pentylenetetrazole caused some signs of convulsions (convulsive twitching, jumps and tonic contractions of the forelimbs). But the introduction of lamotrigine statistically significantly – 6.25 times – extended the latent period, significantly reduced the severity and duration of seizures compared to those of the control group, and the lethality was 14.3%. As shown in the previous studies (Severina et al. 2013), Epirimil prevented convulsions development, completely protected the test animals from seizures and absolutely prevented lethality.

Anticonvulsant activity on the model of MES-induced seizures

Under MES test conditions (Table 4), the reference drugs – lamotrigine and carbamazepine – exerted a pronounced anticonvulsant activity: the number of animals having MES-induced seizures reduced by 30% and 20%, respectively; duration of seizures reduced by 82.3% and 86.9%, respectively, prevented tonic hind limbs extension, and prevented lethality by 90% and 100%, respectively ($p < 0.05$).

Epirimil also exerted well-pronounced anticonvulsant properties, not being inferior to the reference drug carbamazepine and slightly exceeding lamotrigine by all the indicators. After its administration, there was no lethality among the animals, whereas in the control group lethality was 20%, and the overall duration of seizure period was 88.5% shorter ($p<0.05$) than that in the animals without pharmacological correction. The MES-induced seizure test showed that the studied Epirimil is effective in reducing the major primary generalized paroxysms.

Determination of the average effective dose of Epirimil

The average effective dose (ED_{50}) of N-(3,4-dimethoxyphenyl)-2-{[2-methyl-6-(pyridine-2-yl)pyrimidine-4-yl] thio}acetamide in mice was determined by the probit analysis method, using the MES model. The obtained results showed that ED_{50} was 12.5 ± 0.14 mg/kg. The upper ED_{50} limit was 19.82 mg/kg, and the lower ED_{50} limit was 1.22 mg/kg (Table 5).

Acute toxicity

The results of the study of Epirimil acute toxicity are shown in Table 5. High doses caused a hypnosedative effect, breathing and locomotor activity disorders were observed, followed by a deep narcotic condition, resulting in the animals'deaths. The deaths of animals after administration of high doses were recorded for three days. The surviving animals were active after 12 and 24 hours, as well as for the next 14 days. There were no differences in either food intake or body weight compared to those in the control; the normal reflexes were present, any no behavioral deviations or clinical symptoms of intoxication were registered. The skin was smooth and shiny, without any redness, flaking, cracks or other symptoms. In addition, there were no significant deviations in the body weight in the surviving mice. The results of determining LD_{50} in white mice after intragastric administration of the tested substance (Table 6) showed that its acute toxicity (LD_{50}) was 522.0 (432÷613) mg/kg. Therefore, according to the toxicity level, the studied

Table 3. Impact of Epirimil on the Pentylenetetrazole-induced Seizures in Rats.

Groups of animals	Dose, mg/kg	Number of rats in group	Duration of latent period, min	Duration of seizures, min	Lethality, abs. units (%)	Severity of seizures, (points)
Control	–	7	4.7 ± 0.30	9.70 ± 0.90	7(100)	4.96
Epirimil	50 (i.g)	7	30.0	0*	0*	0*
Phenobarbital	20 (i.g)	7	30.0	0*	0*	0*
Lamotrigine	20 (i.g)	7	29.4 ± 2.4*	1.86 ± 0.34*	1(14.3)	2.0

Note: * – statistically significant result ($p\leq0.05$) relatively to the control group animals.

Table 4. Impact of Epirimil, Carbamazepine and Lamotrigine on MES-induced Seizures in Mice.

Experiment conditions	Number of mice in group	Dose, mg/kg	Number of mice with seizures	Duration of seizures (clonic seizures + tonic existence), sec.	Lethality, abs. units (%)
Control	10	–	9	47.3 ± 0.48	2(20%)
Epirimil	10	50	1	5.4 ± 0.31* (-88.5%)	0*
Lamotridgine	10	20	3	8.4 ± 0.39* (-82.3%)	1(10%)
Carbamazepine	10	15	2	6.2 ± 0.56* (-86.9%)	0*

Note: * – statistically significant differences of the obtained results in the experimental group ($p < 0.05$) relatively to the control.

Table 5. Parameters of Average Effective Doses of Epirimil on MES Model in Mice.

Dose, mg/kg intragastrically	The number of animals in group	The number of survived animals	Statistical index M ± m			
			ED_{16} mg/kg	ED_{50} mg/kg	ED_{84} mg/kg	ED_{99} mg/kg
10	10	40%				
20	10	70%				
30	10	80%	4.03 ± 0.31	12.5 ± 0.14	38.7 ± 0.13	176.1 ± 0.36
40	10	80%				
50	10	90%				

Table 6. Results of Studying Acute Toxicity of Epirimil.

Compound	Dose, mg/kg	Number of animals	Lethality within 14 days males/females	LD_{50}, mg/kg and its confidence interval
Epirimil	398.0	6	0/3	522.0 (432÷613)
	447.0	6	1/3	
	500.0	6	1/3	
	562.0	6	2/3	
	630.0	6	3/3	
Solvent	–	4	0/2	

Table 7. Influence of Epirimil on Motion Coordination – rotarod test.

Dose, mg/kg intragastrically	Number of animals in group	Number of animals able to remain on the rod for 60 seconds
10	10	8
30	10	7
50	10	5
70	10	4
90	10	3
130	10	3
140	10	2
180	10	2
200	10	1
Control	10	10

compound can be referred to Class IV– low-toxic substances according to Hodge and Sterner (1949).

Neurotoxicity

Neurotoxicity manifested by motion and coordination dysfunctions in mice was evaluated using a rotating rod test (rotarod test) (Table 7).

According to the probit analysis results, the main parameters of the compound's neurotoxicity were determined and calculated. It was found that the average toxic dose of Epirimil in mice was TD_{50} = 45.89 mg/kg. The standard TD_{50} error was 1.66 mg/kg. The lowest TD_{50} was 23.97 mg/kg. The highest TD_{50} was 67.26 mg/kg. Besides, such important Epirimil's parameters as protective (PI = TD_{50}/ED_{50} = 3.67) and therapeutic indices (TI = LD_{50}/ED_{50} = 41.76) were calculated.

The influence on psycho-emotional state

One of the manifestations of neurotoxicity of psychotropic drugs, including AEDs, is behavioral changes (anxiety, depression, fear); therefore, it was necessary to evaluate the neurotropic properties of Epirimil at an effective anticonvulsant dose of 12.5 mg/kg. The obtained results of the open field test in rats are shown in Table 8.

Table 8. Impact of Epirimil and Lamotrigine on Psycho-emotional State in Rats in the Open-field Test (M ± m, n = 7).

Criteria	Control	Epirimil	Lamotrigine
Number of holes examined	5.29	4.43 (P = 0.535)	2.0 (P = 0.011)
Horizontal activity	18.4	17.6 (P = 0.751)	4.71 (P = 0.001)
Vertical activity	7.71	3.57 (P = 0.017)	1.43 (P = 0.001)
Grooming	4.43	3.43 (P = 0.442)	0.86 (P = 0.001)
Number of defecations and urinations	0.43	0	0

Table 9. Anticonvulsant Activity Parameters of Epirimil after Intragastric Administration.

Compound	LD_{50}, mg/kg	TD_{50}, mg/kg	ED_{50} mg/kg	TI, LD_{50}/ED_{50}	PI, TD_{50}/ED_{50}
Epirimil	522.0 (432 ÷ 613)	45.89 ± 1.66	12.5 ± 0.14	41.76	3.67

The results showed that Epirimil practically did not change the psycho-emotional activity of the animals and did not increase the level of anxiety. The studied parameters were almost similar to the ones of the control animals, except a decrease in horizontal and vertical activities. These indicators were 4.3% and 53.7% lower, respectively, compared to those in the control. However, these changes did not reach statistically significant values (except for horizontal activity and the number of defecations and urinations). But the reference drug lamotrigine showed a significantly greater sedative effect on the CNS, as evidenced by a statistically significant decrease in all the studied parameters of the psycho-emotional state in the animals.

The generalized numeric data of the anticonvulsant activity of Epirimil – the dose dependent parameters (ED_{50}) in the MES test, acute (LD_{50}) and neurotoxicity (TD_{50}) in the rotarod test indices as well as the therapeutic (TI) and protective (PI) indices are shown in Table 9.

Conclusion

Thus, as a result of the study undertaken, the synthesis method of a promising anticonvulsant N-(3,4-dimethoxyphenyl)-2-[2-methyl-6-(pyridin-2-yl)pyrimidin-4-yl] thioacetamide (Epirimil) was improved, and its additional spectral parameters (^{13}C NMR, LS/MS) were described. The use of *in silico* methods (ADMET, docking study) made it possible to rationally design the experiment and predict possible mechanisms of the anticonvulsant effect: inhibition of GABA-aminotransferase and antagonism to glutamate ionotropic receptors – NMDA and AMPA. The obtained results of the complex *in vivo* studies proved the feasibility and perspectives of further studying Epirimil as a promising anticonvulsant, effective on two models of seizures – PTZ-induced seizures in rats and MES-induced seizures in mice. According to this ability, Epirimil is comparable to with the known anticonvulsants – carbamazepine, phenobarbital and exceeds lamotrigine. Toxicity class IV, lack of influence on behavioral responses in the open-field test and motor functions in the rotarod test are positive features of a potential anticonvulsant. There were no statistically significant changes in any of the studied indicators of inquisitive, locomotor and psycho-emotional activities, except for decreased vertical activity. It is also worth mentioning that the *in silico* results were compared with the obtained *in vivo* results, and confirmed the possibility of a multifactor mechanism of the anticonvulsant activity.

Conflict of interest

The authors declare no conflict of interest.

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