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Preclinical evaluation of 1,2,4-triazole-based compounds targeting voltage-gated sodium channels (VGSCs) as promising anticonvulsant drug candidates

Barbara Kaproń[a], Jarogniew J. Łuszczki[b], Agata Siwek[c], Tadeusz Karcz[d], Gabriel Nowak[c], Mirosław Zagaja[e], Marta Andres-Mach[e], Anna Stasiłowicz[f], Judyta Cielecka-Piontek[f], Janusz Kocki[a], Tomasz Plech[g,*]

[a] Department of Clinical Genetics, Medical University of Lublin, Lublin, Poland
[b] Department of Pathophysiology, Medical University of Lublin, Lublin, Poland
[c] Department of Pharmacobiology, Jagiellonian University Medical College, Cracow, Poland
[d] Department of Technology and Biotechnology of Drugs, Jagiellonian University Medical College, Cracow, Poland
[e] Isobolographic Analysis Laboratory, Institute of Rural Health, Lublin, Poland
[f] Department of Pharmacognosy, Poznan University of Medical Sciences, Poznań, Poland
[g] Department of Pharmacology, Medical University of Lublin, Lublin, Poland

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ABSTRACT

Epilepsy is a chronic neurological disorder affecting nearly 65–70 million people worldwide. Despite the observed advances in the development of new antiepileptic drugs (AEDs), still about 30–40% of patients cannot achieve a satisfactory seizure control. In our current research, we aimed at using the combined results of radioligand binding experiments, PAMPA-BBB assay and animal experimentations in order to design a group of compounds that exhibit broad spectrum of anticonvulsant activity. The synthesized 4-alkyl-5-substituted-1,2,4-triazole-3-thione derivatives were primarily screened in the maximal electroshock-induced seizure (MES) test in mice. Next, the most promising compounds (**17**, **22**) were investigated in 6 Hz (32 mA) psychomotor seizure model. Protective effect of compound **22** was almost similar to that of levetiracetam. Moreover, these compounds did not induce genotoxic and hemolytic changes in human cells as well as they were characterized by low cellular toxicity. Taking into account the structural requirements for good anticonvulsant activity of 4-alkyl-5-aryl-1,2,4-triazole-3-thiones, it is visible that small electron-withdrawing substituents attached to phenyl ring have beneficial effects both on affinity towards VGSCs and protective activity in the animal models of epilepsy.

1. Introduction

Epilepsy is a chronic neurological disorder affecting nearly 65–70 million people worldwide [1]. Epileptics not only suffer from the disease's symptoms, but they are also at an increased risk of death [2]. Treatment of epilepsy is based mainly on the properly selected pharmacotherapy. However, despite the observed advances in the development of new antiepileptic drugs (AEDs), still about 30–40% of patients cannot achieve a satisfactory seizure control [3]. New AEDs usually have milder side-effects, better pharmacokinetic profile and slightly improved efficiency. Unfortunately, these drugs have not significantly improved the outcome of treatment in patients with therapy-resistant epilepsy. Hence, there is an immense need for new AEDs exhibiting efficiency against a broad spectrum of seizures.

In our recent papers, we have identified 4-alkyl-5-aryl-1,2,4-triazole-3-thiones as a promising group of antiepileptic drug candidates [4–7]. It has been proved that anticonvulsant activity of these compounds is due to their ability to interact with the voltage-gated sodium channels (VGSCs) [6,7]. Moreover, 4-alkyl-5-aryl-1,2,4-triazole-3-thione derivatives possessed favorable pharmacological and toxicological profile, including low neurotoxicity and toxicity against human cells, lack of genotoxic properties, fast onset of action, sustainable activity over time, and ability to potentiate the anticonvulsant activity of valproate [7,8]. The effect of the alkyl chain structure on the pharmacological activity, toxicity, ability to penetrate the blood-brain barrier, and the affinity towards VGSCs has been well-investigated and discussed in our previous articles [4–7]. 1,2,4-Triazole derivatives with unbranched alkyl chains, containing from 4 to 7 carbon atoms, turned

* Corresponding author at: Department of Pharmacology, Faculty of Health Sciences, Medical University of Lublin, Chodźki 4a, 20-093 Lublin, Poland.
E-mail address: tomasz.plech@umlub.pl (T. Plech).

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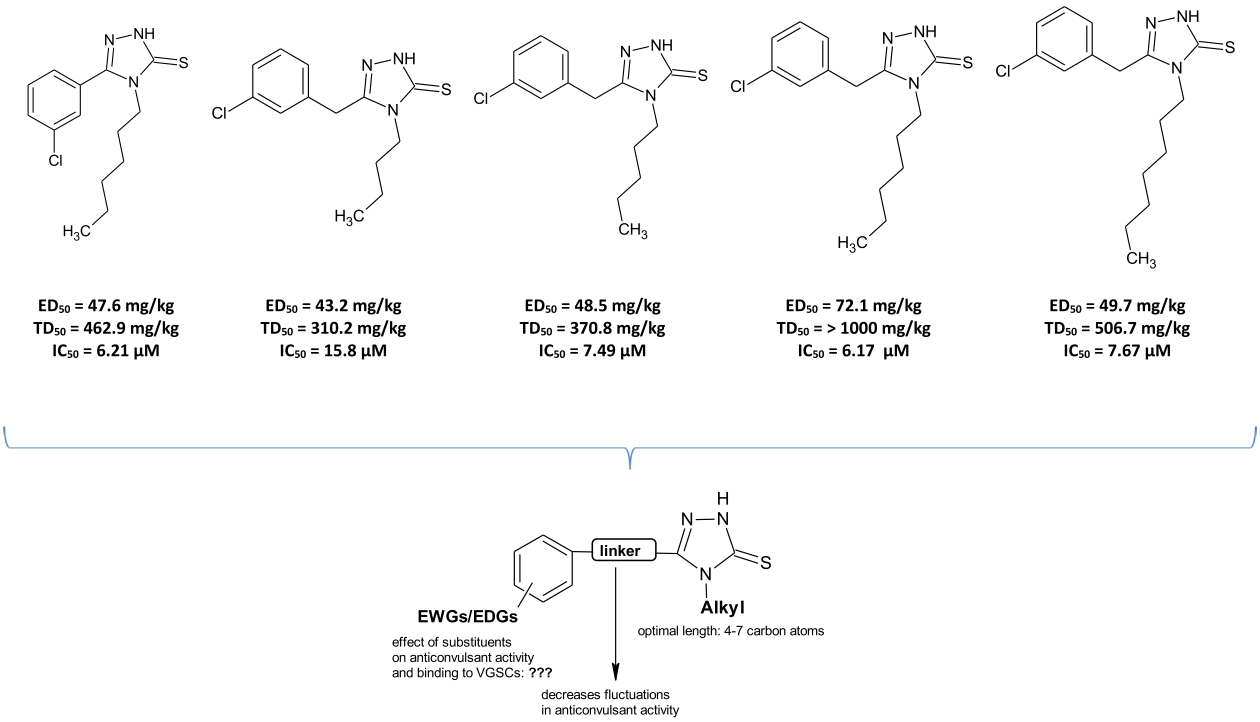


Fig. 1. Design of the target compounds based on the results of anticonvulsant activity (ED_{50}), neurotoxicity (TD_{50}) and affinity towards batrachotoxin-binding sites of VGSCs (IC_{50}) of the most promising 4-alkyl-5-substituted-1,2,4-triazole-3-thione derivatives published so far.

out to possess the most beneficial activity during preclinical studies. However, still little is known about the effects of structural modifications of the aryl moiety attached to 1,2,4-triazole-3-thione core on the anticonvulsant activity since only compounds with 3-chlorophenyl substituent (with or without a methylene linker) have been tested thus far (Fig. 1).

Additionally, these derivatives were tested only in the mouse maximal electroshock-induced seizure (MES) test, which is recognized model of generalized tonic-clonic seizures in humans. The ability of these compounds to inhibit seizures in other types of epilepsy would significantly improve the chances of 1,2,4-triazole derivatives to be investigated in further stages of drug development process. Therefore, in our current studies, we aimed to comprehensively analyze the role of aryl moiety attached to 1,2,4-triazole-3-thione in binding to molecular target and anticonvulsant effect of the synthesized compounds. The most promising 1,2,4-triazole derivatives were also evaluated in the mouse 6 Hz (32 mA) model of psychomotor seizures. We believe that the results presented in this manuscript will provide valuable insights on the design and development of new AED candidates.

2. Results and discussion

2.1. Chemistry

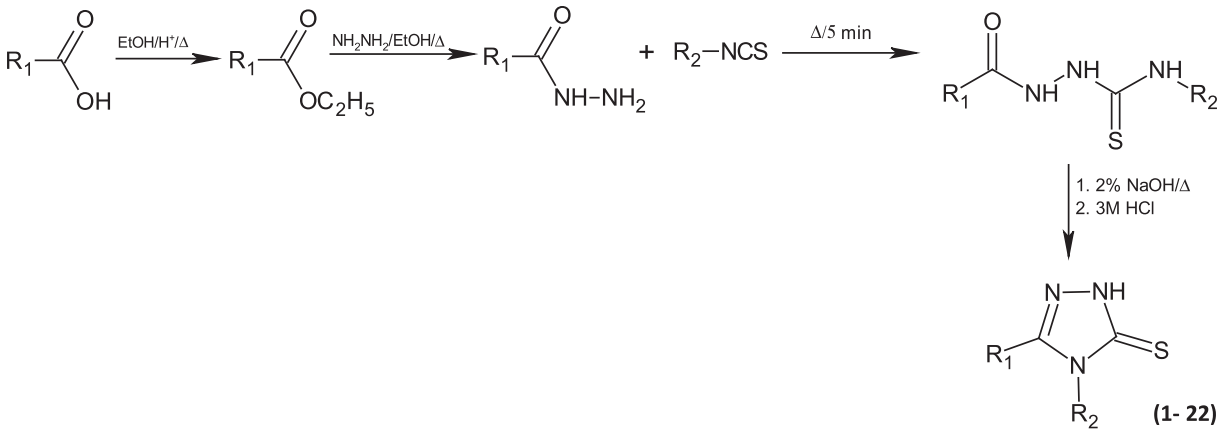
The investigated compounds were synthesized in moderate to high yields (i.e., 48–90%), starting from the respective carboxylic acids (Scheme 1). These acids were converted into the corresponding esters, which subsequently were subjected to hydrazinolysis in order to obtain carboxylic acid hydrazides. Reaction between respective hydrazides and alkyl isothiocyanates resulted in 1,4-disubstituted thiosemicarbazide derivatives. Dehydrocyclization of the respective thiosemicarbazides in an alkaline environment led to the formation of 4-alkyl-5-substituted-1,2,4-triazole-3-thiones (**1–22**). The purity of the title compounds was checked using elemental analysis (C, H, N), while their structures were elucidated on the basis of ^{1}H- and ^{13}C NMR spectra. The obtained results of the elemental analyses were within ± 0.4% of the theoretical values. The results of spectral analyses of the intermediates (i.e., 1,4-disubstituted thiosemicarbazides) are given as Supplementary Material.

2.2. Anticonvulsant activity

From the hitherto synthesized 4-alkyl-5-substituted-1,2,4-triazole-3-thiones, which had protective effect in mouse MES model of epilepsy, all possessed electron-withdrawing groups (EWGs) at the phenyl ring attached to 1,2,4-triazole core [4–7]. Therefore, in order to examine the effect of structural modifications of aryl substituent on the anticonvulsant activity of 1,2,4-triazole-based derivatives, a series of compounds (**1–8**) with electron-donating groups (EDGs) were obtained in the first stage of the study. EDGs differed in their electron-donating properties (−OH, $-OCH_3$) and position in the phenyl ring.

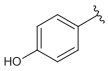
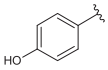
Among 1,2,4-triazoles (**1–8**), only a few compounds exhibited weak protective activity against MES-induced seizures (Table 1). This indicates that EDGs (regardless of their location) negatively affect their anticonvulsant activity measured in the MES test in mice. Therefore, during further studies the EWGs were selected for structure modifications. Only bromine and fluorine derivatives were synthesized (Table 2) since the role of chlorophenyl moiety, with or without a methylene linker, was extensively investigated in our previously published studies [4,5,7]. To better understand the mechanism of the anticonvulsant effect (or its lack) of the compounds **1–22**, an initial evaluation of their affinity towards the batrachotoxin-binding site of the VGSCs and their ability to permeate through the blood-brain barrier (BBB) has been performed (Table 3). These results confirmed that both compounds containing EDGs and EWGs can be classified as BBB + (or CNS +) since good permeation through BBB is expected for compounds with $P_e > 5.19$ cm/s [9]. Therefore weak activity of compounds **1–8** in mouse MES test was associated rather with low or no affinity towards VGSCs (see Table 3).

The anticonvulsant effect of the compounds containing EWGs (**9–22**) was clearly dependent on the type of halogen atom. Smaller substituents (-F, Cl) appear to have beneficial effect on the protective



Scheme 1. Synthetic route to compounds **1–22**. Structures of R_1 and R_2 substituents are given in Tables 1 and 2.

Table 1
Anticonvulsant activity of compounds **1–8** (containing EDGs) in the mouse maximal electroshock-induced seizure (MES) test.

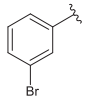
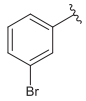
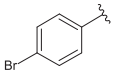
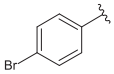
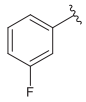
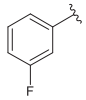
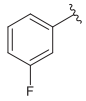
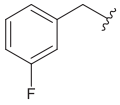
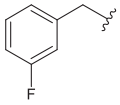
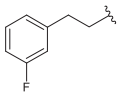
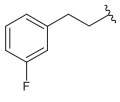
	R_1	R_2	Pretreatment time [min]	MES test ED_{50} [mg/kg] ± SEM
1.		hexyl	15	inactive
			30	
			60	
			120	
2.		heptyl	15	> 500
			30	430.6 ± 29.6
			60	361.8 ± 27.8
			120	330.2 ± 21.4
3.		hexyl	15	inactive
			30	
			60	
			120	
4.		heptyl	15	inactive
			30	
			60	
			120	
5.		hexyl	15	374.4 ± 10.9
			30	317.2 ± 9.5
			60	356.4 ± 19.8
			120	370.1 ± 9.6
6.		heptyl	15	inactive
			30	
			60	
			120	
7.		butyl	15	inactive
			30	
			60	
			120	
8.		hexyl	15	462.9 ± 20.0
			30	429.5 ± 19.0
			60	454.8 ± 24.1
			120	460.8 ± 22.6
	Valproate		15	189.0 ± 17.3
			30	216.9 ± 9.4
			60	218.4 ± 18.9
			120	246.6 ± 21.4

activity of 1,2,4-triazole-3-thiones in MES test in mice. This can be associated with their higher affinity towards VGSCs (lower IC_{50} values for the chlorine [6,7] and fluorine derivatives). Compounds with bromophenyl substituents (**9–14**) mostly possessed weak or no anticonvulsant activity (Table 2). Notably, fluorophenyl derivatives (**15–17**) were characterized by much stronger activity than valproate. The elongation of the alkyl substituent in compounds **15–17** had significant impact on their affinity towards batrachotoxin-binding site in VGSCs. Interestingly, both butyl and heptyl derivatives exhibited the same anticonvulsant potency at their peak activity (ED_{50} = 67.8 mg/kg), although these compounds showed apparently different affinity for VGSCs (i.e., 190 vs 18.2 μM). This may suggest the existence of an additional molecular mechanism responsible for anticonvulsant effect. Such phenomenon is very common in the case of AEDs. For instance, the antiepileptic activity of valproic acid and its salts is associated with blockade of Na^+ and *T*-type Ca^{2+} channels, inhibition of NMDA receptors-mediated excitation, enhancement of GABA-ergic neurotransmission and stimulation of K^+ currents [10].

In our recent studies, we have observed that introduction of a methylene linker into the 4-alkyl-5-aryl-1,2,4-triazole-3-thione derivatives significantly improved their pharmacological properties and decreased neurotoxic effects [5,7]. With this in mind, we analyzed the influence of two types of linkers, i.e., methylene and ethylene ones, on the anticonvulsant activity of 4-alkyl-5-(3-fluorophenyl)-1,2,4-triazole-3-thiones. Contrary to our expectations, the replacement of 3-fluorophenyl moiety by 3-fluorobenzyl group did not result in an increased efficiency of the obtained compounds (**18–20**) in the MES test in mice. Moreover, in the case of heptyl derivative (**20**) significant decrease in anticonvulsant activity has been observed. However, the introduction of methylene linker led to a beneficial time-course profile of anticonvulsant potency, similarly as in the case of previously investigated 4-alkyl-5-(3-chlorobenzyl)-1,2,4-triazole-3-thiones [5]. Respective hexyl and heptyl derivatives (**19**, **20**) lacked significant fluctuations of anticonvulsant activity at consecutive time points (i.e., 15, 30, 60, 120 min). Since *in-vitro* studies have shown that introduction of the ethylene linker leads to a further increase in affinity towards VGSCs, respective (3-fluorophenyl)ethyl derivatives (**21, 22**) were also evaluated against MES-induced seizures. These compounds were also characterized by the highest ability to cross the blood-brain barrier as shown in PAMPA-BBB model (Table 3). Both compounds (**21, 22**) exhibited stronger anticonvulsant activity than valproate. Additionally, 5-[(3-fluorophenyl)ethyl]-4-hexyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**22**) produced stable anticonvulsant effect, devoid of fluctuations after its administration to mice. This may confirm the hypothesis formulated in our earlier paper [5] that the presence of linker between aryl (i.e., halogenphenyl) moiety and 1,2,4-triazole core results in a slower elimination of the compounds from the central nervous system.

The most promising compounds (i.e., **17** and **22**), which activity was above 2-fold higher than that of valproate, were subsequently investigated in the mouse 6 Hz (32 mA) psychomotor seizure model of partial epilepsy. The mentioned test is one of a preclinical screening tool for evaluation of novel AEDs with diverse mechanisms of action. It has been used for several years and by numerous groups as a model of refractory epilepsy to evaluate preclinical AEDs pharmacology [11–13]. However, according to Metcalf et al. [14] the 6 Hz (44 mA) seizure test

Table 2
Anticonvulsant activity and neurotoxicity of compounds **9–22** (containing EWGs) measured in the mouse MES and rotarod tests.

	R_1	R_2	Pretreatment time [min]	MES test ED_{50} [mg/kg] ± SEM	Rotarod test TD_{50} [mg/kg] ± SEM
9.		butyl	15	inactive	nd
			30		
			60		
			120		
10.		pentyl	15	inactive	nd
			30		
			60		
			120		
11.		butyl	15	inactive	nd
			30		
			60		
			120		
12.		pentyl	15	322.3 ± 22.4	nd
			30	353.9 ± 23.9	
			60	341.0 ± 24.8	
			120	365.3 ± 24.1	
13.		butyl	15	283.0 ± 18.4	nd
			30	201.0 ± 20.3	
			60	256.5 ± 29.5	
			120	316.6 ± 21.9	
14.		pentyl	15	195.5 ± 21.2	nd
			30	263.7 ± 23.9	
			60	340.8 ± 22.6	
			120	370.8 ± 21.5	
15.		butyl	15	67.8 ± 7.6	171.7 ± 21.2
			30	123.8 ± 13.3	144.2 ± 21.6
			60	144.3 ± 9.5	130.4 ± 17.6
			120	153.3 ± 13.6	122.5 ± 19.4
16.		hexyl	15	65.2 ± 7.6	120.0 ± 20.3
			30	72.1 ± 10.8	204.0 ± 22.9
			60	136.0 ± 11.1	246.6 ± 21.5
			120	161.1 ± 15.9	320.1 ± 23.6
17.		heptyl	15	67.8 ± 7.6	135.6 ± 15.2
			30	88.6 ± 9.2	210.9 ± 21.0
			60	93.6 ± 9.4	269.2 ± 23.6
			120	119.0 ± 9.5	344.7 ± 25.5
18.		butyl	15	77.3 ± 16.7	181.7 ± 18.0
			30	135.6 ± 15.2	204.6 ± 20.1
			60	153.1 ± 20.4	238.0 ± 18.9
			120	181.7 ± 18.0	243.0 ± 26.7
19.		hexyl	15	127.7 ± 10.0	204.6 ± 20.1
			30	119.0 ± 9.5	246.6 ± 21.5
			60	130.8 ± 10.3	288.5 ± 19.0
			120	134.6 ± 11.8	312.1 ± 20.4
20.		heptyl	15	263.7 ± 24.0	356.4 ± 19.8
			30	252.4 ± 23.6	347.6 ± 21.4
			60	297.2 ± 21.4	339.0 ± 19.0
			120	320.1 ± 23.6	362.5 ± 20.3
21.		butyl	15	94.4 ± 17.3	246.6 ± 21.8
			30	121.3 ± 14.5	255.4 ± 19.9
			60	172.5 ± 26.0	269.2 ± 19.3
			120	> 200	304.1 ± 24.0
22.		hexyl	15	87.5 ± 12.1	309.3 ± 30.0
			30	98.3 ± 13.2	288.6 ± 19.0
			60	100.0 ± 14.6	288.6 ± 19.0
			120	89.6 ± 14.3	290.4 ± 24.5
	Valproate		15	189.0 ± 17.3	363.3 ± 14.2
			30	216.9 ± 9.4	372.8 ± 16.8
			60	218.4 ± 18.9	417.3 ± 9.5
			120	246.6 ± 21.4	512.3 ± 20.2

nd – not determined.

Table 3
In-vitro measurement of the ability of the investigated 1,2,4-triazole derivatives to interact with the batrachotoxin-binding sites of VGSCs and to permeate through the blood–brain barrier (BBB).

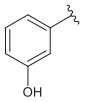
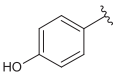
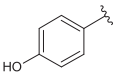
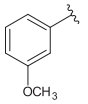
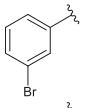
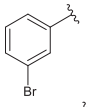
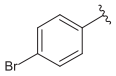
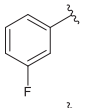
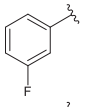
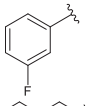
Compound	R_1	R_2	Na^+ channel (site 2) affinity IC_{50} [μM] ± SEM	Permeability coefficients ($P_e \times 10^{-6}$) ± SD [cm/s]
1.		hexyl	> 1000	nd
2.		heptyl	740.0 ± 33.0	38.37 ± 2.46
3.		hexyl	> 1000	nd
5.		hexyl	400.0 ± 15.0	56.61 ± 4.72
6.		heptyl	> 1000	nd
8.		hexyl	560.0 ± 12.0	nd
9.		butyl	> 1000	nd
11.		butyl	> 1000	nd
12.		pentyl	450.0 ± 21.2	nd
13.		butyl	286.6 ± 11.7	nd
15.		butyl	190.0 ± 11.7	14.50 ± 1.22
16.		hexyl	35.0 ± 1.4	nd
17.		heptyl	18.2 ± 0.8	8.19 ± 0.41
18.		butyl	36.2 ± 3.6	13.87 ± 1.04
19.		hexyl	12.2 ± 0.6	14.72 ± 0.81
20.		heptyl	19.3 ± 0.6	nd
21.		butyl	18.7 ± 1.8	25.54 ± 0.81
22.		hexyl	8.8 ± 0.2	54.68 ± 2.14
Valproate			nd	15.37 ± 4.71

Table 3 (*continued*)

Compound	R_1	R_2	Na^+ channel (site 2) affinity IC_{50} [μM] ± SEM	Permeability coefficients ($P_e \times 10^{-6}$) ± SD [cm/s]
Veratridine			17.8 ± 0.9	nd

Permeability coefficients were obtained from PAMPA-BBB assay. Affinity towards Na+ channels was evaluated on the basis of the displacement of [^{3}H] batrachotoxin from its binding site. nd – not determined.

Table 4
Quantitative analysis of anticonvulsant potential of compounds **17** and **22** in the mouse 6 Hz (32 mA) seizure test.

Compound	Pretreatment time [min]	ED_{50} ± SEM [mg/kg]	TD_{50} ± SEM [mg/kg]	Protective index (TD_{50}/ED_{50})
17	15	39.3 ± 13.2	135.6 ± 15.2	3.4
	30	46.5 ± 9.6	210.9 ± 21.0	4.5
	60	53.1 ± 12.1	269.2 ± 23.6	5.1
	120	45.3 ± 10.8	344.7 ± 25.5	7.6
22	15	18.6 ± 4.9	309.3 ± 30.0	16.6
	30	24.9 ± 7.3	288.6 ± 19.0	11.6
	60	33.5 ± 8.9	288.6 ± 19.0	8.6
	120	33.5 ± 8.9	290.4 ± 24.5	8.7
Levetiracetam	60	19.4	> 500 (1601)	> 25.8 (82.5)

Data for levetiracetam are taken from [11,25].

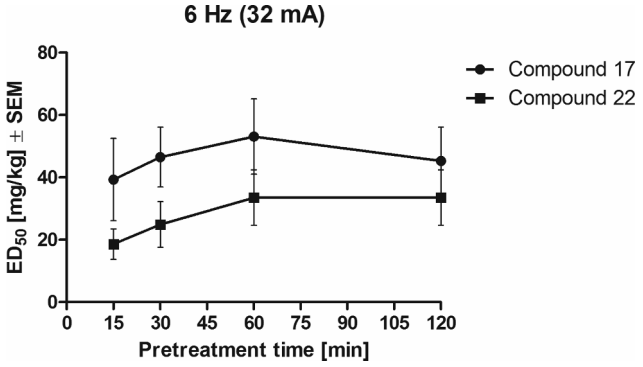


Fig. 2. Time-course effect of compounds **17** and **22** in the 6 Hz (32 mA) psychomotor seizure test.

is much more discriminatory pharmacoresistant screening model when compared to 32 mA.

The latest cohort studies have shown that despite the introduction of over a dozen of new AEDs, the overall seizure control in patients with epilepsy has not essentially changed [15]. Vast majority of these new AEDs are “me-too drugs”, i.e. they are structurally similar to already known ones. Although “me-too drugs” show a better profile of side effects and usually offer beneficial pharmacokinetics, it is highly unlikely that they may constitute an effective alternative in the treatment of refractory epilepsy. Therefore, 4-alkyl-5-substituted-1,2,4-triazole-3-thione derivatives, that are structurally unrelated to currently marketed AEDs have a chance to become promising drug candidates for further clinical testing. As presented in Table 4, both **17** and **22** turned out to be highly effective in 6 Hz psychomotor seizure model. It is worth mentioning that the anticonvulsant activities observed were quite stable over the investigated periods of time (i.e., 15, 30, 60, 120 min) (Fig. 2).

Importantly, the protective effect of compound **22** in the above-mentioned model of psychomotor seizures was much stronger when

Table 5
Comparison of the anticonvulsant activity, neurotoxicity and PI of compounds **17** and **22** with known AEDs.

Compound	TPE[a] [min]	ED_{50} MES [mg/kg]	ED_{50} 6 Hz (32 mA) [mg/kg]	TD_{50} [mg/kg]	PI (TD_{50}/ED_{50})
17	15	67.8	39.3	135.6	2.0 (MES) 3.4 (6 Hz)
22	15	87.5	18.6	309.3	3.5 (MES) 16.6 (6 Hz)
VPA	15	189.0	130.6	363.3	1.9 (MES) 2.8 (6 Hz)
CBZ	30	7.81[b]	47.9[b]	45.4[b]	5.8 (MES) 0.95 (6 Hz)
LEV	60	> 500[b]	19.4[b]	> 500[b]	> 25.8 (6 Hz)
				1601[c]	82.5 (6 Hz)

Reference AEDs: valproate (VPA), carbamazepine (CBZ), levetiracetam (LEV); if not otherwise stated – data taken from own experiments.
[a] TPE – time to peak of anticonvulsant activity.
[b] Data taken from [11].
[c] Data taken from [25] – experiments performed in our laboratory.

compared to valproate and carbamazepine and was almost similar to that of levetiracetam, which is considered a model drug in 6 Hz (32 mA) test [11,16,17] (Table 5). Compound **22** had also substantially better benefit-to-risk ratio than valproate and carbamazepine as indicated by the respective PIs values. Out of the AEDs presented in Table 5, only levetiracetam was found to be much safer than **22** in terms of the impairment of motor coordination evaluated in the rotarod test. According to Barton et al. [11] TD_{50} of levetiracetam was higher than 500 mg/kg, while the experiments performed in our laboratory by Luszczki et al. [25] gave TD_{50} as high as 1601 mg/kg.

Considering the structure-activity (and toxicity) relationships, it is visible that the exchange of 3-fluorophenyl substituent by (3-fluorophenyl)ethyl moiety resulted in a more potent activity against 6 Hz-induced seizures. Introduction of ethylene linker resulted also in an increase of PI values to 16.6, which indicates that such a structural modification has beneficial effect not only on the anticonvulsant activity but also on the safety of compound **22**.

2.3. ADME-Tox properties of compounds *17* and *22*

Since toxicity studies constitute an important part of preclinical testing of new drug candidates, the most promising drug candidates (**17, 22**) were analyzed for their possible cytotoxic, genotoxic and hemolytic effects. Cytotoxicity was monitored after 24 h incubation of liver cells (NMu3Li, HepG2) and human embryonic kidney cells (HEK-293) with the increased concentrations (0–125 μg/ml) of the investigated compounds. Although HepG2 cells have cancerous characteristics, they retain many liver-specific functions and are widely used for studying hepatotoxic properties of drugs and drug-candidates [18]. According to the literature, toxicity threshold was defined as the concentration of the compound at which the viability of cells decreased by at least 30% [19]. Exceeding of this threshold for all of the tested cell lines was observed above 16 μg/ml (Fig. 3). Pharmacokinetic (PK) studies for compounds **17** and **22** were not performed yet, thus their *in-vivo* concentrations are unknown. However, the maximal concentration of the previously examined 5-(3-chlorobenzyl)-4-hexyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione, structurally related to **17** and **22**, in mouse plasma was 4.232 μg/ml [7]. Therefore, it is likely that the above-mentioned 1,2,4-triazole derivatives would be non-toxic for living cells in concentrations necessary to generate the anticonvulsant effect *in-vivo*.

Evaluation of genotoxic potential of **17** and **22** was performed using a single cell gel electrophoresis (SCGE) technique, widely known as comet assay. This method allows detection of DNA damages (e.g., single- and double-strand breaks, AP sites, DNA-DNA and DNA-protein cross-links) in living cells exposed to the investigated factors [20]. The main principle of the comet assay is based on different migration speeds of damaged and undamaged DNA under electrophoretic conditions. The presence and intensity of the comet tail reflect the number of DNA breaks. As can be seen in Fig. 4, there have not been observed any genotoxic changes in HEK-293 cells exposed to compounds **17** and **22** (10 μg/ml) for 24 h.

Finally, the assessment of hemolytic potential of these compounds was performed by spectrophotometric measurement of hemoglobin released from red blood cells (RBCs). Free hemoglobin may cause serious damage to kidneys, liver, heart and spleen [21]. It also exerts neurotoxic activity which is mediated by heme [22]. Drug-induced lysis of RBCs not only prevents *i.v.* administration of the drug but also intensifies its toxicity when administered by other routes [23]. Although toxic hemolysis caused by the antiepileptic drugs is rather rare [24], it is desirable to exclude the risk of such side-effects in the case of each drug candidate. Compounds **17** and **22** in concentrations non-toxic for the cells evaluated in MTT assay (i.e. 5, 10, 15 μg/ml), were tested during *in-vitro* hemolysis study. The investigated derivatives did not produce statistically significant changes in the level of hemoglobin released to the medium (Table 6). Therefore, it can be concluded that the most promising anticonvulsant drug candidates have no tendency to disrupt membranes of RBCs.

3. Conclusions

Based on the results of MES and 6 Hz (32 mA) tests, a group of 4-alkyl-5-substituted-1,2,4-triazole-3-thione derivatives with broad anticonvulsant activity have been selected for further mechanistic and ADME-Tox studies. The most promising compounds (**17**, **22**) exhibited strong anticonvulsant activity both in generalized tonic-clonic and psychomotor seizures. These compounds did not induce genotoxic and hemolytic changes in human cells as well as they were non-toxic for living cells in concentrations less than 16 μg/ml. Taking into account the structural requirements for good anticonvulsant activity of 4-alkyl-5-aryl-1,2,4-triazole-3-thiones, it is visible that small EWGs attached to phenyl ring have beneficial effects both on affinity towards VGSCs and protective activity in the animal models of epilepsy.

4. Materials and methods

4.1. Chemistry

All reagents and solvents were purchased from Sigma-Aldrich and were used without further purification. Melting points of the compounds were measured using Fisher-Johns apparatus. NMR spectra of the compounds dissolved in DMSO-d_6 were recorded on a 700 MHz Bruker Avance II Plus spectrometer (Bruker BioSpin, Germany). Chemical shifts were reported in parts per million (δ) in relation to tetramethylsilane (TMS) as an internal standard. Elemental analyses were performed on AMZ 851 CHX analyzer (PG, Gdańsk, Poland) and the results were within ± 0.4% of the theoretical values. The results of spectral characterization of the intermediates (i.e., 1,4-disubstituted thiosemicarbazides) are given as Supplementary Material.

4.1.1. Synthesis of the compounds *1–22*

Equimolar amounts of various alkyl isothiocyanates and carboxylic acid hydrazides (obtained from the respective carboxylic acids) were mixed together and heated to 110 °C until a product was formed (i.e., for 5 min). The obtained thiosemicarbazide derivatives were washed with diethyl ether, filtered and crystallized from anhydrous ethanol. These compounds were subsequently dissolved in 2% NaOH and heated under reflux for 2 h. After cooling to room temperature, the solvent was neutralized with 3 M HCl to form a solid of appropriate 4-alkyl-5-substituted-1,2,4-triazole-3-thione derivative, which was filtered, dried

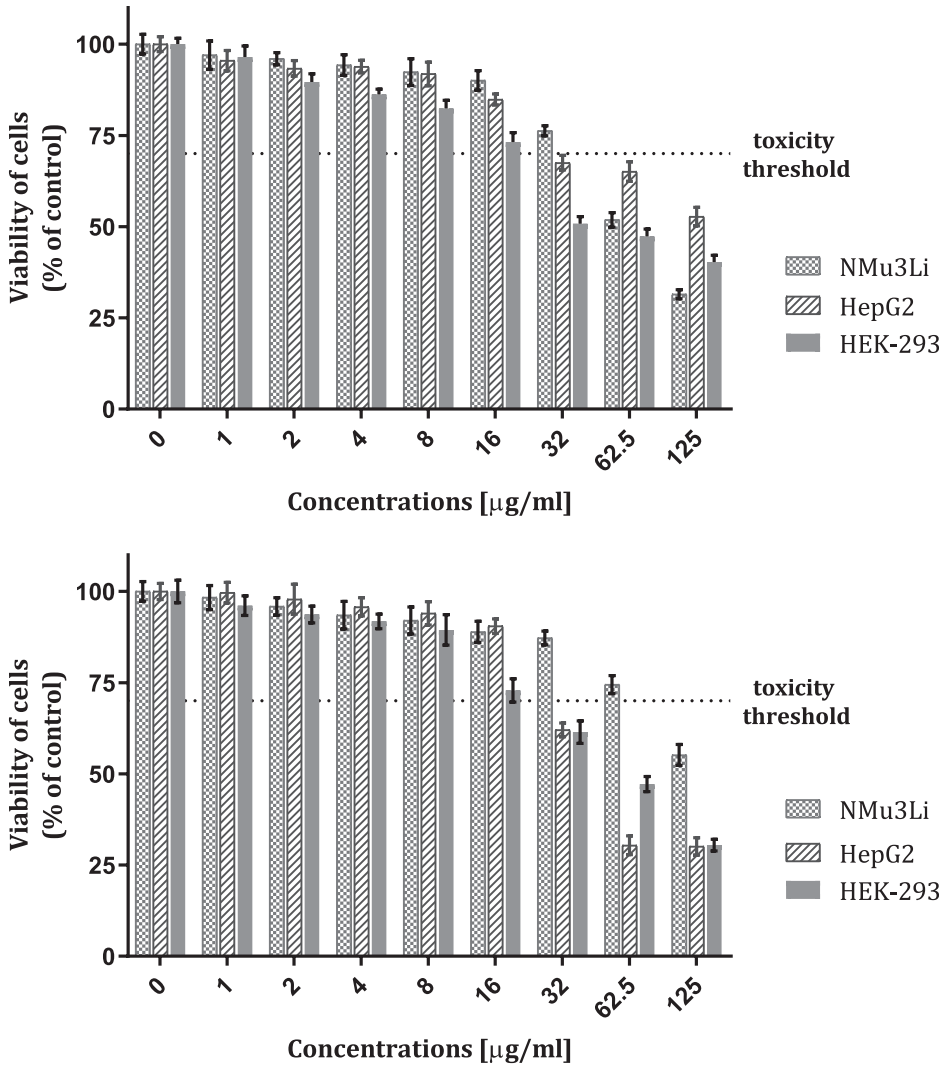


Fig. 3. Toxicity of compounds **17** (upper graph) and **22** (lower graph) against NMu3Li, HepG2 and HEK-293 cells evaluated in MTT assay after 24 h incubation.

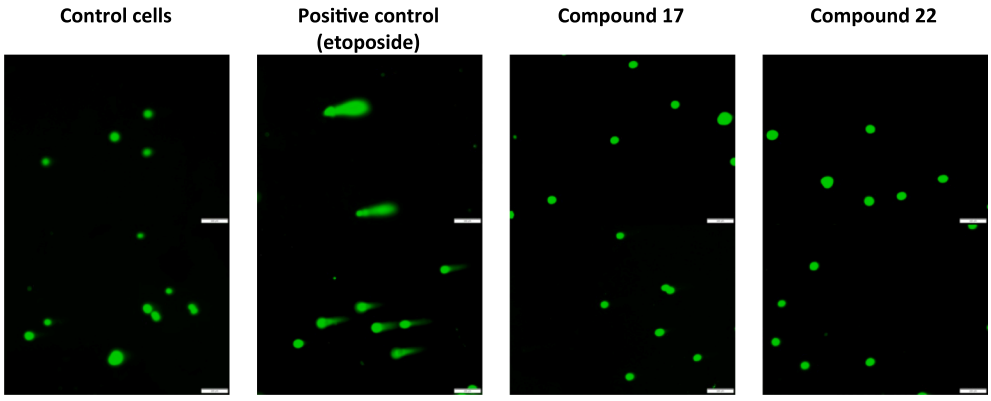


Fig. 4. Genotoxicity evaluation after 24 h treatment of HEK-293 cells with compounds **17** and **22** (10 μg/ml).

and crystallized from anhydrous ethanol.

4.1.1.1. 4-Hexyl-5-(2-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (1). Yield: 59%, m.p. 86–88 °C, ^{1}H NMR (700 MHz): 0.74 (t, 3H, CH_3, J = 7.2 Hz), 1.00–1.26 (m, 6H, $3CH_2$), 1.44–1.49 (m, 2H, CH_2), 3.85 (t, 2H, CH_2, J = 7.5 Hz), 6.95 (td, 1H, ArH, J = 7.4 Hz, J = 1.1 Hz), 7.02 (dd, 1H, ArH, J = 8.2 Hz, J = 0.9 Hz), 7.30 (dd, 1H, ArH, J = 7.6 Hz, J = 1.7 Hz), 7.40–7.43 (m, 1H, ArH), 10.30 (s, 1H, OH), 13.78 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.20, 22.16, 25.76, 27.64, 30.81, 43.92, 113.90, 116.49, 119.79, 131.81, 132.83, 150.36, 156.28, 166.88. Anal. $C_{14}H_{19}N_3OS$ (C, H, N).

4.1.1.2. 4-Heptyl-5-(2-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (2). Yield: 48%, m.p. 72–74 °C, ^{1}H NMR (700 MHz): 0.87 (t, 3H, CH_3, J = 7.1 Hz), 1.08–1.38 (m, 8H, $4CH_2$), 1.45–1.49 (m, 2H, CH_2), 3.79 (t, 2H, CH_2, J = 7.1 Hz), 7.04–7.54 (m, 4H, ArH), 10.26 (s, 1H,

Table 6
Hemolytic activity of compounds **17** and **22** in human red blood cells.

	Hemolysis (%)
Compound 17 (μg/ml)	
5	0.05 ± 0.035
10	0.174 ± 0.070
15	0.187 ± 0.053
Compound 22 (μg/ml)	
5	0.028 ± 0.067
10	0.133 ± 0.004
15	0.182 ± 0.059
PBS treated erythrocytes (control group)	0.025 ± 0.001
0.1% Triton-X	100.21 ± 0.31

Results were expressed as mean ± SD from 3 independent experiments. Results were designated as statistically significant (ANOVA with post-hoc Tukey test) when $p < 0.05$ (vs. control group).

OH), 13.78 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.06, 22.53, 25.22, 27.63, 27.92, 31.65, 47.81, 115.11, 117.60, 119.79, 122.90, 131.01, 145.44, 156.28, 170.27. Anal. $C_{15}H_{21}N_3OS$ (C, H, N).

*4.1.1.3. 4-Hexyl-5-(3-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3**).* Yield: 64%, m.p. 90–92 °C, ^{1}H NMR (700 MHz): 0.78 (t, 3H, CH_3, J = 7.2 Hz), 1.10–1.19 (m, 6H, $3CH_2$), 1.46–1.51 (m, 2H, CH_2), 3.95 (t, 2H, CH_2, J = 7.3 Hz), 6.72–6.82 (m, 2H, ArH), 7.29–7.33 (m, 2H, ArH), 9.90 (s, 1H, OH), 14.00 (s, 1H, NH). ^{13}C NMR (175 MHz): 12.11, 19.37, 21.65, 24.05, 27.79, 43.47, 111.78, 113.21, 118.30, 125.07, 127.97, 150.03, 156.87, 165.91. Anal. $C_{14}H_{19}N_3OS$ (C, H, N).

*4.1.1.4. 4-Heptyl-5-(3-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**4**).* Yield: 52%, m.p. 86–88 °C, ^{1}H NMR (700 MHz): 0.85 (t, 3H, CH_3, J = 7.2 Hz), 1.06–1.24 (m, 6H, $3CH_2$), 1.58–1.64 (m, 4H, $2CH_2$), 3.92 (t, 2H, CH_2, J = 7.4 Hz), 6.94–6.97 (m, 1H, ArH), 7.25–7.35 (m, 3H, ArH), 10.05 (s, 1H, OH), 14.02 (s, 1H, NH). ^{13}C NMR (175 MHz): 11.79, 19.64, 23.85, 25.05, 27.28, 32.54, 113.77, 115.40, 120.21, 124.81, 127.70, 150.06, 156.65, 166.58. Anal. $C_{15}H_{21}N_3OS$ (C, H, N).

*4.1.1.5. 4-Hexyl-5-(4-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**5**).* Yield: 55%, m.p. 86–88 °C, ^{1}H NMR (700 MHz): 0.73 (t, 3H, CH_3, J = 7.2 Hz), 1.08–1.18 (m, 6H, $3CH_2$), 1.49–1.54 (m, 2H, CH_2), 4.00 (t, 2H, CH_2, J = 7.4 Hz), 6.92 (dd, 2H, ArH, J = 6.3 Hz, J = 1.9 Hz), 7.47 (dd, 2H, ArH, J = 6.3 Hz, J = 1.9 Hz), 10.07 (s, 1H, OH), 13.75 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.21, 22.26, 25.78, 27.80, 30.85, 43.91, 116.23, 117.33, 130.61, 152.00, 159.95, 167.29. Anal. $C_{14}H_{19}N_3OS$ (C, H, N).

*4.1.1.6. 4-Heptyl-5-(4-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**6**).* Yield: 61%, m.p. 76–78 °C, ^{1}H NMR (700 MHz): 0.90 (t, 3H, CH_3, J = 7.2 Hz), 1.07–1.27 (m, 6H, $3CH_2$), 1.64–1.72 (m, 4H, $2CH_2$), 4.10 (bs, 2H, CH_2), 6.92 (dd, 2H, ArH, J = 6.3 Hz, J = 2.1 Hz), 7.42 (d, 2H, ArH, J = 6.2 Hz, J = 2.2 Hz), 10.21 (s, 1H, OH), 13.75 (s, 1H, NH). ^{13}C NMR (175 MHz): 19.23, 25.19, 25.62, 25.94, 29.41, 32.36, 57.57, 114.47, 116.51, 117.73, 119.57, 132.42, 149.96, 157.09, 166.07. Anal. $C_{15}H_{21}N_3OS$ (C, H, N).

*4.1.1.7. 4-Butyl-5-(3-methoxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**7**).* Yield: 80%, m.p. 94–96 °C, ^{1}H NMR (700 MHZ): 0.71 (t, 3H, CH_3, J = 7.2 Hz), 1.10 (sext, 2H, CH_2, J = 7.3 Hz), 1.40 (quint, 2H, CH_2, J = 7.1 Hz), 3.71 (s, 3H, OCH_3), 3.96 (t, 2H, CH_2, J = 7.3 Hz), 7.04–7.43 (m, 4H, ArH), 13.92 (s, 1H, NH). ^{13}C NMR (175 MHz): 11.91, 19.58, 29.07, 43.46, 55.21, 112.11, 114.12, 118.44, 125.38, 127.30, 147.52, 159.84, 166.18. Anal. $C_{13}H_{17}N_3OS$ (C, H, N).

*4.1.1.8. 4-Hexyl-5-(3-methoxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**8**).* Yield: 89%, m.p. 118–120 °C, ^{1}H NMR (700 MHz): 0.75 (t, 3H, CH_3, J = 6.8 Hz), 1.01–1.20 (m, 6H, $3CH_2$), 1.40–1.58 (m, 2H, CH_2), 3.79 (s, 3H, OCH_3), 4.01 (t, 2H, CH_2, J = 7.5 Hz), 7.10–7.25 (m, 3H, ArH), 7.42–7.52 (m, 1H, ArH), 13.90 (s, 1H, NH). ^{13}C NMR (175 MHz): 11.39, 19.44, 22.97, 24.95, 28.05, 41.24, 53.07, 111.49, 114.18, 118.45, 125.12, 127.96, 148.71, 157.10, 164.67. Anal. $C_{15}H_{21}N_3OS$ (C, H, N).

*4.1.1.9. 5-(2-Bromophenyl)-4-butyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**9**).* Yield: 81%, m.p. 144–146 °C, ^{1}H NMR (700 MHz): 0.67 (t, 3H, CH_3, J = 7.3 Hz), 1.08 (sext, 2H, CH_2, J = 7.3 Hz), 1.43 (quint, 2H, CH_2, J = 7.3 Hz), 3.76 (t, 2H, CH_2, J = 7.4 Hz), 7.54 (m, 2H, ArH), 7.66–7.70 (m, 1H, ArH), 7.83–7.88 (m, 1H, ArH), 14.02 (s, 1H, NH). ^{13}C NMR (175 MHz): 13.66, 19.38, 29.96, 43.69, 123.81, 127.79, 128.69, 133.13, 133.50, 133.55, 150.33, 167.13. Anal. $C_{12}H_{14}BrN_3S$ (C, H, N).

*4.1.1.10. 5-(2-Bromophenyl)-4-pentyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**10**).* Yield: 88%, m.p. 154–156 °C, ^{1}H NMR (700 MHz): 0.70 (t, 3H, CH_3, J = 6.9 Hz), 1.01–1.10 (m, 4H, $2CH_2$), 1.39–1.50 (m, 2H, CH_2), 3.76 (t, 2H, CH_2, J = 7.5 Hz), 7.53–7.63 (m, 2H, ArH), 7.66–7.70 (m, 1H, ArH), 7.83–7.88 (m, 1H, ArH), 14.02 (s, 1H, NH). ^{13}C NMR (175 MHz): 13.99, 21.76, 27.43, 28.14, 43.89, 123.80, 127.80, 128.68, 133.12, 133.51, 133.55, 150.31, 167.11. Anal. $C_{13}H_{16}BrN_3S$ (C, H, N).

*4.1.1.11. 5-(3-Bromophenyl)-4-butyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**11**).* Yield: 81%, m.p. 106–108 °C, ^{1}H NMR (700 MHz): 0.74 (t, 3H, CH_3, J = 7.3 Hz), 1.05–1.19 (m, 2H, CH_2), 1.43–1.55 (m, 2H, CH_2), 4.02 (t, 2H, CH_2, J = 7.6 Hz), 7.53 (t, 1H, ArH, J = 7.8 Hz), 7.68–7.73 (m, 1H, ArH), 7.79–7.84 (m, 1H, ArH), 7.91 (t, 1H, ArH, J = 1.7 Hz), 14.00 (s, 1H, NH). ^{13}C NMR (175 MHz): 13.69, 19.45, 29.95, 43.82, 122.56, 128.21, 128.88, 131.65, 131.69, 134.12, 150.29, 167.70. Anal. $C_{12}H_{14}BrN_3S$ (C, H, N).

*4.1.1.12. 5-(3-Bromophenyl)-4-pentyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**12**).* Yield: 88%, m.p. 108–110 °C, ^{1}H NMR (700 MHz): 0.74 (t, 3H, CH_3, J = 6.9 Hz), 1.03–1.19 (m, 4H, $2CH_2$), 1.53 (t, 2H, CH_2, J = 7.3 Hz), 4.01 (t, 2H, CH_2, J = 7.6 Hz), 7.53 (t, 1H, ArH, J = 7.9 Hz), 7.68–7.73 (m, 1H, ArH), 7.79–7.84 (m, 1H, ArH), 7.90 (t, 1H, ArH, J = 1.6 Hz), 14.00 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.06, 21.76, 27.45, 28.23, 44.00, 122.66, 128.21, 128.89, 131.67, 134.10, 134.11, 150.28, 167.69. Anal. $C_{13}H_{16}BrN_3S$ (C, H, N).

*4.1.1.13. 5-(4-Bromophenyl)-4-butyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**13**).* CAS: 701946-13-6, yield: 68%, m.p. 128–130 °C, ^{1}H NMR (700 MHz): 0.74 (t, 3H, CH_3, J = 7.3 Hz), 1.12 (sext, 2H, CH_2, J = 7.3 Hz), 1.49 (quint, 2H, CH_2, J = 7.2 Hz), 4.02 (t, 2H, CH_2, J = 7.5 Hz), 7.64 (dd, 2H, ArH, J = 2.0 Hz, J = 6.6 Hz), 7.77 (dd, 2H, ArH, J = 1.9 Hz, J = 6.6 Hz), 13.99 (s, 1H, NH). ^{13}C NMR (175 MHz): 13.75, 19.48, 30.03, 43.87, 124.93, 125.92, 131.10, 132.61, 150.78, 167.73. Anal. $C_{12}H_{14}BrN_3S$ (C, H, N).

*4.1.1.14. 5-(4-Bromophenyl)-4-pentyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**14**).* Yield: 81%, m.p. 124–126 °C, ^{1}H NMR (700 MHz): 0.74 (t, 3H, CH_3, J = 7.1 Hz), 1.02–1.19 (m, 4H, $2CH_2$), 1.50 (quint, 2H, CH_2, J = 7.3 Hz), 4.01 (t, 2H, CH_2, J = 7.6 Hz), 7.64 (dd, 2H, ArH, J = 2.1 Hz, J = 6.6 Hz), 7.78 (dd, 2H, ArH, J = 1.9 Hz, J = 6.6 Hz), 13.98 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.10, 21.85, 27.52, 28.27, 44.03, 124.92, 125.93, 131.11, 132.60, 150.77, 167.71. Anal. $C_{13}H_{16}BrN_3S$ (C, H, N).

*4.1.1.15. 4-Butyl-5-(3-fluorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**15**).* Yield: 78%, m.p. 70–72 °C, ^{1}H NMR (700 MHz): 0.74 (t, 3H, CH_3, J = 7.4 Hz), 1.13 (sext, 2H, CH_2, J = 7.3 Hz), 1.49 (quint, 2H, CH_2, J = 7.4 Hz), 4.02 (t, 2H, CH_2, J = 7.5 Hz), 7.44–7.66 (m, 4H, ArH), 14.00 (s, 1H, ArH). ^{13}C NMR (175 MHz): 13.69, 19.42, 29.98, 43.82, 116.12 (d, J = 23.52 Hz), 118.24 (d, J = 21.09 Hz), 125.40 (d,

J = 2.9 Hz), 128.69 (d, J = 8.5 Hz), 131.84 (d, J = 8.6 Hz), 150.47, 161.80 (d, J = 245.4 Hz), 167.80. Anal. $C_{12}H_{14}FN_3S$ (C, H, N).

*4.1.1.16. 5-(3-Fluorophenyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**16**)*. CAS: 54543-33-8, yield: 76%, m.p. 76–78 °C, ^{1}H NMR (700 MHz): 0.77 (t, 3H, CH_3, J = 7.1 Hz), 1.07–1.17 (m, 6H, $3CH_2$), 1.47–1.54 (m, 2H, CH_2), 3.88 (t, 2H, CH_2, J = 7.3 Hz), 7.45–7.67 (m, 4H, ArH), 13.99 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.19, 22.20, 25.70, 27.68, 30.80, 43.98, 116.17 (d, J = 23.1 Hz), 118.24 (d, J = 21.0 Hz), 125.44 (d, J = 2.9 Hz), 128.80 (d, J = 8.6 Hz), 131.83 (d, J = 8.5 Hz), 150.44, 162.48 (d, J = 243.8 Hz), 167.75. Anal. $C_{14}H_{18}FN_3S$ (C, H, N).

*4.1.1.17. 5-(3-Fluorophenyl)-4-heptyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**17**)*. Yield: 79%, m.p. 64–66 °C, ^{1}H NMR (700 MHz): 0.80 (t, 3H, CH_3, J = 7.3 Hz), 1.06–1.20 (m, 8H, $4CH_2$), 1.50 (quint, 2H, CH_2, J = 7.2 Hz), 4.05 (t, 2H, CH_2, J = 7.5 Hz), 7.44–7.66 (m, 4H, ArH), 13.99 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.31, 22.38, 25.97, 27.71, 28.26, 31.34, 43.98, 116.19 (d, J = 24.0 Hz), 118.23 (d, J = 22.4 Hz), 125.44 (d, J = 2.9 Hz), 128.80 (d, J = 8.6 Hz), 131.81 (d, J = 8.5 Hz), 150.48, 162.49 (d, J = 246.7 Hz), 167.77. Anal. $C_{15}H_{20}FN_3S$ (C, H, N).

*4.1.1.18. 4-Butyl-5-[(3-fluorophenyl)methyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione (**18**)*. Yield: 76%; m.p. 76–78 °C, ^{1}H NMR (700 MHz): 0.81 (t, 3H, CH_3, J = 7.3 Hz), 1.22 (sext, 2H, CH_2, J = 7.5 Hz), 1.31–1.37 (m, 2H, CH_2), 3.84 (t, 2H, CH_2, J = 7.8 Hz), 4.16 (s, 2H, CH_2), 7.10–7.41 (m, 4H, ArH), 13.60 (s, 1H, NH). ^{13}C NMR (175 MHz): 13.91, 19.71, 29.87, 30.86, 43.46, 114.41 (d, J = 20.6 Hz), 116.24 (d, J = 22.3 Hz), 125.46 (d, J = 2.8 Hz), 131.08 (d, J = 8.5 Hz), 138.38 (d, J = 2.8 Hz), 151.26, 162.67 (d, J = 243.2 Hz), 167.22. Anal. $C_{13}H_{16}FN_3S$ (C, H, N).

*4.1.1.19. 5-[(3-Fluorophenyl)methyl]-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**19**)*. Yield: 82%, m.p. 122–124 °C, ^{1}H NMR (700 MHz): 0.83 (t, 3H, CH_3, J = 7.2 Hz), 1.12–1.25 (m, 6H, $3CH_2$), 1.34 (quint, 2H, CH_2, J = 7.7 Hz), 3.83 (t, 2H, CH_2, J = 7.8 Hz), 4.15 (s, 2H, CH_2), 7.09–7.42 (m, 4H, ArH), 13.60 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.28, 22.31, 26.00, 27.67, 30.83, 31.18, 43.69, 114.43 (d, J = 20.9 Hz), 116.22 (d, J = 21.7 Hz), 125.42, 131.08 (d, J = 8.4 Hz), 138.42 (d, J = 7.8 Hz), 151.46, 162.74 (d, J = 245.2 Hz), 167.22. Anal. $C_{15}H_{20}FN_3S$ (C, H, N).

*4.1.1.20. 5-[(3-Fluorophenyl)methyl]-4-heptyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**20**)*. Yield: 84%, m.p. 82–84 °C, ^{1}H NMR (700 MHz): 0.85 (t, 3H, CH_3, J = 7.3 Hz), 1.13–1.19 (m, 6H, $3CH_2$), 1.23 (sext, 2H, CH_2, J = 7.1 Hz), 1.26–1.35 (m, 2H, CH_2), 3.83 (t, 2H, CH_2, J = 7.9 Hz), 4.16 (s, 2H, CH_2), 7.05–7.19 (m, 3H, ArH), 7.37–7.42 (m, 1H, ArH), 13.60 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.39, 22.43, 26.29, 27.71, 28.61, 30.82, 31.48, 43.69, 114.42 (d, J = 21.1 Hz), 116.19 (d, J = 21.7 Hz), 125.40 (d, J = 2.5 Hz), 131.08 (d, J = 8.3 Hz), 138.42 (d, J = 7.8 Hz), 151.17, 162.75 (d, J = 244.8 Hz), 167.23. Anal. $C_{16}H_{22}FN_3S$ (C, H, N).

*4.1.1.21. 4-Butyl-5-[2-(3-fluorophenyl)ethyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione (**21**)*. Yield: 88%, m.p. 86–88 °C, ^{1}H NMR (700 MHz): 0.88 (t, 3H, CH_3, J = 7.4 Hz), 1.28 (sext, 2H, CH_2, J = 7.5 Hz), 1.54–1.59 (m, 2H, CH_2), 2.98–3.04 (m, 4H, $2CH_2$), 3.88 (t, 2H, CH_2, J = 7.7 Hz), 7.00–7.04 (m, 1H, ArH), 7.11–7.17 (m, 2H, ArH), 7.31–7.34 (m, 1H, ArH), 13.50 (s, 1H, NH). ^{13}C NMR (175 MHz): 14.00, 19.80, 26.37, 30.23, 31.23, 43.04, 113.44 (d, J = 21.2 Hz), 115.70 (d, J = 21.2 Hz), 125.09 (d, J = 2.6 Hz), 130.60 (d, J = 8.5 Hz), 143.76 (d, J = 7.5 Hz), 151.90, 162.70 (d, J = 242.7 Hz), 166.80. Anal. $C_{14}H_{18}FN_3S$ (C, H, N).

*4.1.1.22. 5-[2-(3-Fluorophenyl)ethyl]-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**22**)*. Yield: 90%, m.p. 64–66 °C, ^{1}H NMR (700 MHz): 0.82 (t, 3H, CH_3, J = 6.4 Hz), 1.15–1.33 (m, 6H, $3CH_2$), 1.43–1.65 (m, 2H, CH_2), 2.90–3.06 (m, 4H, $2CH_2$), 3.85 (t, 2H, CH_2, J = 7.6 Hz), 6.94–7.94 (m, 4H, ArH), 13.50 (s, 1H, NH). ^{13}C NMR (175 MHz): 11.54, 19.63, 23.32, 23.56, 25.25, 28.40, 40.40, 110.69 (d, J = 20.9 Hz), 112.89 (d, J = 20.9 Hz), 122.28 (d, J = 2.7 Hz), 127.82 (d, J = 8.4 Hz), 141.00 (d, J = 7.6 Hz), 149.11, 159.76 (d, J = 242.4 Hz), 163.97. Anal. $C_{16}H_{22}FN_3S$ (C, H, N).

4.2. Animal experimentations

All animal experiments were performed in accordance with EU Directive 2010/63/EU for animal experiments and complied with the ARRIVE guidelines. All the procedures were also approved by the Local Ethics Committee (Lublin, Poland, No. of approvals: 102/2017, 19/2018, 65/2018, 87/2018).

4.2.1. MES test

Anticonvulsant activity of the investigated compounds was determined according to the same procedure as described in [4,5,7]. Experiments were conducted on adult Swiss albino mice weighing 20–25 g. The animals were kept in colony cages with free access to food and tap water. After one week of acclimatization, the animals were randomly divided into groups consisting of 8 mice. The investigated compounds were suspended in a 1% solution of Tween 80 in distilled water and administered *i.p.* in a volume of 5 ml/kg. During experiment, the compounds were administered *i.p.* in a fixed dose of 300 mg/kg at four pretreatment times (15, 30, 60, 120 min) before the MES test. Electroconvulsions were produced by an alternating current (0.2 s stimulus duration; 500 V, 50 Hz, fixed current intensity of 25 mA) delivered *via* ear-clip electrodes by a Type 221 Rodent Shocker generator (Hugo Sachs Elektronik, Freiburg, Germany). The abolition of a hindlimb tonic extension was taken as a criterion of the anticonvulsant activity. Next, the active compounds (i.e., the compounds that exhibited anticonvulsant effect in a dose of 300 mg/kg) were administered in increasing doses in order to calculate ED_{50} values using the log-probit method by Litchfield and Wilcoxon.

4.2.2. 6 Hz test

Psychomotor (limbic) seizures in mice were evoked by current (6 Hz, 0.2 ms rectangular pulse width, 32 mA, 3 s duration) generated by an S48 Square Pulse Stimulator and CCU1 Constant Current Unit (Grass Technologies, West Warwick, RI, USA). After application of ocular anesthetic (0.5% solution of tetracaine hydrochloride) to the mouse corneas, the animals treated with the increasing concentrations of **17** and **22** underwent corneal stimulation and were placed separately in plexiglas cages (25 × 15 × 10 cm) for the observation of the presence or absence of psychomotor seizures. Immediately following the 6 Hz corneal stimulation the animals exhibited a “stunned” posture associated with rearing and automatic movements that lasted from 60 to 120 s in untreated animals [11]. The mice were considered to be protected from seizures if they resumed their normal exploratory behavior within 20 s after stimulation. The anticonvulsant activity was evaluated as the ED_{50} value (median effective dose of the compound, which protects 50% of mice against convulsions) calculated from the log-probit method according to Litchfield and Wilcoxon.

4.2.3. Rotarod test

Motor coordination impairment (acute neurotoxicity) after *i.p.* administration of the investigated compounds to mice was examined using the rotarod test as described in [25]. Neurotoxicity of the tested compounds is presented as median toxic doses (TD_{50}) that impair motor coordination in 50% of the mice challenged with the rotarod test.

4.3. PAMPA-BBB assay

Blood-brain barrier (BBB) permeability of the compounds was investigated using a PAMPA method (parallel artificial membrane

permeability assay). The PAMPA system consisted of a 96-well microfilter plate was divided into two chambers: a donor at the bottom and an acceptor at the top, separated by a 120-μm-thick microfilter disc coated with BBB lipid solution (Pion, Inc.). The solutions of each compound were prepared in dimethyl sulfoxide (DMSO) at 4 mg/ml concentration and then diluted with Prisma buffer (pH = 7.4) to obtain the donor drug solution with the final nominal concentration of 20 μg/ml. The donor solutions were placed in donor plate. Acceptor plate contained Brain Sink Buffer (BSB). The plates were put together and incubated at 37 °C for 180 min in a humidity-saturated atmosphere. The concentrations of the respective compounds were determined with a UV-reader (Multiskan GO, Thermo Scientific) at 254 nm in the donor and acceptor compartments.

The permeability coefficient values (P_e) were calculated by using the following equation:

$$P_e = \frac{-ln\left(1 - \frac{C_A}{C_{equilibrium}}\right)}{S \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t}$$

where V_D– donor volume, V_A – acceptor volume, $C_{equilibrium}$ – equilibrium concentration, $C_{equilibrium} = \frac{C_D \times V_D + C_A \times V_A}{V_D + V_A}$, C_D – donor concentration, C_A – acceptor concentration, S – membrane area, t – incubation time (in seconds).

4.4. Radioligand binding assay

Radioligand binding studies with [^{3}H]batrachotoxin were performed using the same procedure as described in our previous paper [6].

4.5. Cellular toxicity evaluation

Toxicity of the compounds was analyzed using MTT assay on murine (NMu3Li) and human (HepG2, HEK-293) cells. Experiments were performed in accordance with the international standard ISO-10993-5:2009. HEK-293 (human embryonic kidney) cells grew in Minimal Essential Medium (MEM; Sigma-Aldrich, Saint Louis, MO, USA), HepG2 (hepatocellular carcinoma) cells were cultured in Eagle's Minimal Essential Medium containing L-glutamine (Sigma-Aldrich), while NMu3Li cells were cultivated in DMEM-high glucose (Sigma Aldrich). All media were supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), 100 U/mL of penicillin and 100 mg/mL of streptomycin (PenStrep, Sigma Aldrich). Procedure of MTT test was described in details in our previously published paper [7]. Experiments were repeated twice, and the measurements in each experiment were run in quadruplicate.

4.6. Hemolytic activity evaluation

Human red blood cells (RBCs) concentrate was obtained from the Regional Blood Donation and Transfusion Centre (Lublin, Poland). About 5 ml of RBCs concentrate was washed three times with sterile PBS and each time centrifuged at 1500 rpm for 3 min. The obtained pellet was resuspended using sterile PBS in order to obtain 2% suspension of RBCs. A volume of 1 ml of RBCs suspension was mixed with 1 ml of different concentrations of the investigated compounds. The mixtures were incubated at 37 °C for 30 min and centrifuged at 2500 rpm for 10 min. The amount of free hemoglobin in supernatants was measured spectrophotometrically at 405 nm. Negative and positive controls were performed by incubating RBCs with sterile PBS and 0.1% Triton-X, respectively. Each experiment was run in triplicate.

4.7. Genotoxicity evaluation

Assessment of DNA damages in HepG2 cells was performed using OxiSelect Comet Assay Kit (Cell Biolabs, Inc.) according to the manufacturer's protocol. HepG2 cells (1×10^5 cells/ml in ice-cold phosphate buffer saline) previously exposed (for 24 h) to compounds **17** and **22** (10 μg/ml) were mixed with liquefied low melting agarose and transferred onto the Oxiselect comet slide. After storing for 15 min at 4 °C, the slides were immersed in a chilled Lysis Buffer (for 45 min at 4 °C, in the dark) and Alkaline Solution (for 30 min at 4 °C, in the dark). Next, the Alkaline Solution was replaced with pre-chilled TBE electrophoresis solution. After immersing for 5 min, the slides were transferred into the horizontal electrophoresis chamber and covered with TBE electrophoresis buffer. The electrophoresis was run for 15 min at 1 V/cm. DNA released from the cells was stained with Vista Green DNA Dye for 15 min at room temparature and observed under fluorescence microscope (Olympus BX63). The images of cells were captured using XM10 digital camera (Olympus).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103355.

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