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Preliminary communication

Synthesis, characterization and preliminary anticonvulsant evaluation of some 4-alkyl-1,2,4-triazoles

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ABSTRACT

Designed and synthesized 4-alkyl-1,2,4-triazole-3-thione derivatives showed significant anticonvulsant activity, determined in the maximal electroshock-induced seizure (MES) test. The chemical structure of all new compounds was confirmed by spectral methods (^{1}H NMR, ^{13}C NMR, IR, MS). A sensitive and selective method was elaborated for the determination of the anticonvulsant compounds levels in mice brain tissue, based on HPLC with diode array detector (DAD). Chromatographic tests showed that lack of anticonvulsant effect of two derivatives (**15**, **16**) with long alkyl chains at N-4 position of the 1,2,4-triazole ring was due to the inability to cross the blood–brain barrier (BBB).

1. Introduction

It is estimated that approximately 50 million people worldwide suffer from epilepsy. Most of them (90%) are residents of the so-called developing countries [1,2]. Despite the introduction of a dozen of novel antiepileptic drugs (AEDs) in the recent years [3], still in almost 30% of those patients the disease remains resistant to standard therapy (using 1st and 2nd generation AEDs) [4,5]. Side effects related to the use of those drugs also seem to be a problem. Therefore, there still is a need for ongoing research of novel antiepileptic medications.

One of the most commonly used classic AED is valproic acid and its salts. The drug is known to increase endogenic GABA synthesis and inhibits its decomposition. Yet it also induces adverse effects, e.g. hepatotoxicity and teratogenicity [6,7]. The relatively simple structure of valproic acid suggests that three fragments of the molecule play an important role in its antiepileptic activity. These are the alkyl, the hydrogen bond donor (HBD) fragment and the hydrogen bond acceptor (HBA) fragment. It is also known that the teratogenic effect of valproic acid is caused by the presence of: (i) a carboxyl group; (ii) a branched aliphatic chain at carbon C-2; and (iii) a hydrogen atom at carbon C-2 [8,9].

Using the above data, a group of compounds was synthesized in which an aliphatic fragment (varying in length) was coupled with the HBA and HBD fragments, while there would be no elements whose presence might induce the teratogenic effect. To this end, the aliphatic (not branched) chain was joined with the 1,2,4-triazole-3-thione ring. The C=S and NH fragments played the role of hydrogen bond acceptor and donor, respectively. An additional reason to use the 1,2,4-triazole ring in designing our compounds was the fact that the ring may bind the GABA-A receptor through nitrogens N-1 and N-2 [10]. Moreover, our previous studies demonstrated that the 3-chlorophenyl fragment bound to the 1,2,4-triazole-3-thione ring improves the antiepileptic activity of the obtained derivatives [11,12].

Anticonvulsant 1,2,4-triazole derivatives designed to date usually have two aryl components in their structure, since it was believed that the second aryl component increases biological activity [13–16]. However, little is known about the anticonvulsant effect of alkyl 1,2,4-triazole derivatives. Therefore, the aim of our study was to elucidate how the presence and the size of the alkyl fragment affect anticonvulsant activity.

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2. Results and discussion

2.1. Chemistry

4-Alkyl-5-(3-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones were synthesized in a two step reaction (Scheme 1). In the first step, a reaction between aliphatic isothiocyanates (with alkyl chains varying in length) and 3-chlorobenzoic acid hydrazide was conducted to obtain 4-alkyl-1-(3-chlorobenzoyl)thiosemicarbazide derivatives (**1**–**8**).

Our earlier studies demonstrated that 1,4-disubstituted thiosemicarbazides may be quickly and efficiently obtained if anhydrous ethanol is used as reaction solvent [17]. Eliminating the need for any kind of solvent would perhaps be a significant simplification of the synthesis procedure. Moreover, such modification would be in accordance with the idea of "green chemistry", constantly growing in popularity. Thus we decided to check whether it is possible to use a solid-state synthesis method to obtain compounds **1**–**8**. Synthesis optimization was performed by selecting appropriate temperature and time of substrate heating. The completion of the reaction was monitored using thin-layer chromatography. The best results were obtained with 3-chlorobenzhydrazide heated with aliphatic isothiocyanates at 120 °C. Under these conditions the reaction was deemed complete in less than 1 min. Reaction efficiency ranged between 80% and 94%. The results are comparable to those usually obtained using microwave generators and much better compared to syntheses conducted in various solvents, even those employing ultrasound [18]. Undeniable advantages of our method are its low cost, rapidity and simplicity. The synthesized 4-alkyl-1-(3-chlorobenzoyl)thiosemicarbazide derivatives (**1**–**8**) were subsequently subjected to alkaline dehydrocyclization which gave rise to appropriate 4-alkyl-5-(3-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**9**–**16**). 4-Unsubstituted analog – 5-(3-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**17**) – was obtained by prolonged heating of 4-benzoyl-1-(3-chlorobenzoyl) thiosemicarbazide in 5% solution of sodium hydroxide. The obtained compounds were fully characterized by elemental analyses, ^{1}H NMR, ^{13}C NMR, IR and MS spectra (details are shown in the Section Experimental; see also Supplementary section).

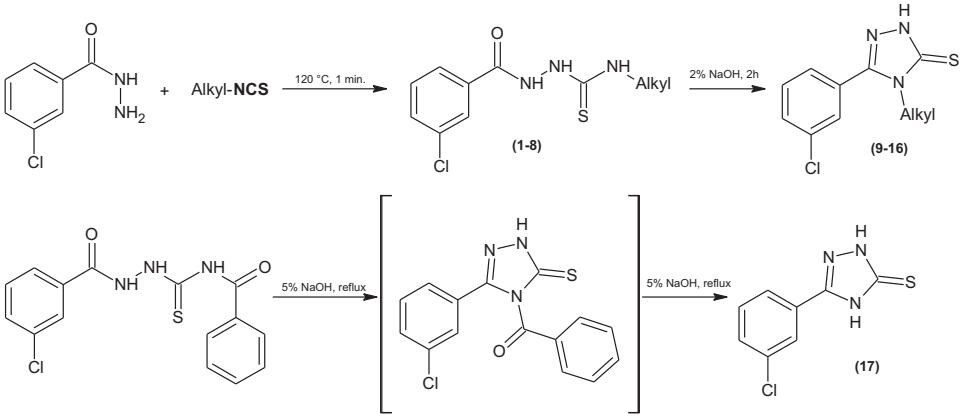
2.2. Anticonvulsant activity

The anticonvulsant activity of compounds **9**–**17** was determined using maximal electroshock-induced seizure (MES) test. This test is thought to be an experimental model of tonic–clonic seizures and of partial convulsions with or without secondary generalization in humans. Compounds **9**–**17** were administered to mice at the dose of 300 mg/kg at four pretreatment times (i.e., 15, 30, 60, 120 min) prior to the test. This method allowed to determine the percentage of animals (in a group consisting of 8 mice), which were protected against seizures induced by electric current (Fig. 1).

5-(3-Chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**17**) having no alkyl substituent, lacked the protective anticonvulsant effect. Whereas, introduction of the alkyl fragment in position 4 of the 1,2,4-triazole nucleus resulted in achievement of a group of derivatives showing strong anticonvulsant activity. The characteristic feature of all active compounds (**9**–**14**) was rapid onset and long lasting effects (up to 2 h). Fifteen minutes after *i.p.* administration of compounds **9**–**14**, 100% of mice were protected against MES-induced convulsions. Slight weakening of the activity, after 30 min of the MES test, was observed for 5-(3-chlorophenyl)-4-ethyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**9**). Whereas, the decyl (**15**) and dodecyl (**16**) derivatives were completely inactive in this test.

In the second stage of the pharmacological research median effective doses (ED_{50}), median toxic doses (TD_{50}) and PI (protective index) values for compounds **9**–**14** were determined (Table 1). When the length of the alkyl chain was increased by two methylene groups, from $-C_2H_5$ to $-C_4H_9$, a distinct increase in the anticonvulsant activity was observed. In such case the strength of anticonvulsant effect increased approximately 4-fold (from 152 mg/kg to 38.5 mg/kg). Further elongation of the alkyl chain did not cause any increase at the maximum activity (in 15 min of the test), but caused the decrease of ED_{50} value in subsequent time intervals tested. This tendency is very beneficial in potentially new drugs, as it means that the drug maintains relatively stable concentration in the body. It was only after the increase of the length of the substituent to $-C_9H_{19}$ that produced the pharmacological activity after 15 min and 30 min following *i.p.* administration to decreased by approximately two-fold. However, there were no significant differences in ED_{50} values among the respective intervals (15, 30, 60, and 120 min). It is also worth mentioning that compounds **9**–**14** displayed much stronger anticonvulsant effect than valproate.

Elongation of the alkyl chain results in the increase of lipophilicity of the subsequent 1,2,4-triazole derivatives. These changes may justify the changes of activity in the respective time intervals. For instance, 4-butyl-5-(3-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**10**), despite the fact that it showed the strongest anticonvulsant activity after 15 min *via* systemic administration ($ED_{50} = 38.5 \pm 3.9$ mg/kg), its ED_{50} value increased to 311.2 mg/kg



Scheme 1. Synthetic route to compounds **9**–**17**. Alkyl substituents: $-C_2H_5$ (**1**, **9**), $-C_4H_9$ (**2**, **10**), $-C_5H_{11}$ (**3**, **11**), $-C_6H_{13}$ (**4**, **12**), $-C_7H_{15}$ (**5**, **13**), $-C_9H_{19}$ (**6**, **14**), $-C_{10}H_{21}$ (**7**, **15**), $-C_{12}H_{25}$ (**8**, **16**).

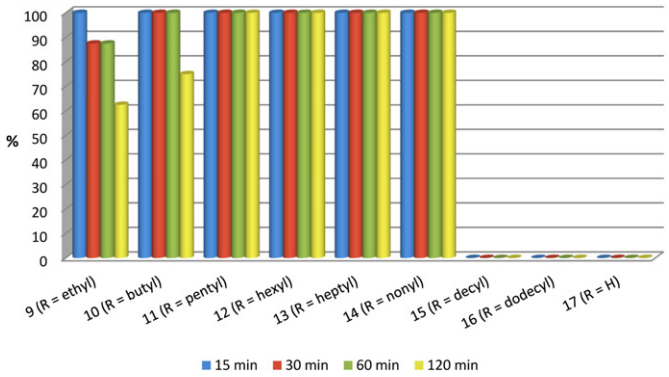


Fig. 1. Time-course of anticonvulsant effects of compounds **9**–**17** against maximal electroshock (MES)-induced seizures in mice. Data are presented as percentage of animals protected against MES-induced seizures out of 8 animals per group. The MES test was performed at various pretreatment times (15, 30, 60, 120 min) after systemic administration of the investigated compounds in a fixed dose of 300 mg/kg.

(120 min). This suggests that the activity decreased approximately eight fold after the concentration of this compound in the central nervous decreased relatively quickly. The increased lipophilicity of subsequent derivatives increases their affinity to brain structures, and hence the elimination of these compounds is slower. Therefore, in the cases of pentyl (**11**) and hexyl (**12**) derivatives no such significant differences in activities were observed at various pretreatment times (15, 30, 60, 120 min).

The acute adverse effects (neurotoxicity) of compounds **9**–**14** and valproate were evaluated and expressed as their median toxic doses (TD_{50}), producing deficits in motor coordination in 50% of animals tested in the chimney test. Dividing the TD_{50} values of the compounds **9**–**14** by those of valproate, one can calculate the potency ratio for these derivatives. The lowest acute adverse-effect (neurotoxic) potential, as compared to valproate, was observed in the cases of ethyl (potency ratio ranged from 1.73 to 2.02) and nonyl (potency ratio from 1.29 to 1.91) derivatives. In contrast, compound **11** (pentyl) was characterized by the highest neurotoxic potential (potency ratio from 0.72 to 0.85) as compared to valproate.

The protective index ($PI = TD_{50}/ED_{50}$) for the alkyl derivatives of 1,2,4-triazole-3-thione administered *i.p.* at various pretreatment times (15, 30, 60, 120 min) ranged considerably, from 1.4 to 10.3. The highest PI values were observed for butyl (**10**), hexyl (**12**) and heptyl (**13**) derivatives administered at 15 min before the MES and chimney tests (PI = 10.3, 9.7, 7.5, respectively). Worth mentioning is also the fact that, in case of compound **14**, the least differences in PI values at different pretreatment times were observed (PI ranged from 4.6 to 6.1).

Taking into account the results of pharmacological studies of 4-alkyl-5-(3-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**9**–**14**), one can suppose that these derivatives may be considered as potential anticonvulsant agents. Obviously, more specific studies are required to determine pharmacological and toxicological profiles of the mentioned compounds.

Table 1
Time-course and dose–response effects of the active compounds in the MES and chimney tests in mice.

Compound	R	Pretreatment time (min)	ED_{50} (mg/kg)	TD_{50} (mg/kg)	PI (TD_{50}/ED_{50})
9	$-C_2H_5$	15	152.6 ± 17.3	730.6 ± 48.1	4.8
		30	203.6 ± 27.0	753.0 ± 45.9	3.7
		60	251.5 ± 27.7	741.6 ± 47.1	2.9
		120	320.1 ± 23.6	887.1 ± 42.8	2.8
10	$-C_4H_9$	15	38.5 ± 3.9	397.9 ± 26.9	10.3
		30	77.8 ± 5.5	405.4 ± 27.2	5.2
		60	156.1 ± 10.2	413.8 ± 27.6	2.7
		120	311.2 ± 11.7	429.2 ± 34.0	1.4
11	$-C_5H_{11}$	15	46.5 ± 3.7	309.8 ± 55.9	6.7
		30	65.2 ± 4.1	309.8 ± 55.9	4.8
		60	69.5 ± 4.3	302.8 ± 38.1	4.4
		120	147.4 ± 15.7	391.5 ± 43.0	2.7
12	$-C_6H_{13}$	15	47.6 ± 3.8	462.9 ± 20.0	9.7
		30	68.3 ± 10.3	462.9 ± 20.0	6.8
		60	98.1 ± 16.4	456.9 ± 19.7	4.7
		120	159.7 ± 21.7	448.1 ± 21.7	2.8
13	$-C_7H_{15}$	15	55.1 ± 5.2	415.8 ± 24.1	7.5
		30	65.2 ± 4.6	422.7 ± 22.8	6.5
		60	101.6 ± 16.8	429.5 ± 19.0	4.2
		120	134.6 ± 11.8	439.5 ± 19.1	3.3
14	$-C_9H_{19}$	15	144.2 ± 21.9	666.8 ± 37.1	4.6
		30	130.4 ± 17.6	712.7 ± 39.5	5.5
		60	104.7 ± 16.5	640.2 ± 47.1	6.1
		120	142.8 ± 14.6	659.1 ± 59.9	4.6
Valproate		15	189.0 ± 17.3	363.3 ± 14.2	1.9
		30	216.9 ± 9.4	372.9 ± 16.9	1.7
		60	218.4 ± 18.9	417.3 ± 9.5	1.9
		120	246.6 ± 21.4	512.3 ± 20.2	2.1

Results are presented as median effective doses (ED_{50} ± S.E.) and median toxic doses (TD_{50} ± S.E.) of the examined compounds. All compounds were administered *i.p.*, at four various pretreatment times (15, 30, 60, 120 min) prior to the appropriate pharmacological test (MES or chimney test). In case of valproate (magnesium salt), the results from the both tests have been presented in our earlier study [25].

2.3. Chromatographic investigations

We have found it interesting that 5-(3-chlorophenyl)-4-decyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**15**) and 5-(3-chlorophenyl)-4-dodecyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**16**) were totally deprived of any activity, despite the fact that compound **14** still showed twice as high activity as valproate. We have considered two possible reasons for this phenomenon: (1) the compounds cross the blood–brain barrier, but do not show affinity to respective receptors; (2) the compounds are not capable of penetrating the brain, and therefore they do not affect the receptors in the central nervous system. To check this, we have developed and validated a chromatographic method determining the concentration of compounds **15** and **16** in the mice brains. Moreover, for comparison purposes, the concentration of the pharmacologically active compound – 5-(3-chlorophenyl)-4-hexyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**12**), has been determined.

The analyses were carried out on a reversed phase column (C18, 150 mm × 4.6 mm, 5 μm), using a mobile phase composed of methanol, acetonitrile, 2-propanol. Investigated compounds administered to mice in the same doses: 300 mg kg^{-1} were extracted from tissue homogenate with solid phase extraction (SPE) on octadecyl cartridges. There is no evidence that conditions used in sample preparation such as $HClO_4$ or heat lead to major degradation of investigated compounds. No impurities were observed during stress studies. The method provided a high extraction yield of the examined compounds from brain tissue homogenate with absolute recovery values greater than 90.0% (Table 2). The detector response was linear over a concentration range of 0.2–100.0 μg mL^{-1}. Precision results, expressed by the intra-day and the inter-day relative standard deviation values, were satisfactory (less than 5%). Accuracy was satisfactory as well. This method proved to be suitable for the analysis of investigated compounds in mice brain tissues and for the study of their distribution and pharmacokinetics in mice brain. All details of the validation procedure will be published in a separate paper [19].

On the grounds of the findings (Table 3) it has been determined that permeability of the blood–brain barrier was the main factor determining the lack of activity of two (C_{10} and C_{12}) alkyl derivatives (**15**, **16**). The C_{10} derivative (**15**) concentration in the brain, was found to be approx. 10 times less than the pharmacologically active

Table 2
Validation parameters.

	LOD (μg/mL)	LOQ (μg/mL)	Concentration range (μg/mL)	r^2 of linearity equation	Mean of extraction yield (%)	Repeatability (RSD%)	Intermediate precision (RSD%)
12 ($-C_6H_{13}$)	0.01039	0.03467	0.2–100.0	0.9958	97.99	2.24	3.60
15 ($-C_{10}H_{21}$)	0.01305	0.04351	0.2–100.0	0.9992	93.67	2.33	3.50
16 ($-C_{12}H_{25}$)	0.1020	0.3400	0.2–100.0	0.9999	93.37	1.50	3.40

LOD – limit of detection; LOQ – limit of quantification.

C_6 (hexyl) derivative (**12**). Also, the dodecyl (C_{12}) analog (**16**) did not permeate to the brain at all. The results obtained confirm the known rule, namely molecules in which the number of rotatable bonds is higher or equal to 10, have little chance to permeate through biological membranes [20]. Data obtained from chromatographic investigations will be helpful during the tests intended to determine the mechanism of action of alkyl derivatives of 1,2,4-triazoles. Further, the suggested methods of determination of 1,2,4-triazole derivatives (**12**, **15**, **16**) may be used to test their pharmacokinetic features.

3. Conclusions

4-Alkyl-5-(3-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**9**–**16**) were obtained in a two step synthesis. The first stage was optimized in such manner that the time of reaction was shorter than 1 min. It has been proven that introduction of an alkyl substituent in N-4 position leads to pharmacologically active compounds. The anticonvulsant effect of the compounds against maximal electroshock-induced seizures in mice was greater than that of valproate. Chromatographic tests allow one to state that the lack of permeability through the blood–brain barrier was the reason for the lack of activity of some compounds in this series.

4. Experimental

4.1. General

All reagents were purchased from Lancaster (Ward Hill, USA) and Merck Co. (Darmstadt, Germany). Melting points (uncorrected) were determined by using Fischer-Johns apparatus (Sanyo, Japan). The ^{1}H NMR (250 MHz) and ^{13}C NMR (90 MHz) spectra were recorded on a Bruker Avance instrument using DMSO-d_6 as a solvent and TMS as an internal standard. The IR spectra were recorded in KBr discs using a Perkin–Elmer 1725X FTIR spectrometer. The mass spectra were obtained on a Finnigan Trace DSQ spectrometer operating at 70 eV. Elemental analyses were performed on an AMZ 851 CHX analyser (PG, Gdańsk, Poland) and the results were within ±0.4% of the theoretical value.

4.2. General procedure for the synthesis of 4-alkyl-1-(3-chlorobenzoyl)thiosemicarbazides (**1**–**8**)

A mixture of 0.01 mol (1.70 g) of 3-chlorobenzhydrazide and equimolar amount of the respective alkyl isothiocyanate was heated in an oil bath at 120 °C for 1 min. After cooling, the product precipitated and was washed with diethyl ether and recrystallized from ethanol.

4.2.1. 1-(3-Chlorobenzoyl)-4-ethylthiosemicarbazide (**1**)
Yield: 84%. CAS Registry Number: 26036-05-05.

4.2.2. 4-Butyl-1-(3-chlorobenzoyl)thiosemicarbazide (**2**)
Yield: 80%, CAS Registry Number: 891073-07-7.

4.2.3. 1-(3-Chlorobenzoyl)-4-pentylthiosemicarbazide (**3**)
Yield: 91%, m.p. 134–135 °C, ^{1}H NMR (DMSO-d_6): 0.84 (t, 3H, CH_3, J = 7.10 Hz), 1.13–1.42 (m, 4H, $2CH_2$), 1.40–1.54 (m, 2H, CH_2), 3.36–3.47 (m, 2H, CH_2), 7.46–8.12 (m, 4H, Ar–H), 9.24, 9.68, 10.40 (3s, 3H, 3NH). ^{13}C NMR (DMSO-d_6): 14.04, 21.99, 28.52, 33.12, 43.60, 126.65, 127.76, 130.38, 131.68, 133.18, 134.62, 164.85, 184.10. IR (KBr, cm^{-1}): 3411 (NH), 3100, 3003 ($CH_{arom.}$), 2958 ($CH_{aliph.}$), 1697 (C=O), 1319 (C=S). MS (*m*/*z*): 299 $[M]^+$, 301 $[M + 2]^+$. Anal. Calc. for $C_{13}H_{18}ClN_3OS$: C 52.08, H 6.05, N 14.02. Found: C 52.10, H 6.16, N 14.21.

4.2.4. 1-(3-Chlorobenzoyl)-4-hexylthiosemicarbazide (**4**)
Yield: 88%, m.p. 141–142 °C, ^{1}H NMR (DMSO-d_6): 0.83 (t, 3H, CH_3, J = 7.11 Hz), 1.13–1.31 (m, 6H, $3CH_2$), 1.37–1.52 (m, 2H, CH_2), 3.35–3.47 (m, 2H, CH_2), 7.36–8.11 (m, 4H, Ar–H), 9.24 (s, 2H, 2NH), 10.42 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 13.99, 22.15, 25.98, 28.74, 31.12, 43.68, 126.63, 127.75, 130.41, 131.73, 133.16, 134.58, 164.53, 181.29. IR (KBr, cm^{-1}): 3356 (NH), 3076 ($CH_{arom.}$), 2989, 2881 ($CH_{aliph.}$), 1676 (C=O), 1331 (C=S). MS (*m*/*z*): 313 $[M]^+$, 315 $[M + 2]^+$. Anal. Calc. for $C_{14}H_{20}ClN_3OS$: C 53.58, H 6.42, N 13.39. Found: C 53.73, H 6.53, N 13.24.

4.2.5. 1-(3-Chlorobenzoyl)-4-heptylthiosemicarbazide (**5**)
Yield: 94%, m.p. 138–139 °C, ^{1}H NMR (DMSO-d_6): 0.83 (t, 3H, CH_3, J = 7.03 Hz), 1.13–1.30 (m, 8H, $4CH_2$), 1.39–1.51 (m, 2H, CH_2), 3.36–3.46 (m, 2H, CH_2), 7.46–8.14 (m, 4H, Ar–H), 9.24 (s, 2H, 2NH), 10.40 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 16.26, 24.33, 28.47, 30.76, 30.98, 33.54, 45.88, 128.83, 129.96, 132.62, 133.93, 135.38, 136.80, 167.02, 183.76. IR (KBr, cm^{-1}): 3420, 3348 (NH), 3121, 3039 ($CH_{arom.}$), 2955, 2809 ($CH_{aliph.}$), 1675 (C=O), 1327 (C=S). MS (*m*/*z*): 327 $[M]^+$, 329 $[M + 2]^+$. Anal. Calc. for $C_{15}H_{22}ClN_3OS$: C 54.95, H 6.76, N 12.82. Found: C 54.89, H 6.78, N 12.95.

4.2.6. 1-(3-Chlorobenzoyl)-4-nonylthiosemicarbazide (**6**)
Yield: 84%, m.p. 126–127 °C, ^{1}H NMR (DMSO-d_6): 0.82 (t, 3H, CH_3, J = 7.00 Hz), 1.12–1.32 (m, 12H, $6CH_2$), 1.38–1.54 (m, 2H, CH_2),

Table 3
Concentration of investigated compounds (**12**, **15**, **16**) in brain of mice treated with a fixed dose of 300 mg/kg body weight.

Compound	μg of compound found (range)	Average value of analyte in 1 mg of the brain tissue homogenate of a mouse treated with the dose of 300 mg/kg	Standard deviation value (±)
12 ($-C_6H_{13}$)	1.8074–13.3031	7.2017	0.1000
15 ($-C_{10}H_{21}$)	0.6939–0.9704	0.7029	0.0900
16 ($-C_{12}H_{25}$)	Not found	Not found	–

3.39 (t, 2H, CH_2, J = 7.35 Hz), 7.44–8.12 (m, 4H, Ar–H), 9.23 (s, 2H, 2NH), 10.41 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 14.06, 22.16, 26.31, 27.34, 28.73, 28.91, 29.06, 31.38, 43.66, 126.61, 127.77, 130.38, 131.71, 133.18, 134.58, 164.80, 181.51. IR (KBr, cm^{-1}): 3370 (NH), 3023 ($CH_{arom.}$), 2834, 2792 ($CH_{aliph.}$), 1680 (C=O), 1340 (C=S). MS (m/z): 355 $[M]^+$, 357 $[M + 2]^+$. Anal. Calc. for $C_{17}H_{26}ClN_3OS$: C 57.37, H 7.36, N 11.81. Found: C 57.38, H 7.46, N 11.95.

4.2.7. 1-(3-Chlorobenzoyl)-4-decylthiosemicarbazide (**7**)

Yield: 87%, m.p. 122–123 °C, ^{1}H NMR (DMSO-d_6): 0.83 (t, 3H, CH_3, J = 6.95 Hz), 1.10–1.31 (m, 14H, 7CH_2), 1.38–1.54 (m, 2H, CH_2), 3.34–3.47 (m, 2H, CH_2), 7.46–8.15 (m, 4H, Ar–H), 9.26 (s, 2H, 2NH), 10.40 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 14.11, 21.46, 25.47, 26.80, 28.15, 28.51, 29.10, 31.62, 44.38, 127.04, 128.20, 129.16, 130.87, 131.38, 134.03, 164.70, 180.23. IR (KBr, cm^{-1}): 3420, 3367 (NH), 3107 ($CH_{arom.}$), 2961, 2821 ($CH_{aliph.}$), 1675 (C=O), 1329 (C=S). MS (m/z): 369 $[M]^+$, 371 $[M + 2]^+$. Anal. Calc. for $C_{18}H_{28}ClN_3OS$: C 58.44, H 7.63, N 11.36. Found: C 58.34, H 7.70, N 11.47.

4.2.8. 1-(3-Chlorobenzoyl)-4-dodecylthiosemicarbazide (**8**)

Yield: 88%, m.p. 108–109 °C, ^{1}H NMR (DMSO-d_6): 0.83 (t, 3H, CH_3, J = 6.86 Hz), 1.14–1.29 (m, 18H, 9CH_2), 1.37–1.52 (m, 2H, CH_2), 3.34–3.45 (m, 2H, CH_2), 7.48–8.12 (m, 4H, Ar–H), 9.23 (s, 2H, 2NH), 10.42 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 12.28, 20.17, 24.86, 25.77, 26.10, 27.19, 27.36, 27.56, 27.78, 30.84, 42.05, 127.14, 127.87, 128.30, 129.08, 129.98, 132.26, 165.87, 181.06. IR (KBr, cm^{-1}): 3390 (NH), 3111, 3027 ($CH_{arom.}$), 2939, 2788 ($CH_{aliph.}$), 1670 (C=O), 1337 (C=S). MS (m/z): 398 $[M]^+$. Anal. Calc. for $C_{20}H_{32}ClN_3OS$: C 60.35, H 8.10, N 10.56. Found: C 60.46, H 8.18, N 10.53.

4.3. General procedure for the synthesis of 4-alkyl-5-(3-chlorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione derivatives (**9**–**16**)

4-Alkyl-1-(3-chlorobenzoyl)thiosemicarbazides (**1**–**8**) were dissolved in 2% NaOH and the resulting solution was refluxed for 2 h. After cooling, the mixture was neutralized with 3 M HCl. The precipitate that formed was filtered and washed with distilled water. The compounds were recrystallized from EtOH.

4.3.1. 5-(3-Chlorophenyl)-4-ethyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**9**)

Yield: 87%. CAS Registry Number: 26028-63-7.

4.3.2. 4-Butyl-5-(3-chlorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**10**)

Yield: 80%. CAS Registry Number: 92696-66-7.

4.3.3. 5-(3-Chlorophenyl)-4-pentyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**11**)

Yield: 90%, m.p. 88–89 °C, ^{1}H NMR (DMSO-d_6): 0.72 (t, 3H, CH_3, J = 6.96 Hz), 0.98–1.17 (m, 4H, 2CH_2), 1.41–1.56 (m, 2H, CH_2), 4.00 (t, 2H, CH_2, J = 7.62 Hz), 7.54–7.82 (m, 4H, Ar–H), 14.00 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 14.14, 21.24, 29.20, 34.14, 43.62, 125.76, 127.50, 130.00, 131.48, 134.19, 134.89, 152.21, 169.76. IR (KBr, cm^{-1}): 3423 (NH), 3058 ($CH_{arom.}$), 2961, 2866 ($CH_{aliph.}$), 1569 (C=N), 1328 (C=S). MS (m/z): 281 $[M]^+$, 283 $[M + 2]^+$. Anal. Calc. for $C_{13}H_{16}ClN_3S$: C 55.41, H 5.72, N 14.91. Found: C 55.50, H 5.70, N 15.06.

4.3.4. 5-(3-Chlorophenyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**12**)

Yield: 83%, m.p. 96–98 °C, ^{1}H NMR (DMSO-d_6): 0.75 (t, 3H, CH_3, J = 6.87 Hz), 0.99–1.18 (m, 6H, 3CH_2), 1.40–1.55 (m, 2H, CH_2), 4.02 (t, 2H, CH_2, J = 7.75 Hz), 7.55–7.78 (m, 4H, Ar–H), 13.88 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 14.23, 23.40, 26.18, 29.87, 34.45, 44.29, 127.56, 129.06, 131.34, 132.90, 133.66, 135.36, 152.77, 170.20. IR (KBr, cm^{-1}): 3461 (NH), 3000 ($CH_{arom.}$), 2925 ($CH_{aliph.}$), 1586 (C=N), 1326 (C=S). MS (m/z): 295 $[M]^+$, 297 $[M + 2]^+$. Anal. Calc. for $C_{14}H_{18}ClN_3S$: C 56.84, H 6.13, N 14.20. Found: C 56.95, H 6.01, N 14.32.

4.3.5. 5-(3-Chlorophenyl)-4-heptyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**13**)

Yield: 87%, m.p. 80–81 °C, ^{1}H NMR (DMSO-d_6): 0.77 (t, 3H, CH_3, J = 7.00 Hz), 0.99–1.19 (m, 8H, 4CH_2), 1.42–1.55 (m, 2H, CH_2), 4.00 (t, 2H, CH_2, J = 7.58 Hz), 7.55–7.78 (m, 4H, Ar–H), 14.00 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 16.16, 24.21, 27.77, 29.49, 30.05, 33.17, 45.78, 129.72, 130.47, 130.69, 133.06, 133.33, 136.04, 152.23, 169.39. IR (KBr, cm^{-1}): 3453 (NH), 3105, 3023 ($CH_{arom.}$), 2968 ($CH_{aliph.}$), 1581 (C=N), 1333 (C=S). MS (m/z): 309 $[M]^+$, 311 $[M + 2]^+$. Anal. Calc. for $C_{15}H_{20}ClN_3S$: C 58.14, H 6.51, N 13.56. Found: C 58.00, H 6.50, N 13.48.

4.3.6. 5-(3-Chlorophenyl)-4-nonyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**14**)

Yield: 86%, 88–90 °C, ^{1}H NMR (DMSO-d_6): 0.81 (t, 3H, CH_3, J = 7.04 Hz), 1.00–1.27 (m, 12H, 6CH_2), 1.39–1.54 (m, 2H, CH_2), 4.00 (t, 2H, CH_2, J = 7.66 Hz), 7.51–7.89 (m, 4H, Ar–H), 13.99 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 14.01, 22.14, 25.56, 27.25, 28.15, 28.57, 28.65, 31.27, 43.56, 127.49, 128.25, 128.45, 130.84, 131.11, 133.82, 150.02, 167.20. IR (KBr, cm^{-1}): 3415 (NH), 3054 ($CH_{arom.}$), 2896 ($CH_{aliph.}$), 1582 (C=N), 1321 (C=S). MS (m/z): 337 $[M]^+$, 339 $[M + 2]^+$. Anal. Calc. for $C_{17}H_{24}ClN_3S$: C 60.42, H 7.16, N 12.44. Found: C 60.51, H 7.01, N 12.30.

4.3.7. 5-(3-Chlorophenyl)-4-decyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**15**)

Yield: 80%, m.p. 91–92 °C, ^{1}H NMR (DMSO-d_6): 0.84 (t, 3H, CH_3, J = 7.01 Hz), 1.08–1.27 (m, 14H, 7CH_2), 1.47–1.52 (m, 2H, CH_2), 4.02 (t, 2H, CH_2, J = 7.85 Hz), 7.57–7.88 (m, 4H, Ar–H), 14.01 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 14.02, 22.15, 25.55, 27.25, 28.14, 28.71, 28.88, 31.33, 43.58, 127.48, 128.25, 128.46, 130.84, 131.10, 133.82, 150.03, 167.20. IR (KBr, cm^{-1}): 3397 (NH), 3015 ($CH_{arom.}$), 2868, 2804 ($CH_{aliph.}$), 1586 (C=N), 1322 (C=S). MS (m/z): 351 $[M]^+$, 353 $[M + 2]^+$. Anal. Calc. for $C_{18}H_{26}ClN_3S$: C 61.43, H 7.45, N 11.94. Found: C 61.55, H 7.52, N 12.05.

4.3.8. 5-(3-Chlorophenyl)-4-dodecyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**16**)

Yield: 81%, m.p. 68–70 °C, ^{1}H NMR (DMSO-d_6): 0.82 (t, 3H, CH_3, J = 6.97 Hz), 1.00–1.29 (m, 18H, 9CH_2), 1.39–1.55 (m, 2H, CH_2), 4.00 (t, 2H, CH_2, J = 7.91 Hz), 7.52–7.81 (m, 4H, Ar–H), 14.01 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 12.57, 20.72, 24.11, 25.80, 26.69, 27.24, 27.31, 27.45, 27.59, 29.91, 42.11, 126.03, 126.80, 126.99, 129.36, 129.64, 132.35, 148.53, 165.72. IR (KBr, cm^{-1}): 3438 (NH), 3105 ($CH_{arom.}$), 2962 ($CH_{aliph.}$), 1569 (C=N), 1341 (C=S). MS (m/z): 379 $[M]^+$, 381 $[M + 2]^+$. Anal. Calc. for $C_{20}H_{30}ClN_3S$: C 63.22, H 7.96, N 11.06. Found: C 63.20, H 8.08, N 11.20.

4.4. Synthesis of 5-(3-chlorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**17**)

4-Benzoyl-1-(3-chlorobenzoyl)thiosemicarbazide (CAS Registry Number: 648860-52-0) was dissolved in 5% NaOH and the resulting solution was refluxed for 10 h. After cooling, the mixture was neutralized with 3 M HCl. The precipitate formed was filtered off and washed with distilled water. Yield: 74%, CAS Registry Number: 117320-61-3.

4.5. Pharmacology

4.5.1. General

Adult male Swiss mice (weighing 22–26 g), that were kept in colony cages with free access to food and tap water, housed

under standardized housing conditions, were used. After seven days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups each comprised of 8 mice. Each mouse was used only once. Procedures involving animals and their care were conducted in accordance with current European Community and Polish legislation on animal experimentation. The experimental protocols and procedures described in this manuscript were approved by the First Local Ethics Committee at the Medical University in Lublin and the Second Local Ethics Committee at the University of Life Sciences in Lublin and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

4.5.2. Maximal electroshock seizure test

The investigated compounds (**9**–**17**) were suspended in a 1% solution of Tween 80 in distilled water and administered intraperitoneally (*i.p.*) as a single injection, in a volume of 5 mL/kg body weight. Valproate (magnesium salt), used as a reference drug, was directly dissolved in distilled water. Fresh drug solutions were prepared on each day of experimentation and administered at 15, 30, 60 and 120 min before the initiation of maximal electroconvulsions. The pretreatment times before testing of the investigated compounds and the route of *i.p.* administration were based upon information from the Anticonvulsant Screening Program [21]. Electroconvulsions were produced by a current (0.2 s stimulus duration; 500 V, 50 Hz, fixed current intensity of 25 mA) delivered *via* ear-clip electrodes by a Rodent Shocker generator (constant-current stimulator Type 221, Hugo Sachs Elektronik, Freiburg, Germany). The criterion for the occurrence of seizure activity was the tonic hind limb extension. The animals were administered with a constant dose of 300 mg/kg of each of the examined compounds and were subjected to MES-induced seizures. The anticonvulsant activity of the active compounds administered *i.p.* at various pretreatment times was determined as their median effective doses (ED_{50} values in mg/kg) in the MES-induced seizure test in mice. The animals were administered with different doses of the tested compounds so as to obtain a variable percentage of protection against maximal electroconvulsions, allowing the construction of dose–response relationship lines for each examined compound administered *i.p.* at various pretreatment times, according to Litchfield and Wilcoxon [22]. Each ED_{50} value represents the dose of the studied compounds that is required to protect 50% of the animals tested against MES-induced seizures.

4.5.3. Chimney test

The chimney test of Boissier et al. [23] was used to quantify the acute adverse-effect potential of compounds **9**–**14** and valproate on motor performance in mice. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 30 cm length), and motor performance impairment was indicated by the inability of the mice to climb backward up the transparent tube within 60 s. The acute adverse (neurotoxic) effects of compounds **9**–**14** and valproate administered alone were expressed as their median toxic doses (TD_{50}), representing the doses, at which the investigated compounds impaired motor coordination in 50% of the animals tested in the chimney test. To evaluate each TD_{50} value, at least four groups of animals (each group consisted of 8 mice) injected with various doses of the appropriate compound were challenged with the chimney test. A dose–response relationship line was calculated on the basis of the percentage of mice showing motor deficits by means of the log-probit method according to Litchfield and Wilcoxon [22].

4.5.4. Protective index (PI)

The protective index for the investigated compounds was calculated by dividing a TD_{50} value, as determined in the chimney test, by the respective ED_{50} value, as determined in the MES test. The protective index is considered as an index of the margin of safety and tolerability between anticonvulsant doses and doses of the compounds exerting acute adverse effects e.g., sedation, motor coordination impairment, ataxia or other neurotoxic manifestations [24].

4.6. Determination of the concentration of compounds *12*, *15*, *16* in the mice brain tissue

4.6.1. Preparation of the mice brain homogenates

Respective 1,2,4-triazole-3-thione derivatives: **12** (pharmacologically active compound) and **15**, **16** (inactive ones) were suspended in a 1% aqueous solution of Tween 80 (Sigma, St. Louis, MO, USA) and administered *intraperitoneally* in a dose of 300 mg/kg in a volume of 5 mL/kg body weight. The control animals received adequate amounts of vehicle. Mice pretreated with appropriate compounds (or with the vehicle only) were decapitated at time reflecting the peak of maximum anticonvulsant effect for the active derivative (**12**). The whole brains of mice were removed from skulls, weighed, harvested and homogenized using Abbott buffer (1:2 weight/volume; Abbott Laboratories, North Chicago, IL, USA) in an Ultra-Turrax T8 homogenizer.

4.6.2. Chromatographic conditions

Experiments were performed using a LaChrom HPLC Merck Hitachi (E. Merck, Darmstadt, Germany) model equipped with diode array detector, L-7350 column oven and L-7612 solvent degasser. The column (150 mm × 4.6 mm I.D.) was packed with 5-μm Zorbax Extend-C18 (pore size: 80 Å, surface area: 180 m^2/g) Agilent Technologies (Santa Clara, CA, USA); its void volume was determined to be 1.31 mL, by the injection of thiourea in acetonitrile–water (50:50) eluent system. The column was thermostated at 20 °C ± 0.1. Mobile phase was a mixture of acetonitrile, methanol or 2-propanol in water. The phase was filtered through a Nylon 66 membrane filter (0.45 μm) Whatman (Maidstone, England). Retention data were recorded at a flow-rate of 1 mL min^{-1} for 80% methanol (retention time of the first compound, **12** was 4.7 min) and 85% acetonitrile (retention time for the second compound, **15** was 6.01 min) and 0.5 mL min^{-1} for 80% 2-propanol (retention time for the third compound, **16** was 5.17 min) used as a mobile phase. The detection of the compounds was set at appropriate wavelength (λ_{max} = 254 for the first and second compound and 260 nm for the third compound) chosen in accord with the recorded spectra. Typical injection volumes were 20 μL.

4.6.3. Solutions

Stock solutions of the analytes at concentration of 300 μg mL^{-1} were prepared in methanol, acetonitrile or 2-propanol for compounds **12**, **15**, **16**, respectively. The stock solutions were stored at refrigerator in glass vials. Standard solutions were prepared daily by diluting the stock solutions by the use of appropriate organic solvent.

4.6.4. Sample preparation

Analyte standard solutions of the first compound (**12**) were prepared as follows: 200 μL of the blank brain tissue homogenate was mixed with 50 μL of standard solution. Obtained mixture was incubated in 37.1 °C for 60 min. After that 1 mL of methanol and 250 μL of 3% $HClO_4$ were added and the sample was heated to 60 °C for 30 min. The mixture was centrifuged at 9000× *g* for 15 min. Aliquot of the supernatant was further analyzed by SPE procedure. The brain homogenate of the mouse treated by the dose of 300 mg/kg was weighted and mixed with 4 mL of methanol and 1 mL of 3%

$HClO_4$ (the ratio of methanol to perchloric acid was 4:1 to receive the best extraction efficiency). An additional procedure was carried out for a blank sample. The preparation of samples of the second compound (**15**) required application of acetonitrile instead of methanol, the ratio of organic solvent to 3% perchloric acid was 2:1 and temperature was established as 70 °C. In the case of the last compound (**16**), the following changes were made in the procedure of sample preparation: 2-propanol was used as organic solvent, the ratio of organic solvent to 3% perchloric acid was 1:1 and incubation temperature was 80 °C.

4.6.5. SPE procedure

Solid-phase extraction (SPE) was carried out using BAKERBOND™ spe Octadecyl (C_{18}) J.T. Baker cartridges (200 mg, 3 mL and 500 mg, 6 mL) on a Baker spe-12G apparatus. The C18 cartridges were activated and conditioned with 2 × 1 mL of methanol and 2 × 1 mL of water respectively and dried applying full vacuum for 1 min (−30 kPa). Aliquots of the supernatant were loaded onto the conditioned cartridge. The whole was then washed with 2 × 1 mL of water in the case of compound (**12**) and additionally by the use of 1 mL of 50% methanol in the case of compound (**15**) or 1 mL of 50% acetonitrile in the case of (**16**). Analyte elution was carried out with 2 mL of methanol, acetonitrile or 2-propanol in the case of the first, the second and the third compound respectively, applying full vacuum for 1 min. 20 μL of the eluate was injected directly into the HPLC column.

4.6.6. Method validation

4.6.6.1. Linearity. Analyte standard solutions at six different concentrations were added to 200 μL of blank sample, and subjected to the SPE and HPLC procedure. The analyte peak area was plotted against the corresponding concentrations and the calibration curves determined by means of the least-squares method. Good linearity values with $r^2 > 0.995$ were found for all examined compounds. Complete results of linearity, limit of detection, limit of quantification are presented in Table 2. Limit of quantification (LOQ) and limit of detection (LOD) values were also determined as the analyte concentrations which gave rise to peaks whose height is 10 and 3 times that of the baseline and noise, respectively.

4.6.6.2. Recovery. Spiked blank samples were prepared as follows: 50 μL of standard solution of different concentrations were added to 200 μL of the blank brain tissue homogenate and incubated at 37.1 °C for 30 min. At least three concentrations were prepared and analyzed for each compound, corresponding to the lower, middle and upper limit of the corresponding linearity curve. These samples were analyzed as described above. The determined analyte peak areas were substituted into calibration curve equation and the percentage extraction yield was calculated.

4.6.6.3. Accuracy. Analyte standard solutions at three different concentrations were added to 200 μL of the brain tissue homogenate and the obtained mixture was subjected to further SPE and HPLC procedure. Recovery values were calculated according to the following formula: 100 × [(after spiking − before spiking)/added]. The procedure was repeated three times at each concentration level to obtain standard deviation values. The mean recovery values were almost always higher than 90% and standard deviation values ≤ 3%. All data are summarized in Table 2.

4.6.6.4. Precision. Spiked blank samples were prepared and analyzed according to procedure described above. The analysis was repeated six times giving intraday precision values and six times in another day giving intermediate precision values both expressed as percentage relative standard deviation value-RSD%. Obtained precision results were satisfactory. RSD% for repeatability was always lower than 3% and for intermediate precision − lower than 5%. All collected precision values are presented in Table 2.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.11.026.

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