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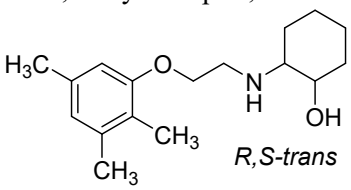

R,S-trans

ED_{50} MES = 15.67 (9.10 - 26.97) mg/kg b.w. (mice, *i.p.*)
TD_{50} rotarod = 78.30 (6.82 - 93.13) mg/kg b.w. (mice, *i.p.*)
PI = 5.00
analgesic at 15 mg/kg b.w. (OXA-induced pain, von Frey test, mice, *i.p.*)

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Anticonvulsant and analgesic in neuropathic pain activity in a group of new aminoalkanol derivatives

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ABSTRACT

As part of the presented research, thirteen new aminoalkanol derivatives were designed and obtained by chemical synthesis. *In vivo* studies (mice, *i.p.*) showed anticonvulsant activity (MES) of nine compounds, and in the case of one compound (*R,S-trans*-2-((2-(2,3,5-trimethylphenoxy)ethyl)amino)cyclohexan-1-ol, **4**) both anticonvulsant (ED_{50} MES = 15.67 mg/kg, TD_{50} rotarod = 78.30 mg.kg, PI = 5.00) and analgesic activity (OXA-induced neuropathic pain, active at 15 mg/kg). For selected active compounds additional *in vitro* studies have been performed, including receptor studies (5-HT_{1A}), evaluation of antioxidant activity (DPPH assay), metabolism studies as well as safety panel (mutagenicity, safety in relation to the gastrointestinal flora, cytotoxicity towards astrocytes as well as impact on their proliferation and cell cycle).

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neuropathic pain, are both health and social challenges. Epidemiological data suggest that neuropathic pain concerns about 7-10% of society,[1] while epilepsy – 1%.[2] Despite availability of a wide range of anticonvulsants and pain killers on the market, a large proportion of patients still does not get satisfying health improvement.[3,4] Lack of full knowledge about the pathophysiology of CNS disorders, and thus the lack of identification of some molecular targets for drugs, as well as the occurrence of adverse effects and drug interactions during treatment with available medications may be responsible for the lack of full effectiveness of therapy.

The above facts are a premise to look for new centrally-active compounds. Research on the mechanisms of action of active compounds and the implementation of safety and metabolism studies in the early stages of research are particularly important. The multidirectional central activity of some compounds is also an interesting research direction. Many drugs on the market show diverse effects depending on the dose and this diversity is sometimes used in the treatment of CNS diseases. For example, antiepileptic drugs are used in the treatment of neuropathic pain (e.g. gabapentin, pregabalin).[5,6] This phenomenon is explained, among others, by the common pathomechanism of epilepsy and neuropathic pain, which also contributes to their frequent comorbidity.[7]

The detection of anticonvulsant activity showed by anti-arrhythmic drugs such as propranolol[8] and mexiletine[9] was important for the study of aminoalkanol derivatives for CNS activity (Figure 1).

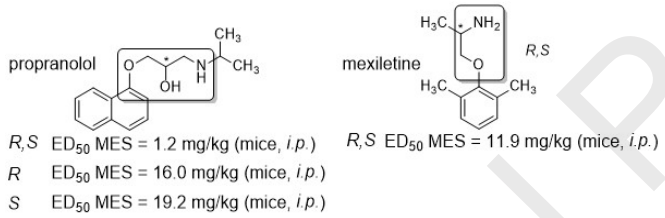

Figure 1. Anticonvulsant activity (maximal electroshock seizure test, MES test) of anti-arrhythmic drugs: propranolol[8] and mexiletine[9] (aminoalkanol component framed).

Compounds obtained so far at the Department of Bioorganic Chemistry were derivatives of the appropriate chiral and achiral aminoalkanols, among which many compounds were characterized by a high anticonvulsant activity in animal models, as well as analgesic activity in neuropathic pain models. Some pharmacological parameters, such as the median effective dose (ED_{50}) in a respective animal test, median toxic dose (TD_{50}) in the rotarod test or protective index (PI), were comparable or more favorable than the results observed for the reference drugs. Among all obtained active compounds in this chemical group, promising results obtained for reference compounds **I** – **IV**[10-12] (Figure 2) became the premise for the synthesis of a series of compounds di- or tri-substituted with methyl groups in the phenyl ring, presented in this paper. The aminoalkanol moieties include among others cyclic aminoalkanols present in the structure of compounds **I** and **II** (i.e. cyclohexanol, piperidinol), while the linker is an alkyl chain (with 2, 3 or 4 carbon atoms), or ethoxyethylene (as in the case of compound **IV**).

The multidirectional central activity of compound **III** is noteworthy - in addition to the anticonvulsant activity of this compound, analgesic activity was also observed in the streptozotocin (STZ)-induced neuropathic pain model, which corresponds to human type I diabetes-induced neuropathic pain. are often used in the treatment of neuropathic pain,[13] which is a premise for the study of anticonvulsants also for the analgesic activity. Compound **III** has also been the subject of extensive research on the mechanism of its action. The data indicate the participation of sigma receptors and the serotonergic system.

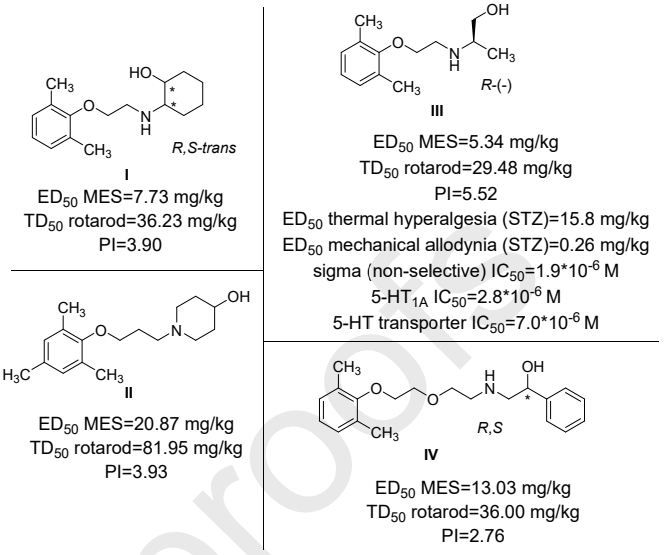

Figure 2. Chemical structures and the central activity (mice, *i.p.*) of previously synthesized reference compounds: **I**,[10] **II**,[11] **III**,[12] **IV**.[11] STZ – streptozotocin-induced neuropathic pain model; reference drugs:[5,14,15] Valproic acid – ED_{50} (MES, mice, *i.p.*) = 272 mg/kg, ED_{50} (MES, rats, *i.p.*) = 212 mg/kg; Carbamazepine – ED_{50} (MES, mice, *i.p.*) = 8.81 mg/kg, ED_{50} (MES, rats, *i.p.*) = 3.4 mg/kg; Phenytoin – ED_{50} (MES, mice, *i.p.*) = 9.50 mg/kg, ED_{50} (MES, rats, *i.p.*) = 11.3 mg/kg.

For the design of the presented new compounds (Scheme 1, Table 1), the aminoalkanol moiety initially included a distance of two carbon atoms between hydroxy and amino groups (e.g. 2-aminopropan-1-ol, 2-aminobutan-1-ol, 2-aminocyclohexan-1-ol, piperidin-3-ol). Longer chains were the most often introduced in order to compare the activity of isomers (e.g. 4-aminocyclohexan-1-ol *vs* 2-aminocyclohexan-1-ol, piperidin-4-ol *vs* piperidin-3-ol). An interesting aspect was also the use of alicyclic aminoalcohol (piperidinol) in place of the chain one (aminocyclohexanol).

An important stage in the design of biologically active compounds is *in silico* research, which allows prediction of selected physicochemical parameters and exclusion of structures with low probability of *in vivo* activity, e.g. due to low bioavailability. The compounds designed in this work were subjected to *in silico* analysis using the Molinspiration platform.[16] Among the calculated values of physicochemical parameters important for oral bioavailability were parameters covered by the Lipinski's rule: molecular weight (MW), logP coefficient, number of hydrogen bond acceptors (N/O), number of hydrogen bond donors (NH/OH). Compounds with a predicted favorable bioavailability profile should meet a minimum of four of the following criteria: MW ≤ 500, logP ≤ 5, N/O ≤ 10, NH/OH ≤ 5, which characterizes all designed compounds. All compounds meet also criteria important for blood-brain barrier penetration (MW < 450, logP < 5, N/O < 7, NH/OH < 3).[17] The exact values of the calculated parameters are presented in the Supplementary material (Table S1).

Chemistry

All compounds were obtained by chemical synthesis (Scheme 1), *via* previously published methods.[11] The substrates were appropriately substituted phenols. In the first step of the synthesis, the corresponding phenoxyalkyl- or phenoxyethoxyethyl

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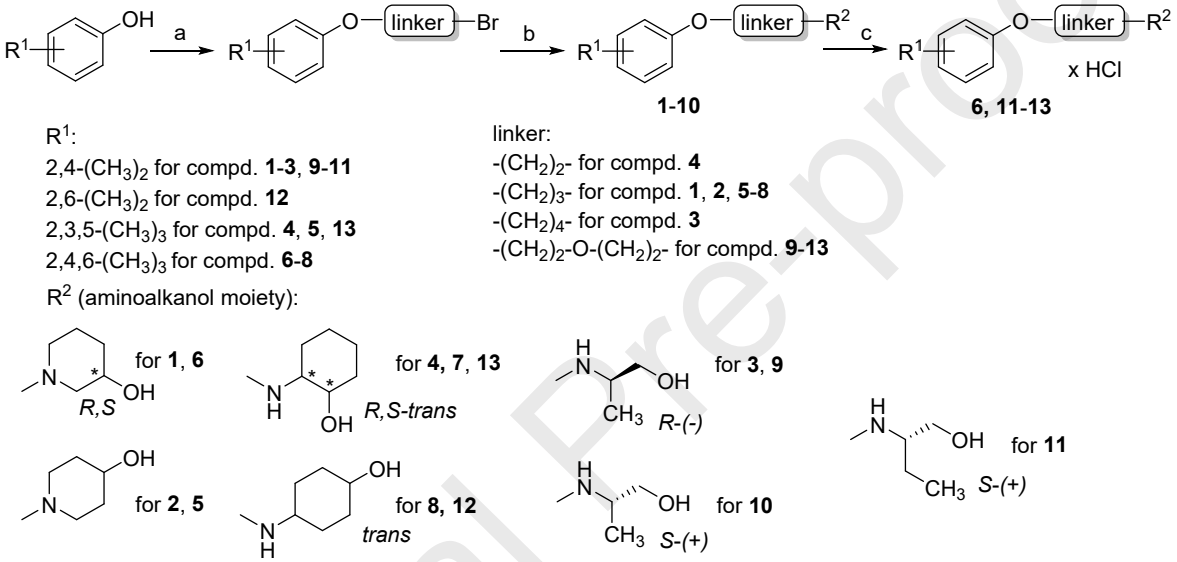
bromination reaction, allowing obtaining an ether from properly substituted phenol and an alkyl halide. Reaction of the corresponding phenols with 2-chloroethan-1-ol, 3-chloropropan-1-ol, 4-chlorobutan-1-ol or 2-(2-chloroethoxy)ethan-1-ol gave alcohols, which were then substrates in the bromination reaction using phosphorus tribromide. The resulting bromides were subjected to aminolysis using the appropriate aminoalkanol. The reaction was carried out in toluene in the presence of K_2CO_3 as a proton acceptor for approximately 5 hours. For compounds **4**, **7** and **13**, the aminoalkanol – *R,S-trans*-2-aminocyclohexan-1-ol – was obtained synthetically *via* reaction of cyclohexene oxide with a 25% aqueous ammonia solution. In other cases, aminoalkanols were obtained from commercial sources. The obtained final products were crystallized from petroleum ether, hexane or heptane (in case of too low solubility, mixtures of these solvents with toluene were used in the proportions 20:1 – 5:1). In order to achieve adequate chromatography column (silica gel, eluent: methylene chloride/methanol, 70:30 v/v) before the crystallization step. In the case of compounds **6**, **11** – **13**, the obtained compounds were converted into the salt form (hydrochloride) by saturating the ethanolic solution of the compound with hydrogen chloride gas. The obtained hydrochlorides were crystallized from a solvent mixture (acetone/methanol 10:1 or acetone/ethanol 5:1). The identity of all obtained compounds was confirmed by spectral methods (NMR), while their purity and homogeneity by means of chromatographic methods (e.g. TLC, LCMS). The melting point of all substances was also determined. For optically active compounds, specific rotation was measured. The detailed description of synthetic procedures as well as physicochemical data of title compounds are included in Supplementary material.



Scheme 1. Synthesis of compounds **1**-**13**. Reagents: a: 1. Cl-linker-OH, K_2CO_3, acetone/EtOH, 2. PBr_3; b: R^2, K_2CO_3, toluene; c: HCl(g), EtOH.

Pharmacology

Most of the antiepileptic drugs available on the market are the result of (1) *in vivo* screening of structurally diverse compounds or (2) structure optimization of the known antiepileptic drugs or active compounds in preclinical studies. Rational molecular target-oriented design was used only for compounds activating GABAergic transmission (e.g. vigabatrin, tiagabine) and glutaminergic receptor antagonists (e.g. perampanel).[18] On the other hand, data from 2011 and 2012 indicate that as much as 71% of all new drugs approved by FDA resulted from the rational design of substances with a specific mechanism of action. The reason for this discrepancy is probably the lack of a sufficient number of well-established molecular targets for anticonvulsants.[19] Regarding the above facts, the presented study uses the strategy of structural modification of anticonvulsant compounds developed previously in the Department of Bioorganic Chemistry, followed by *in vivo* screening of all obtained compounds. A preliminary assessment of the potential mechanism of action is planned only for selected compounds active *in vivo*. The described procedure increases the chances of developing new compounds active *in vivo*, at the same time being in line with the current trend of searching for potential molecular targets for new antiepileptic drugs.

In vivo screening of the anticonvulsant activity was performed with a use of the maximal electroshock seizure (MES) test (mice, *i.p.*, Table 1). Among the 13 tested compounds, 10 showed anticonvulsant activity (ED_{50} ranging from 14.79 mg/kg for the compound **7** to 52.49 mg/kg for the compound **3**). All compounds were also preliminary screened for potential neurotoxicity in the rotarod test (mice, *i.p.*). Taking into account both anticonvulsant activity and results of the rotarod test, compound **4** showed the most advantageous pharmacological profile (PI = 5.00). For selected compounds characterized by $ED_{50} < 20$ mg/kg in MES test (**4** – **7**, **13**) additional pharmacological studies were planned, including the assessment of their antiallodynic and antihyperalgesic properties in oxaliplatin (OXA)- or streptozotocin (STZ)-induced neuropathic pain models (compounds **4**, **5**, **13**). Compound **4** attenuated tactile allodynia but not cold hyperalgesia in both phases of OXA-induced neuropathy (von Frey test: activity at a dose of 15 mg/kg, $p < 0.0001$ in the early phase and $p < 0.05$ in the late one, mice, *i.p.*, Table 2). Tested compounds were inactive in the STZ-induced neuropathy model. The detailed results are presented in Supplementary material (Table S2).

Compounds **4** – **7** and **13** were also tested *in vitro* for potential antioxidant activity (microplate method using DPPH free radical).[20] The premise for this type of research was the fact that

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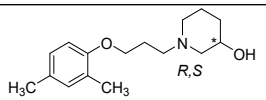
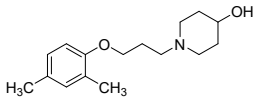
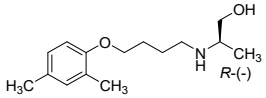
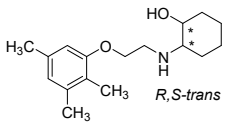
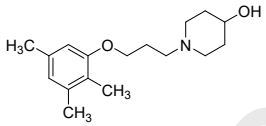
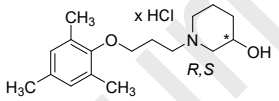
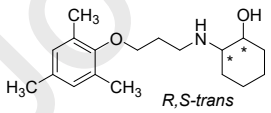
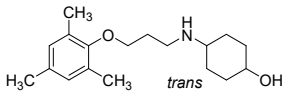
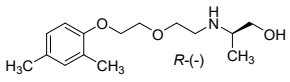
the pathological factor in the development of some central nervous system diseases.[21] An example of a registered drug whose main mechanism of action is antioxidant activity is idebenone (a coenzyme Q_{10} derivative). The compound was initially developed towards the therapy of Alzheimer's disease and then neuromuscular diseases. At present, its main indication is Leber's hereditary optic neuropathy.[22] The role of reactive oxygen species is also emphasized in the context of the pathomechanism of epilepsy, e.g. antioxidants have been postulated to potentially slow

sted compounds showed antioxidant activity in this test in the concentration range of 125-1000 μM (test results available in Supplementary material, Figure S1).

Table 1. Anticonvulsant activity of compounds **1** – **13** in the maximal electroshock seizure (MES) test and their neurotoxicity in the rotarod test (mice, *i.p.*).

Compd	Chemical structure	Dose [mg/kg]	MES[a]	Lethality in MES[b]	Rotarod[c]	ED_{50} (confidence interval) TD_{50} (confidence interval) [mg/kg]	PI[d]
1		30	2/6	NT	NT	ED_{50} = 34.82 (27.46 – 44.16)	3.09
		40	4/6	NT	NT	TD_{50} = 107.66 (95.29 – 121.64)	
		50	5/6 (4/4)	0/4	1/4		
		100	NT	NT	2/6		
		115	NT	NT	4/6		
		130	NT	NT	5/6		
2		50	0/4	0/4	0/4	NT	NT
3		30	1/6	1/6	2/6	ED_{50} = 52.49 (35.50 – 77.61)	2.59
		50	3/6	0/6	1/6	TD_{50} = 135.99 (106.74 – 173.28)	
		70	4/6	0/6	1/6		
		120	NT	NT	2/6		
		150	NT	NT	4/6		
		200	NT	NT	5/6		
4		10	2/6	NT	2/6	ED_{50} = 15.67 (9.10 – 26.97)	5.00
		20	3/6	NT	3/6	TD_{50} = 78.30 (6.82 – 93.13)	
		30	5/6	NT	5/6		
		50	4/4	0/4	1/4		
		60	NT	NT	1/6		
		80	NT	NT	3/6		
		100	NT	NT	5/6		
5		10	0/6	0/6	NT	ED_{50} = 17.71 (15.75 – 19.91)	3.58
		20	4/6	1/6	NT	TD_{50} = 63.40 (58.07 – 69.21)	
		30	6/6	0/6	0/6		
		45	NT	NT	0/6		
		60	NT	NT	4/6		
		100	NT	NT	6/6		
6		10	0/6	0/6	NT	ED_{50} = 18.17 (16.14 – 20.46)	3.86
		20	3/6	2/6	NT	TD_{50} = 70.19 (64.55 – 76.32)	
		30	6/6	0/6	0/6		
		60	NT	NT	1/6		
		80	NT	NT	4/6		
		100	NT	NT	6/6		
7		10	1/6	NT	NT	ED_{50} = 14.79 (12.24 – 17.88)	2.90
		20	3/6	NT	NT	TD_{50} = 42.85 (36.69 – 50.05)	
		30	6/6	NT	NT		
		40	NT	NT	2/6		
		45	NT	NT	4/6		
		50	4/4	0/4	4/4		
		55	NT	NT	5/6		
8		30	0/6	6/6	0/6	NT	NT
9		20	1/6	0/6	0/6	ED_{50} = 27.43 (23.75 – 31.69)	3.40
		30	2/6	0/6	1/6	TD_{50} = 93.24 (84.84 – 102.47)	
		50	6/6	0/6	0/6		
		60	NT	NT	0/6		
		80	NT	NT	1/6		
		100	NT	NT	3/5 (1 death)		

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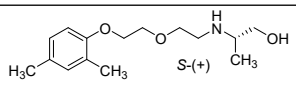
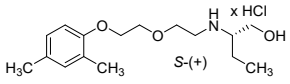
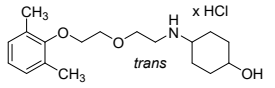
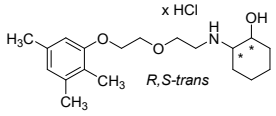
Co		[mg/kg]		y in MES[b]		TD_{50} (confidence interval) [mg/kg]	d
10		30	0/6	2/6	1/6	NT	NT
11		30	1/6	NT	NT	ED_{50} = 40.51 (31.90 – 51.44)	2.33
		40	3/6	NT	NT	TD_{50} = 94.38 (84.99 – 104.81)	
		50	4/6 (2/4)	1/4	0/4		
		60	5/6	NT	NT		
		80	NT	NT	1/6		
		90	NT	NT	2/6		
		100	NT	NT	4/6		
12		30	1/6	NT	NT	ED_{50} = 33.03 (31.52 – 34.62)	3.99
		35	3/6	NT	NT	TD_{50} = 131.88 (123.87 – 140.40)	
		40	6/6	NT	NT		
		50	4/4	1/4	0/4		
		100	NT	NT	0/6		
		130	NT	NT	4/6		
		150	NT	NT	5/6		
13		10	0/6	NT	NT	ED_{50} = 18.17 (16.14 – 20.46)	2.91
		20	3/6	NT	NT	TD_{50} = 54.21 (44.76 – 65.66)	
		30	6/6	NT	NT		
		40	NT	NT	1/6		
		50	4/4	0/4	2/6 (4/4)		
		60	NT	NT	4/6		
Phenytoin[e]						ED_{50} = 6.65 (5.42 – 8.16) TD_{50} = 56.91 (48.53 – 66.74)	8.56
Lacosamide[24]						ED_{50} = 9.24(8.28– 10.30) TD_{50} = 46.20(44.48–48.00)	5.00

MES – maximal electroshock seizure test; [a] number of animals protected/number of animals tested; [b] number of lethal cases/number of animals tested; [c] TD_{50} [mg/kg b.w.] or number of mice in which motor impairment was observed/number of mice tested (10 rpm); [d] PI = TD_{50}/ED_{50}; [e] 60 min. after drug administration; NT – not tested.

Table 2. Effect of the test compounds on tactile allodynia and cold hyperalgesia in a mouse model of chemotherapy-induced peripheral neuropathy caused by intraperitoneally administered OXA.

Compd	Dose [mg/kg]	Test	Effect±SEM For cold plate test: mean latency to nocifensive response [s] For von Frey test: mean paw withdrawal threshold [g]				
			1st day (early phase of neuropathy)			7th day (late phase of neuropathy)	
			Before OXA – healthy mice	Before compd administration (3 h after OXA)	1 h after compd administration	Before compd administration	1 h after compd administration
Vehicle[a]	-	Cold plate	57.64±1.267	59.76 ±0.16	58.48±0.856	57.64±1.267	57.96±0.634
		Von Frey	3.36±0.23	3.144±0.169	3.209±0.156	3.192±0.181	3.068±0.177
4[b]	15	Cold plate	50.433±2.888	25.189±7.641###	22.333±6.748	11.133±2.178	9.278±2.443
		Von Frey	2.867±0.019	1.797±0.039####	**2.222±0.086****	1.866±0.036	**2.122±0.072***
5[b]	18	Cold plate	56.822±1.69	16.222±7.176####	15.167±6.135	16.344±5.226	8.833±3.473
		Von Frey	2.795±0.05	1.791±0.038####	1.921±0.058	1.81±0.065	2.027±0.054
6[b]	18	Cold plate	46.95±3.672	22.82±5.766####	18.18±4.47	12.81±3.916	10.65±3.169
		Von Frey	2.714±0.058	1.837±0.05####	1.958±0.066	1.855±0.043	1.947±0.029
7[b]	15	Cold plate	56.73±2.604	34.27±7.511###	19.35±5.399	11.77±5.692	7.09±1.525
		Von Frey	2.985±0.079	1.765±0.038####	1.999±0.055	1.843±0.055	1.958±0.047
13[b]	18	Cold plate	54.25±2.472	20.45±6.407####	20.92±6.57	16.1±5.289	8.86±2.05
		Von Frey	2.714±0.081	1.9±0.045####	2.09±0.036	1.822±0.042	1.907±0.027

[a] vehicle-treated mice did not receive OXA; [b] the test compounds were injected 3 h after OXA or 7 days after OXA. OXA – oxaliplatin; statistical analysis – two-way ANOVA and post-hoc Tukey's test; statistical significance compared values before OXA administration: ### $p < 0.001$, #### $p < 0.0001$; statistical significance compared to values before compound administration: * $p < 0.05$, **** $p < 0.0001$.

In order to evaluate the mechanism of compounds action, the most active compounds have been checked for affinity to 5-HT_{1A} receptors. The choice of this receptor based on results previously obtained for reference compounds (Figure 2). The results are presented in Table 3. Compounds **4**, **6**, **7**, **9** and **13** showed binding to the tested receptors. The K_i values ranged 1006 – 4769 nM.

Table 3. Receptor studies performed for compounds **1**, **3** – **7**, **9**, **12**, **13**.

Compd	5-HT_{1A} K_i [nM]
1	-
3	-
4	1006.0 ± 113.0
5	-
6	3387.0
7	2370.0 ± 250.0
9	4796.0
12	-
13	1200.0 ± 118.0
DPAT	1.0 ± 0.05

- – no binding at 10^{-5} M

10 anticonvulsant compounds were obtained in a group of 13 aminoalkanol derivatives. It seems that substitution of the phenyl

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ring ties. positive effect on anticonvulsant activity. Of the 6 compounds of this type obtained, only compound **8** was inactive (substituted at positions 2,4,6, high mortality of experimental animals), while the remaining five (**4** – **7** and **13**) had ED_{50} values below 20 mg/kg b.w. Of the seven compounds disubstituted at positions 2,4 or 2,6, five were found to be active, but they showed lower activity compared to the trisubstituted derivatives (ED_{50} values in the range of 27.43 – 52.49 mg/kg b.w.). Considering the neurotoxicity of the compounds in this group, compound **4** (substituted in positions 2,3,5), was characterized by the highest protective index. However, no relationship was observed between the phenyl ring substitution pattern and protective index value, which indicates a greater influence of other structural elements of the molecule.

Among the active compounds were 6 compounds possessing an alkyl linker 2 – 4 carbon atoms long, and 3 compounds possessing an ethoxyethyl linker. Comparing test results for compounds differing only in linker structure (**3** and **9**, **4** and **13**) as well as experience in conducting research in similar groups of compounds provide some valuable information on an influence of linker's structure on pharmacological profile. The use of an ethylene or ethoxyethyl linker seems to be advantageous in terms of both anticonvulsant activity and protective index. Despite the significant difference in the length of these linkers, it cannot be clearly determined which of them influences the pharmacological profile favorably. This phenomenon can be explained by the significant influence of other fragments of the molecule (e.g. the amino component), by a different mechanism of anticonvulsant activity of compounds with an ethylene or ethoxyethyl linker, but also by a different binding mode to the molecular target or conformation changes occurring within a molecule in the molecular target environment.

The compounds obtained include molecules differing only in the amine component, which makes it possible to analyze the structure – activity relationship (SAR) considering this structural fragment. For compounds **1** and **2**, changing the position of the piperidinol's hydroxy group from position 3 to 4 causes a loss of activity. Also, in the case of compounds **6**, **7** and **8** (derivatives of piperidinol – **6** or aminocyclohexanol – **7** and **8**), the distance between the oxygen and nitrogen atoms equal to 2 carbon atoms seems favorable (active compounds **6** and **7**). Among the remaining cyclic aminoalkanol derivatives, anticonvulsant activity was demonstrated by compounds **4**, **13** (2-aminocyclohexan-1-ol derivatives), **12** (4-aminocyclohexan-1-ol derivative) and **5** (piperidin-4-ol derivative), and among chain aminoalkanols, compounds **3**, **9** (*R*-(-)-2-aminopropan-1-ol derivatives) and **11** (*S*-(+)-2-aminobutan-1-ol derivative). The distance between the oxygen and nitrogen atoms within the aminoalkanol component does not seem to determine the anticonvulsant activity of the compound, as evidenced by the activity of compounds **5** and **12**. Nevertheless, the position of the hydroxy group undoubtedly affects the pharmacological profile, including the ED_{50} value and protective index. Moreover, it is worth to note that all compounds possessing ethoxyethyl linker are also colamine derivatives, however -NH-CH_2-CH_2-O- moiety is present within linker, not only within aminoalkanol moiety.

The chirality of the used aminoalkanols allows consideration of the role of stereoisomerism. Among the active compounds were both *R* (compounds **3** and **9**) and *S* (compound **11**) isomers. It can be concluded that the case of each of the tested chiral compounds should be considered separately. For example, in the case of compounds **9** and **10**, which are derivatives of 2-aminopropan-1-ol, the active enantiomer had the *R* configuration. Derivatives of these compounds containing *R*-2-aminobutan-1-ol[11] and *S*-2-

The *R*-enantiomer was only active at a high dose (100 mg/kg b.w.) in which high neurotoxicity was also observed,[11] whereas **11** had moderate anticonvulsant activity and a relatively favorable PI value of 2.33.

Receptor studies have shown that interaction with the 5-HT_{1A} receptor is not a must for anticonvulsant activity. However, its involvement in modulation of the pharmacological profile cannot be completely excluded. It is worth noting that the highest affinity for the receptor was characterized by compound **4**, which showed multidirectional anticonvulsant and analgesic activity. Considering also the results of receptor studies carried out for reference compounds, among the potential molecular targets of this group of compounds, sigma receptors and/or other elements of the serotonergic system may be mentioned. At the same time, based on the studies carried out, the joint or separate mechanism of anticonvulsant and analgesic activity of compound **4** cannot be clearly confirmed. Perhaps the mechanism of both activities involves modulation of the activity of a number of therapeutic targets, including 5-HT_{1A} receptors. It is possible, however, that anticonvulsant and analgesic activity are determined by separate mechanisms.

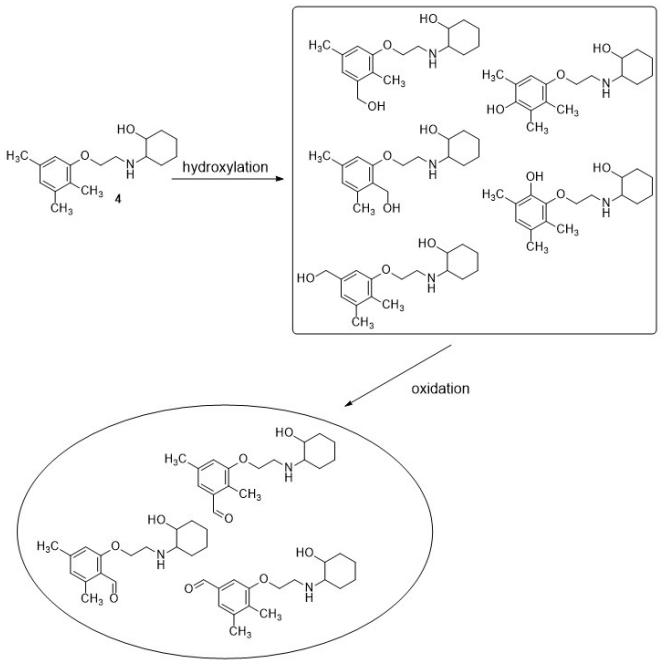
Metabolism and safety characterization *in vitro*

Accurate characterization of the pharmacokinetic and toxicity parameters of the active compounds at the early stages of new drugs' development reduces the time and cost of conducting extensive research for less promising compounds. Therefore, fast and low-budget screening methods, including *in silico* and *in vitro* assays, are used in the drug design process.[25]

Metabolism studies

Metabolism of compounds **1**, **4**, **6**, **7**, **12** and **13** was studied *in vitro* using murine microsomes. For all compounds, the main pathway of metabolism was the hydroxylation reaction, which resulted in the formation of monohydroxylated metabolites (m/z = M+16), and in the case of compounds **7** and **13** – also dihydroxylated (m/z = M+32). For compound **4**, oxidation of the previously formed hydroxylated derivative to the aldehyde group (m/z = M+14) was observed. The detailed results are presented in Supplementary material (Table S3).

To determine the structure of metabolites, LC/MS fragmentation analysis was performed. Analysis of the results confirmed the hydroxylation of methyl groups substituted to aromatic ring, or – in the case of a larger number of metabolites formed – also the aromatic ring itself (exemplum spectra available in Supplementary material, Figure S2). A similar direction of metabolic changes is observed for the previously described



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com ... ility and bupranolol.[27] It was not possible to determine exactly where the hydroxylation reaction takes place in the case of monohydroxylated metabolites for compounds substituted with more than one methyl group. Figure 3 shows the most probable structures of the resulting metabolites as exemplified by compound **4**. Considering safety of the tested compounds after *in vivo* administration, the aldehyde metabolite of compound **4** deserves attention. As reactive compounds, aldehydes can interact with nucleic acids and proteins and as a consequence cause mutagenicity or cytotoxicity. Among known antiepileptic drugs, felbamate is metabolized to aldehyde (2-phenyl-prop-2-en-1-al). This metabolite may be responsible for the hepatotoxicity of this drug as well as the risk of aplastic anemia as a result of its use.[28]

Figure 3. Possible metabolites of compound **4**.

In addition to determining the direction of metabolic changes and indicating the possible structures of the resulting metabolites, *in vitro* studies using microsomes allow comparison of phase I metabolic rate. Half-life ($t_{1/2}$) was calculated for each compound, as well as internal clearance (Cl_{int}), which parameter does not depend on the concentration of microsomes and describes the metabolic stability of the compounds (Table 4). According to the classification used by CYPROTEX,[29] compound **12** is characterized by slow metabolism, while the remaining ones are classified as metabolically unstable. Compounds **4** and **7**, which have the highest Cl_{int} values, are 2-aminocyclohexan-1-ol derivatives. It can be assumed that this aminoalkanol moiety may increase the metabolic lability of compounds, despite the fact that metabolism occurs within the substituents of the aromatic system. According to the classification published by B. Williamson et al.,[30] all tested compounds are classified as moderately metabolically stable.

Table 4. Metabolic stability of tested compounds – values of half-life ($t_{1/2}$) and internal clearance (Cl_{int}).

Compd	Protein concentration (mg/mL)	$t_{1/2}$ (min)	Cl_{int} (µL/mg/min)
1	0.4	28.6	60.5
4	0.2	27.2	127.5
6	0.4	38.9	44.5
7	0.2	27.2	127.5
12	0.4	87.7	19.8
13	0.2	37.2	93.0
Imipramine[31]	0.5	11.0	125.5

In silico safety evaluation

All compounds obtained were evaluated *in silico* for potential toxicity. Mutagenicity, carcinogenicity and reproductive toxicity were included, due to their significance in the case of orally administered drugs, as well as serious health consequences in case of occurrence. Two alternative programs available for free – Osiris Property Explorer[32] and Lazar[33] – were used. Programs differ in the method of predicting toxic properties. Osiris Property Explorer enables risk prediction (low, medium, high) of selected types of toxicity based on the analysis of the chemical structure and detection of fragments predisposing to particular type of toxicity. The list of these groups bases on the analysis of the structures of all compounds present in the RTECS database (Registry of Toxic Effects of Chemical Substances),[34] which are known to show the particular type of toxicity (e.g. mutagenic or carcinogenic compounds). The Lazar program enables prediction of selected types of toxicity on the basis of experimental literature data available for structurally similar compounds, the so-called "neighbors". The results of the prediction consist not only of the potential toxicity alert, but also of the extensive characteristics of the calculations performed, including the list of compounds ... assessment). The results of the analyzes carried out are presented in Table 5.

Table 5. *In silico* prediction of selected types of toxicity performed for compounds **1** – **13**.

Type of toxicity	Software	
	Osiris Property Explorer	**Lazar** [a]
Mutagenicity	Compounds: **4**, **5**, **13** (high risk)	-
Carcinogenicity	-	Compounds: **1 – 3**, **9 – 11**
Reproductive toxicity	-	[b]

[a] for compounds **4**, **8**, **12** (carcinogenicity) and **7** (carcinogenicity and mutagenicity) prediction in Lazar was not possible due to low amount of "neighbor" compounds in the database; for all other compounds similarity threshold < 0.5 – prediction may be out of applicability domain; [b] lack of reproductive toxicity predictions in Lazar

The Osiris Property Explorer program identified 3 potentially mutagenic compounds. The structural alert for this type of toxicity was substitution at the aromatic ring with three methyl groups at positions 2, 3 and 5. The Lazar program identified several substances as potentially carcinogenic compounds. However, for some compounds, prediction was not possible due to the insufficient number of "neighbors" for which experimental data are available. Prediction for other compounds may be affected by an error due to the low threshold of similarity of "neighbors" to the initial test compound. For the above-mentioned reasons, the Lazar program does not seem to be a preferential tool for predicting toxicity in the group of phenoxyalkyl- and phenoxyethoxyethyl derivatives of aminoalkanols. Perhaps in the future an update of the experimental database will increase the reliability of the prediction.

Mutagenicity tested in vitro

The Ames[35] test was used to verify the potential mutagenic activity of compounds *in vitro* and assess the effectiveness of prediction of the Osiris Property Explorer program. The test uses genetically modified *Salmonella enterica* sv. Typhimurium strains, which are devoid of histidine synthesis and do not grow on medium lacking this amino acid. In the presence of the mutagen, a reverse mutation occurs that restores this ability and increased growth of bacterial colonies compared to the control can be observed. Depending on the strain used, the test can detect compounds that cause different types of mutations, e.g. point mutations (TA100 strain) or frameshift mutations (TA98 strain).[35] The selection of compounds for mutagenicity testing was based on the results of predictions using the Osiris Property Explorer program. Compounds with high risk of mutagenicity (**4**, **5** and **13**) were tested, as well as compounds **1** and **12** in order to include compounds with various cyclic aminoalkanol components. Each compound was tested at five concentrations (10 – 750 or 10 – 1000 µg/plate). The concentration range depended on the solubility of tested compounds. Concentrations above 750 µg/plate caused precipitation on the dish, making the results difficult to read. Therefore, at further stages of the study, the concentration of 750 µg/plate was the highest one used. All compounds were tested using strains TA100 and TA98.

The results are presented in Table 6. Only compound **13** was safe at all concentrations tested, despite being indicated by Osiris Property Explorer. All other compounds were characterized by MI (mutagenicity index) ≥ 2 at a minimum of one concentration for one or both bacterial strains. A clearly marked relationship

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betv [illegible] o observed for compound **5**. For the remaining compounds, this relationship is absent, mutagenic activity was only observed at the highest concentration tested (compound **1**), or MI decreases with increasing concentration (compound **4**). The latter case may be the result of the compound's toxicity at high concentrations, which is manifested by the complete absence of bacterial growth at concentrations of 500 µg/plate and higher (strain TA100). It should also be noted that at higher concentrations, the results obtained for some compounds were characterized by a high standard deviation and the morphology of the bacteria changed (in some cases making reading difficult – e.g. compounds **12**, **13**). This indicates possible additional effects produced by the tested compounds, in addition to the mutagenic effect.

Table 6. Mutagenicity of compounds **1**, **4**, **5**, **12**, **13** (Ames test).

Compd	Conc. [µg/plate][a]	Mean number of revertants ± SD (MI)[b]	
		Strain	
		TA100	**TA98**
1	DMSO	72.5±9.2	8.0±1.4
	10	89.5±6.4 (1.2)	6.3±5.9 (0.4)
	75	69.5±10.6 (1.0)	8.0±1.0 (0.8)
	200	88.5±0.7 (1.2)	6.7±2.1 (1.2)
	500	81.0±9.9 (1.1)	4.7±1.5 (1.0)
	750	3869.5±1253.7 (**53.4**)	3.3±1.5 (1.0)
4	DMSO	130.7±11.0	|
	0.1	147.0±15.6 (1.1)	|
	1	138.3±20.0 (1.1)	|
	5	134.7±10.7 (1.0)	|
	DMSO	72.5±9.2	8.0±1.4
	10	185.5±57.3 (**2.6**)	7.3±3.1 (0.9)
	75	105.0±58.1 (1.4)	6.3±2.1 (0.8)
	200	49.0±13.9 (0.7)	6.7±3.2 (0.8)
	500	-	8.7±2.5 (1.1)
	750	-	9.3±2.3 (1.2)
5	DMSO	64.3±10.4	8.3±2.1
	10	66.0±2.0 (1.0)	12.0±3.5 (1.4)
	75	52.3±5.1 (0.8)	15.3±5.5 (1.8)
	200	42.0±8.7 (0.7)	11.0±3.6 (1.3)
	500	944.0±859.1 (**14.7**)	6.7±5.8 (0.8)
	750	|	364.3±84.6 (**43.7**)
	1000	1190.0±1031.2 (**18.5**)	|
12	DMSO	72.5±9.2	8.0±1.4
	10	226.7±50.0 (**3.1**)	8.0±2.0 (1.0)
	75	312.3±120.2 (**4.3**)	8.3±3.5 (1.0)
	200	55.0±5.7 (0.8)	9.3±2.5 (1.2)
	500	274.3±119.1 (**3.8**)	6.7±2.1 (0.8)
	750	/	3.0±2.6 (0.4)
13	DMSO	72.5±9.2	8.0±1.4
	10	86.0±5.0 (1.2)	7.0±2.6 (0.9)
	75	80.7±27.1 (1.1)	8.0±4.6 (1.0)
	200	70.5±7.8 (1.0)	11.0±1.4 (1.4)
	500	/	9.0±2.8 (1.1)
	DMSO	130.7±11.0	|
	750	56.0±14.1 (0.4)	/
Sodium azide	DMSO	64.3±10.4	|
	5	533.7±9.9 (**8.3**)	
4-nitro-*o*-phenylene--diamine	DMSO	|	8.3±2.1
	2.5		519.0±10.6 (**62.3**)

[a] all tests performed in triplicate; [b] SD – *standard deviation*; MI – mutagenicity index – the quotient of the number of revertants present on the plate with the test compound at a given concentration and the number of revertants present on the negative control; dose-dependent increase (MI ≥ 2) in the number of revertants for a minimum of one strain – compound considered mutagenic; - – no growth; / – equivocal result (including e.g. significant change in bacterial morphology); | – not tested

For four active aminoalkanol derivatives (compounds **3**, **5**, **6** and **9**), safety tests were performed in relation to the gastrointestinal flora.

The preferred route of administration of anticonvulsants and analgesics is oral, which is associated with the risk of dysbiosis if they are active against the bacteria in the microbiome. Until now, disorders of the natural gastrointestinal flora have been considered primarily as an adverse effect of the use of antibiotics. Recent reports, however, prove that dysbiosis can also be caused by other groups of drugs, including centrally active ones (e.g. some antidepressants).[36,37] Dysbiotic conditions can cause many complications, including inflammatory bowel disease, but also inflammation of other organs and systems. Due to the weakening of the blood-brain barrier, inflammation can even affect the central nervous system. The relationship between the pathophysiology of some central nervous system diseases and the state of the microbiome has been documented, including Alzheimer's disease, Parkinson's disease and depression.[37,38]

The study was carried out using a variety of bacterial strains living on different sections of the digestive tract, including aerobic (*Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* – a clinical strain, *Salmonella enterica* ATCC 51957) – by serial dilution method in liquid medium, as well as anaerobic (*Bacteroides fragilis* ATCC 25285, *Lactobacillus paracasei* ATCC 25302, *Prevotella loescheii* ATCC 15930, *Bifidobacterium* spp. – a clinical strain, *Clostridium difficile* – a clinical strain) – by dilution method in solid medium and by diffusion-disk method.

All the strains listed showed resistance to the tested compounds at the concentrations tested, i.e. from 0.195 µg/mL to 100 µg/mL for aerobic strains and 50 µg/mL for anaerobic strains. Therefore, the tested compounds can be considered safe in relation to the gastrointestinal flora. The detailed results are presented in Supplementary material (Tables S4-S11).

Cell culture studies

Compound **4** was selected for comprehensive studies on the effects on astroytes, including cytotoxicity as well as impact on proliferation and cell cycle. Such studies are an important element of safety evaluation panel for compounds targeting central nervous system. However, glial cells are also interesting in the context of epilepsy and seizures' inhibition.

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cytotoxicity *in vitro*. These are especially carbamazepine, topiramate and oxcarbazepine, but in concentration 50 μg/mL or higher also gabapentin, lamotrigine, tiagabine and levetiracetam.[39] Moreover, valproate proved to exhibit antiproliferative effect in C6 glioma model by restriction of the glial cell cycle at a defined point in the mid-G1 phase, which fact may explain its teratogenicity and specificity in inducing neural tube defects.[40] The above data could be taken into account in the therapeutic use of particular drugs as well as in design of new compounds characterized by high tolerability. On the other hand, astrogliosis is considered a part of pathophysiological processes accompanying epileptogenesis, which was observed e.g. in animal models of temporal lobe epilepsy.[41] Astrocytes became a potential therapeutic target for anticonvulsants, although their role in epilepsy has not yet been fully understood. Among the most frequently mentioned potential astrocytic targets for anticonvulsants are modulation of glutamate uptake, release of neurotransmitters (including glutamate, GABA or ATP) and suppressing Ca^{2+} signalling.[42,43] Noteworthy, pharmacological activity of some known anticonvulsants (e.g. valproate, gabapentin, phenytoin) may be partially mediated by direct depression of astrocytic activity.[43] The above facts are a premise for screening new anticonvulsant compounds for their influence on astrocytes.

As part of the current work a panel of cytotoxicity tests was conducted, including tests based on both metabolic impairment evaluation (MTT test, Figure 4A and 4B) as well as detection of

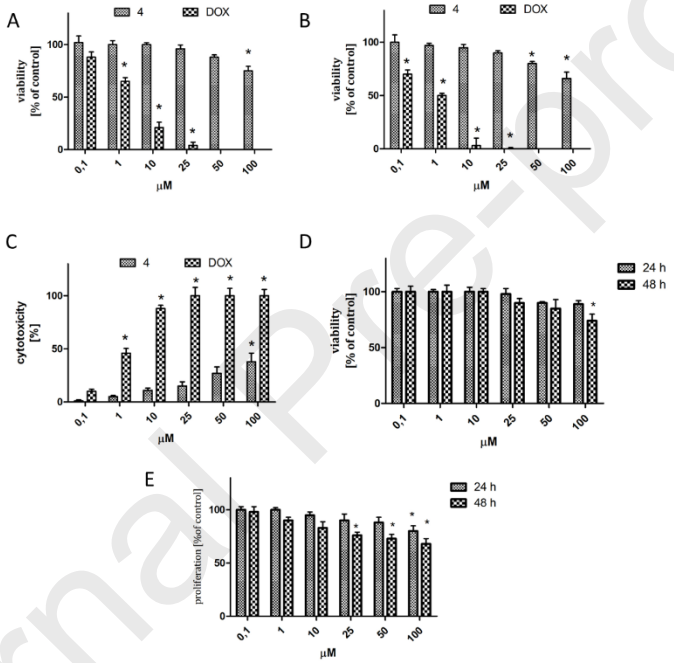

Figure 4. Cell culture studies performed for compound **4**: cytotoxicity (A – MTT assay, murine astrocytes; B – MTT assay, human astrocytes derived from iPSC; C – LDH assay, murine astrocytes; D – trypan blue assay, murine astrocytes) and antiproliferative effect (E – crystal violet assay, murine astrocytes); DOX – doxorubicin.

In summary, 13 new aminoalkanols were designed and synthesized, including 9 compounds possessing anticonvulsant activity and 1 compound (**4**) exhibiting both anticonvulsant and analgesic in neuropathic pain activities. The introduction of an ethylene or ethoxyethyl linker seems to be advantageous in terms of both anticonvulsant activity and protective index. The distance between the oxygen and nitrogen atoms within the aminoalkanol component does not seem to determine the anticonvulsant activity of the compound, however the position of the hydroxy group undoubtedly affects the pharmacological profile. Major differences were observed within enantiomers' pharmacological activity, however among the active compounds were both *R* and *S* isomers. Receptor studies have shown that interaction with the 5-HT_{1A} receptor is not a must for anticonvulsant activity, however

after 24 and 48 h incubation – Figure 4D). For all tests murine astrocytes were used in order to make results relevant to previously observed anticonvulsant activity (mice, *i.p.*). However, the MTT test was also performed comparatively on human astrocytes obtained by means of iPSC cells induction. All assays performed on murine cells showed cytotoxicity of compound **4** in the highest tested concentration equal to 100 μM (in trypan blue test the effect was observed only after 48 h incubation). However, in the case of the MTT test performed with a use of human astrocytes, cytotoxicity has already been observed from a concentration of 50 μM.

Additionally, influence of compound **4** on murine astrocytes' proliferation was evaluated in crystal violet assay (Figure 4E). The dye is accumulated in a cell nucleus. After extraction, the number of cells can be detected calorimetrically. The assay showed antiproliferative activity of compound in concentration 100 μM after 24 h incubation and 25 μM and higher after 48 h incubation. According to results obtained using crystal violet assay we decided to check if compound **4** have influence on cell cycle distribution of astrocytes using flow cytometry analysis (propidium iodide staining). This approach allowed determination of the total amount of DNA per cell and stoichiometrically determined to obtain cell cycle distributions. Flow cytometry analysis do not show any statistically significant differences between control cells (non-treated) and cells incubated in the presence of compound **4** (25μM) for 24 hours. The data are presented in Supplementary material (Figure S3).

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its i , K.; distinct mechanism of anticonvulsant and analgesic activity remains unclear. A wide range of metabolism and safety studies performed within the presented study revealed that presented group of compounds is safe with relation to gastrointestinal flora. Aspects that are worth paying attention to when designing new active compounds are potential mutagenicity, cytotoxicity, as well as metabolism to aldehydes.

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Supplementary Material

Supplementary material is available as a separate file.