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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · JULY 2015

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European Journal of Medicinal Chemistry 102 (2015) 14–25

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Research paper

Design, synthesis and biological activity of new amides derived from 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetic acid

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ARTICLE INFO

Article history:
Received 18 December 2014
Received in revised form
7 July 2015
Accepted 8 July 2015
Available online 13 July 2015

Keywords:
Anticonvulsant activity
Antinociceptive properties
Succinimides
In vivo and *in vitro* studies
Mutagenicity and antimutagenicity studies

ABSTRACT

A series of new 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetamides (**6**–**23**) has been synthesized and evaluated for their anticonvulsant activity in the maximal electroshock (MES) and subcutaneous pentylenetetrazole (*sc*PTZ) seizure tests after intraperitoneal injection in mice. The acute neurological toxicity was determined using the rotarod test. The *in vivo* preliminary pharmacological results showed that in the whole series only two compounds (**15**, **21**) were devoid of activity, whereas other molecules revealed protection in at least one animal model of epilepsy (MES or/and *sc*PTZ). The *in vivo* quantitative studies in mice showed that in the MES test the most active were 1-{2-[4-(2-methoxyphenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**17**), 1-{2-[4-(4-fluorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**8**), and its 2-fluorophenyl analog (**7**) with ED_{50} values of 97.51 mg/kg (**17**), 104.11 mg/kg (**8**), and 114.68 mg/kg (**7**), respectively. In the *sc*PTZ screen the most potent were compound **6** with an $ED_{50} = 40.87$ mg/kg, and 4-benzylpiperidine derivative **22** – $ED_{50} = 60.00$ mg/kg. Furthermore, selected compounds **8**, **14**, **17**, and **23** were tested in the psychomotor seizure 6-Hz test. Compounds **7**, **8**, and **17** revealed significant analgesic activity in the formalin model of tonic pain in mice, without impairment of the motor coordination in the chimney test. The *in vitro* binding studies showed that the mechanism of anticonvulsant activity may be partially related with the influence on the voltage-gated sodium and calcium channels. The mutagenic and antimutagenic effects of **13**, **17**, and **22** were evaluated using the novel *Vibrio harveyi* assay.

1. Introduction

Epilepsy is a chronic medical disorder or condition, usually resulting in unpredictable, unprovoked recurrent seizures that affect a variety of mental and physical functions. Treatment of epilepsy often imposes an exposure to various antiepileptic drugs (AEDs) and requires long term commitment and compliance from the patient. The vast majority of patients are maintained through chronic medical management for appropriate seizure control. Despite the advent of new AEDs over the past 25 years, approximately 30% of epileptics experience recurrent seizures as well as many undesirable side effects most notably related to CNS exposure like diminished attention, executive function, intelligence, language skills, memory and processing speed [1–3]. Therefore, there remains a substantial need for the development of more efficacious AEDs especially for patients with refractory seizures.

Numerous compounds are synthesized and screened for their anticonvulsant activities each year. To make the discovery of new anticonvulsants more rational, several investigators identified chemical fragments which presence in the structure may enhance anticonvulsant properties. One of the structural features that play a significant role in relation to antiepileptic activity is an amide function. This moiety may be the part of heterocyclic ring e.g. ethosuximide, phenytoin or may appear as linear as amide bond e.g. levetiracetam, and its analogs brivaracetam or seletracetam [4–6] (Fig. 1).

The previous research from our laboratory have demonstrated diversified anticonvulsant activity of differently substituted pyrrolidine-2,5-diones [7–15]. Among these compounds the most promising were *N*-Mannich bases derived from 3,3-disubstituted-

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http://dx.doi.org/10.1016/j.ejmech.2015.07.017
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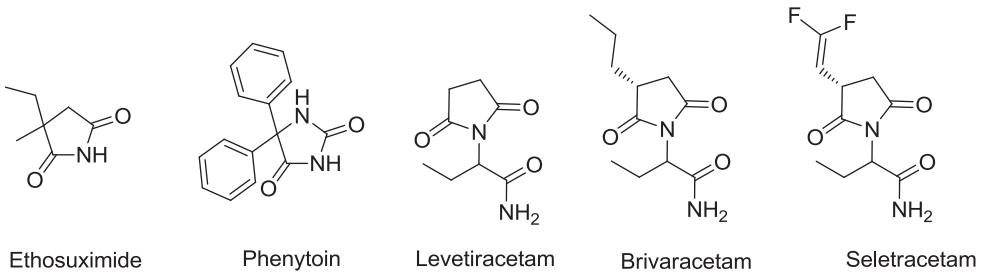


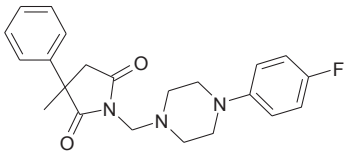
Fig. 1. Structures of known AEDs containing five-member heterocyclic ring and amide function.

pyrrolidine-2,5-diones with 4-phenylpiperazine as a basic fragment. The structure–anticonvulsant activity relationship studies (SAR) revealed that their efficiency was highly dependent on the substitution mode of the phenylpiperazine moiety thus the most favorable was the presence of electron-withdrawing chlorine, fluorine atoms or trifluoromethyl group [15]. These molecules were especially effective in the maximal electroshock seizure test (MES). Among aforementioned derivatives the most active were compounds **1**–**3** depicted in Fig. 2.

The subsequent SAR discussion revealed high anticonvulsant protection for analogs of *N*-Mannich bases containing in their structures an alkylamide moiety between the pyrrolidine-2,5-dione ring and the amine function [13,14]. Following these findings, in aim to search for new effective anticonvulsants as well as to continue systematic SAR studies among these series of derivatives, in the current work we have synthesized a new series of 3-methyl-3-phenyl-2,5-dioxo-pyrrolidine-1-yl-acetamides (**6**–**23**). These compounds have been designed as analogs of model compounds **1**–**3** in which methylene spacer has been replaced for acetamide moiety. The proposed structural modification enables to assess the influence of the presence of aforementioned amide function on anticonvulsant properties in this group of derivatives. With the aim of ensuring the reliable SAR discussion as an amine function variously substituted piperazines, benzylpiperidine or morpholine have been introduced.

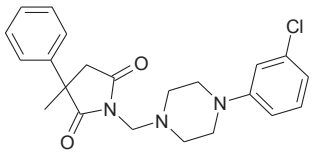
Pharmacological and clinical studies on epilepsy and neuropathic pain have documented, that both of them are chronic neurological disorders arising from an excessive neuronal activity and hypersynchronous neuronal firing [16,17]. Thus, nowadays antiepileptic drugs such as valproic acid, carbamazepine, gabapentin, pregabalin or lamotrigine are the first-line treatment of neuropathic pain. In view of the above facts, many candidates on new AEDs are often evaluated for their effectiveness in the neuropathic pain models [18]. Bearing in mind the aforementioned facts, several compounds with the most potent activity in the maximal electroshock seizures (MES) were subsequently tested for their antinociceptive activity in the formalin model of tonic pain in mice.

Taking into consideration that mutagenic activity is one of the most important endpoints for risk assessment of chemical compounds including drugs and drug candidates [19,20], the *Vibrio harveyi* assay was used to evaluate the mutagenic properties of selected 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetamides. In addition, the antimutagenic potential of these compounds was also tested, in order to identify agents that can protect the genetic material against damage.



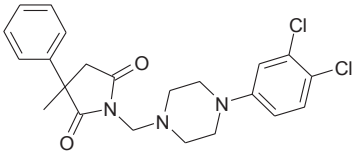
1

ED_{50} = 120.15 mg/kg (MES, mice *i.p.)*
TD_{50} > 500 mg/kg (MES, mice *i.p.*)
PI (TD_{50}/ED_{50}) > 4.16



2

ED_{50} = 41.10 mg/kg (MES, mice *i.p.*)
TD_{50} = 496.38 mg/kg (MES, mice *i.p.*)
PI (TD_{50}/ED_{50}) = 12.08



3

ED_{50} = 37.27 mg/kg (MES, mice *i.p.*)
TD_{50} > 500 mg/kg (MES, mice *i.p.*)
PI (TD_{50}/ED_{50}) > 13.41

Fig. 2. Structures of model compounds **1**–**3** obtained in the previous studies.

2. Results and discussion

2.1. Chemistry

Compounds **6**–**23** were synthesized according to Scheme 1. The starting 3-methyl-3-phenyl-succinic acid (**4**) was prepared as previously reported [21]. In the next step the cyclocondensation of **4** with 2-amineacetic acid yielded in 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetic acid (**5**). Final compounds **6**–**23** were obtained in the coupling reaction of intermediate **5** with equimolar amounts of appropriate secondary amines in the presence of carbonyldiimidazole (CDI) reagent. The reaction was carried out at room temperature in dry DMF for 24 h. The crude products were crystallized from 2-propanol. The final compounds were obtained in good yields. Their purities were assessed by TLC and HPLC chromatography. The structures of compounds synthesized were confirmed by both spectral (^{1}H NMR, ^{13}C NMR, LC–MS) and elemental (C, H, N) analyses. The detailed physical and analytical data are listed in the Experimental section.

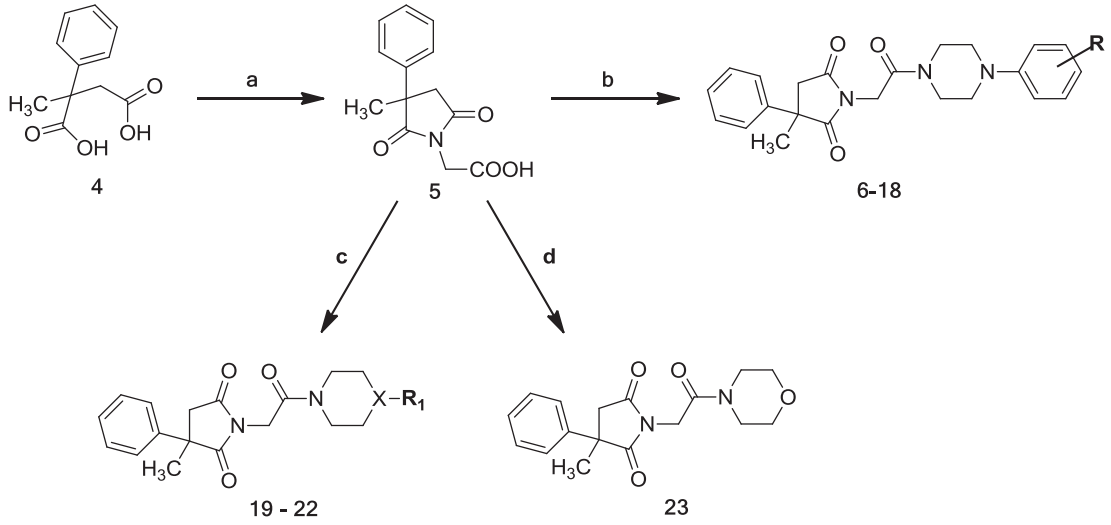
2.2. Anticonvulsant activity

The preliminary anticonvulsant screening of final molecules **6**–**23** involved two mechanism-independent animal seizure models, namely maximal electroshock seizure (MES) and the subcutaneous pentyleneterazole seizure (*sc*PTZ) test. Despite the diversity of models that could potentially be used to screen for anticonvulsant activity, the MES model and the *sc*PTZ model remain the "gold standards" in the early stages of testing. The MES test is the mechanism-independent animal seizure model which enables identification of compounds preventing seizure spread. This test uses an electrical stimulus to produce generalized tonic–clonic seizures, and thus is thought to be an experimental model of tonic–clonic epilepsy and of partial convulsions with or without secondary generalization in humans. The *sc*PTZ test employs chemically induced myoclonic seizures and is proposed to identify the agents raising the seizure threshold. This test is related to human generalized absence seizures [22,23].

In the first step of pharmacological studies the anticonvulsant activity of **6**–**23** was established after intraperitoneal (*i.p.*) injection in mice at doses of 30, 100 and 300 mg/kg. An observation was carried out at two different time intervals, namely 0.5 h and 2 h. The acute neurological toxicity was determined in the rotarod test (NT) in the same conditions. The *in vivo* screening results are shown in Table 1.

Except of inactive compounds **15** and **21**, all other derivatives showed activity in at least one model of epilepsy. Compounds **7**, **8**, **16**, and **17** revealed protection in the MES test at a dose of 100 mg/kg, whereas **6**, **10**, **13**, **14**, **22**, and **23** were active at a dose of 300 mg/kg. The MES-active molecules showed activity at 0.5 h (**6**, **7**), 2 h (**13**, **22**) or in both time points (**8**, **10**, **14**, **16**, **17**, and **23**). In the whole series 10 compounds were effective in the *sc*PTZ seizures. Among these molecules **6**, **7**, **9**, **18**–**20**, and **22** showed comparable activity to ethosuximide and valproic acid which are known as the model AEDs effective in *sc*PTZ seizures. Other compounds (**11**, **12** and **23**) revealed activity at higher dose – 300 mg/kg. The different anticonvulsant potencies of the derivatives synthesized are not directly correlated to their relative lipophilicity (clog *P*, Table 1), thus suggesting the influence of other parameters. Results from the rotarod toxicity evaluations demonstrate that **6**, **9**–**15**, **18**, **20**, **22**, and **23** did not show neurotoxicity at the maximum dose administrated (300 mg/kg). The other derivatives showing neurotoxicity at a dose of 300 mg/kg include **7**, **8**, **16**, **17**, **19**, and **21**.

Based on the preliminary results, in the next step of the pharmacological studies, the median effective doses (ED_{50}, in the MES and *sc*PTZ tests) and the median neurotoxic doses (TD_{50}, in the rotarod test) were determined for compounds **6**, **7**–**10**, **17**, **19**, and **22**. The quantitative studies were performed at previously estimated time of peak effect (TPE) for MES and *sc*PTZ protection. The results for compounds tested along with the data for standard AEDs



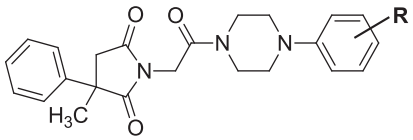
R = H, 2-F, 4-F, 2-Cl, 3-Cl, 4-Cl, 2,3-Cl, 3,4-Cl, 3-CF_3, 2-CH_3, 3-CH_3, 2-OCH_3, 3-OCH_3

X = N, C

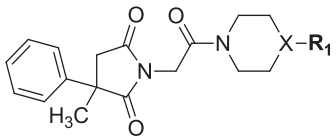
R^1= Benzyl, 2-pyrimidinyl, cyclohexyl

Scheme 1. Synthetic routes to intermediates **4**, **5** and final compounds **6**–**23**. Reagents and conditions: (a) 2-amineacetic acid, 190–200 °C, 1.5 h, (b) 4-phenylpiperazine derivatives, CDI, DMF, room temperature, 24 h, (c) 4-substituted piperazines, benzylpiperidine, (d) morpholine, CDI, DMF, room temperature, 24 h.

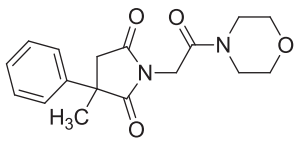
Table 1
The screening results after intraperitoneal administration in mice (**6**–**23**).



6-18



19-22



23

No	R	X	R^1	MES[a]		*sc*PTZ[b]		NT[c]		*clog P*[d]
				0.5 h	2 h	0.5 h	2 h	0.5 h	2 h	
6	H	–	–	300	–	100	300	–	–	2.10
7	2-F	–	–	100	–	100	300	300	300	2.41
8	4-F	–	–	100	100	–	–	300	300	2.41
9	2-Cl	–	–	–	–	–	100	–	–	2.98
10	3-Cl	–	–	300	300	–	–	–	–	2.98
11	4-Cl	–	–	–	–	300	300	–	–	2.98
12	2,3-Cl	–	–	–	–	300	–	–	–	3.63
13	3,4-Cl	–	–	–	300	–	–	–	–	3.63
14	3-CF_3	–	–	300	300	–	–	–	–	3.28
15	2-CH_3	–	–	–	–	–	–	–	–	2.60
16	3-CH_3	–	–	100	300	–	–	300	300	2.60
17	2-OCH_3	–	–	100	300	–	–	300	300	2.12
18	3-OCH_3	–	–	–	–	100	–	–	–	2.12
19	–	N		–	–	100	300	300	300	3.44
20	–	N		–	–	–	100	–	–	0.39
21	–	N		–	–	–	–	300[f]	–	3.54
22	–	C		–	300	100	–	–	–	3.89
23	–	–	–	300	300	300	300	–	–	1.16
ETX[e]				–	–	100	300	–	–	
PHT				30	30	–	–	100	100	
VPA[e]				300	–	300	–	–	–	

Doses of 30, 100, and 300 mg/kg were administered *i.p.* The data indicate the minimum dose whereby bioactivity was demonstrated. The animals were examined at 0.5 and 2.0 h. A dash indicates the absence of anticonvulsant activity and neurotoxicity at the maximum dose administered (300 mg/kg).
[a] Maximal electroshock test.
[b] Subcutaneous pentylenetetrazole test.
[c] Neurotoxicity screening – rotarod test.
[d] *clog P* values calculated using a log *P* module of ChemDraw Ultra program, version 7.0.1 (Cambridge Soft Corporation, Cambridge, MA, USA).
[e] AEDs: PHT – Phenytoin; ETX – Ethosuximide; VPA – Valproic acid, screening data Ref. [44].
[f] Death of animals.

– phenytoin (MES active), ethosuximide (*sc*PTZ active), and valproic acid (MES/*sc*PTZ active) are shown in Table 2.

The quantitative data revealed that **7**, **8**, **10**, and **17** displayed higher activity than valproic acid in the MES test. Furthermore **7** was also more potent in the PTZ seizures. The mentioned compounds caused a weaker motor impairment in the rotarod test that resulted in more favorable protection indexes (TD_{50}/ED_{50}) in comparison with mentioned drug. Distinctly better results were observed in the *sc*PTZ test. In this case, all compounds were more potent than both model PTZ-active AEDs ethosuximide and valproic acid. As shown in Table 2 the highest PTZ-activity revealed **6** that was 5.8-fold more effective in comparison with valproic acid and 3.4-fold than ethosuximide. Moreover, compound **6** showed clearly better protective index (PI) comparing with both ethosuximide and valproic acid. The potent PTZ-activity, low acute neurotoxicity and favorable PI value revealed also benzylpiperidine derivative **22**. All compounds tested displayed lower protection in the MES seizures than model MES-active AED – phenytoin.

During further biological characterization, in accordance with ASP dispositions, 4 compounds **8**, **14**, **17**, and **23** were selected for the evaluation of anticonvulsant activity in the 6-Hz test in mice (*i.p.*). It should be stressed that 6-Hz stimulation is known as a useful model of therapy-resistant limbic seizures. Moreover, the 6-Hz model is suggested to be capable for identifying anti-seizure agents with a novel spectrum of activity and unknown mechanism of anticonvulsant action. One example supporting this hypothesis is provided by levetiracetam, which has demonstrated efficacy in refractory human partial epilepsies. It was found to be inactive against classical MES and *sc*PTZ seizures even at high doses, whereas showed high efficacy in the 6-Hz psychomotor seizure model of partial epilepsy [24]. The results obtained are presented in Table 3.

Among derivatives tested the most potent was **23**, which protected mice at time points of 0.5 h and 2 h at a dose of 100 mg/kg. Compound **17** was also active at a dose of 100 mg/kg but only 0.5 h after *i.p.* administration. Other compounds (**8**, **14**) were less active and protected animals at dose of 300 mg/kg in both time intervals. The data obtained herein give an opportunity to find chemically related molecules with potent activity in the 6-Hz seizures. Moreover, compounds **8**, **14**, **17**, and **23** which revealed protection both in MES and 6-Hz tests may be recognized as prototype substances for wide-spectrum anticonvulsants effective in generalized tonic-

Table 2
Quantitative data following *i.p.* administration in mice for compounds **6**–**10**, **17**, **19**, and **22**.

No	TPE (h)[a]	ED_{50}[b] MES (mg/kg)	ED_{50}[b] *sc*PTZ (mg/kg)	TD_{50}[b] (mg/kg)	PI[c] (TD_{50}/ED_{50})
6	0.5	NT	40.87 (21.23–78.69)	>500	>12.23 (*sc*PTZ)
7	0.5	114.68 (99.99–131.52)	97.12 (88.09–107.08)	302.47 (207.59–440.72)	2.64 (MES) 3.11 (*sc*PTZ)
8	2.0	104.11 (65.41–165.72)	NT	248.10 (210.19–292.85)	2.37 (MES)
9	2.0	NT	120.06 (76.92–187.40)	>500	>4.16 (*sc*PTZ)
10	2.0	215.31 (164.88–281.18)	NT	>500	2.32 (MES)
17	0.5	97.51 (88.89–106.96)	NT	239.98 (209.56–274.81)	2.46 (MES)
19	0.5	NT	96.55 (65.30–142.75)	205.20 (150.63–279.53)	2.12 (*sc*PTZ)
22	0.5	NT	60.00 (46.65–77.19)	>500	>8.33 (*sc*PTZ)
PHT[d]	1.0	6.65 (4.42–8.16)	>500	59.9 (48.5–66.7)	5.56 (MES)
ETX[d]	0.25	>500	140.40 (115.81–170.21)	318.01 (295.80–341.89)	2.26 (*sc*PTZ)
VPA[d]	0.5	252.74 (220.10–290.22)	239.45 (209.18–274.10)	430.77 (407.92–454.90)	1.70 (MES) 1.80 (PTZ)

[a] Time to peak effect.
[b] Results are represented as mean ± SEM at 95% confidence limit (MES – maximal electroshock test; *sc*PTZ – subcutaneous pentylenetetrazole test; NT – acute neurotoxicity – rotarod test).
[c] Protective index (TD_{50}/ED_{50}).
[d] AEDs: PHT – Phenytoin; ETX – Ethosuximide; VPA – Valproic acid tested in the same conditions.

Table 3
Anticonvulsant evaluation, psychomotor seizure test (6-Hz) current 32 mA.

No	Intraperitoneal injection to mice[a]	
	0.5 h	2 h
8	300	300
14	300	300
17	100	–
23	100	100

[a] Dose of 30, 100, and 300 mg/kg was administrated *i.p.* The data indicate the minimum dose whereby activity was demonstrated.

clonic and partial convulsions with or without secondary generalization, as well as in therapy-resistant epilepsy.

Taking into consideration the prominent anticonvulsant activity of **7**, **8** and **17** in the MES test, their analgesic properties were investigated at doses equal to the ED_{50} values from the MES model. The results of the antinociceptive studies are summarized in Table 4 and depicted in Figs. 3–5.

A significant antinociceptive activity was observed for each of the test compounds (Table 4). In the first (neurogenic) phase of the test all active compounds reduced the duration of the licking response up to 85%. In this phase compound **17**, which was tested at the dose of 97.51 mg/kg had the highest antinociceptive efficacy of all derivatives (85%, $p < 0.0001$). Molecules **7** and **8**, tested at the doses of 114.68 mg/kg and 104.11 mg/kg, respectively also exerted analgesic activity. They reduced duration of licking response by about 73% ($p < 0.0001$) (**7**) and 60% ($p < 0.01$) (**8**). In the second (late) phase of the formalin test, a statistically significant antinociceptive effect was observed between 15 min and 20 min of the test for all compounds: **7** (98%, $p < 0.01$), **8** (95%, $p < 0.05$), and **17** (100%, $p < 0.001$). Additionally, compounds **8** and **17** significantly reduced the duration of the licking response between 20 and 25 min: **8** by 95% ($p < 0.05$) and **17** by 100% ($p < 0.01$) *vs.* control. Moreover, between 25 and 30 min after formalin injection, a statistically significant antinociceptive effect was observed for compounds **7** (81%, $p < 0.01$) and **17** (98%, $p < 0.001$), (see Figs. 3–5). In parallel to the antinociceptive studies the neurotoxicity for compounds **7**, **8**, and **17** was assessed in the chimney test (Table 5). All molecules given at a doses equal to the respective ED_{50} values obtained in MES test, as well as tested in the formalin model, did not affect motor coordination. All animals were able to perform the test within 60 s.

Summing up, the comparison of the anticonvulsants activity of respective analogs, namely N-Mannich bases (**1**–**3**, Fig. 1) and compounds **8**, **10** (Table 2), **13** (Table 1) revealed that exchange of the methylene linker with the acetamide moiety decreased anticonvulsant efficacy or made the compounds more neurotoxic in the

Table 4
Antinociceptive activity of the tested compounds in the formalin test.

No	Dose [mg/kg]	Duration of licking response [s] ± SEM (phase I)	Antinociceptive activity (%)	Duration of licking response [s] ± SEM (phase II)	Antinociceptive activity (%)
Vehicle	–	83.2 ± 9.4	–	149.0 ± 32.7	–
7	114.68	22.2 ± 4.4****	73.3	19.2 ± 18.7**	87.1
8	104.11	33.3 ± 7.2**	60.0	26.3 ± 14.6**	82.3
17	97.51	12.1 ± 4.1****	85.5	1.0 ± 0.4***	99.3

Results are shown as mean time of licking response in phase I (0–5 min after intraplantar injection of 5% formalin) and in phase II (15–30 min after formalin injection). For each compound the dose tested in the formalin test was its median effective dose (ED_{50}) determined in MES test. Each value represents the mean ± SEM obtained from 8 mice. The compounds and the vehicle were administered *i.p.* 30 min (cmpds **7**, **17**) or 2 h (cmpd **8**) before the assay. Significant difference compared to the vehicle-treated group – Student's *t*-test: $^{**}p < 0.01$, $^{***}p < 0.001$, $p < 0.0001$.

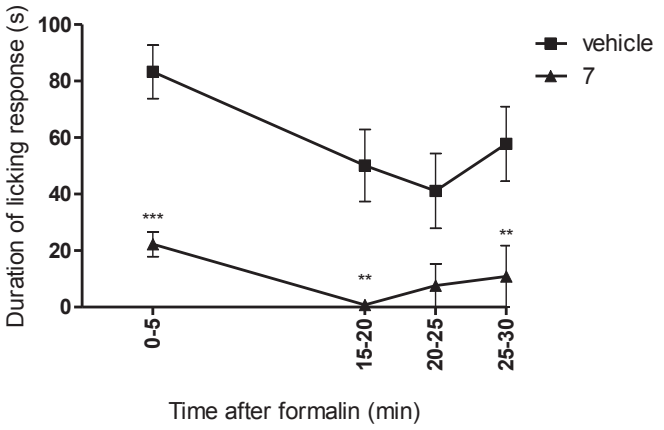


Fig. 3. Time course of the antinociceptive activity of compound **7** in the formalin test. Statistical analysis of the result was conducted using two-way ANOVA, followed by Bonferroni's multiple comparison. Results compared to vehicle-treated mice at the same time points: $^{***}p < 0.001$, $^{**}p < 0.01$. Drug effect: F[1,42] = 40.10; $p < 0.001$; time effect: F[3,42] = 9.12; $p < 0.01$; interaction: F[3,42] = 1.68; NS.

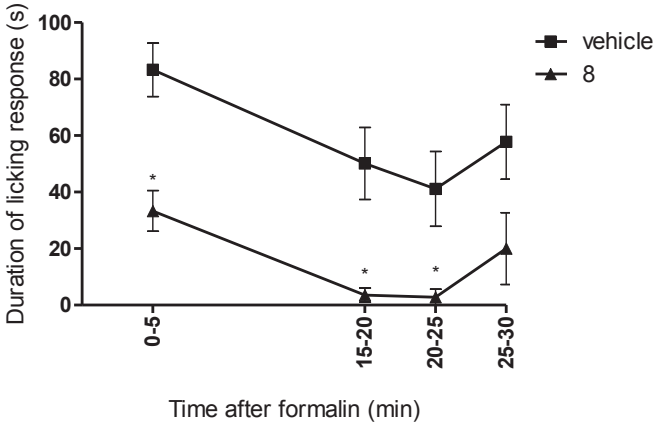


Fig. 4. Time course of the antinociceptive activity of compound **8** in the formalin test. Statistical analysis of the result was conducted using two-way ANOVA, followed by Bonferroni's multiple comparison. Results compared to vehicle-treated mice at the same time points: $^{*}p < 0.05$. Drug effect: F[1,42] = 32.88; $p < 0.001$; time effect: F[3,42] = 13.87; $p < 0.01$; interaction: F[3,42] = 0.49; NS.

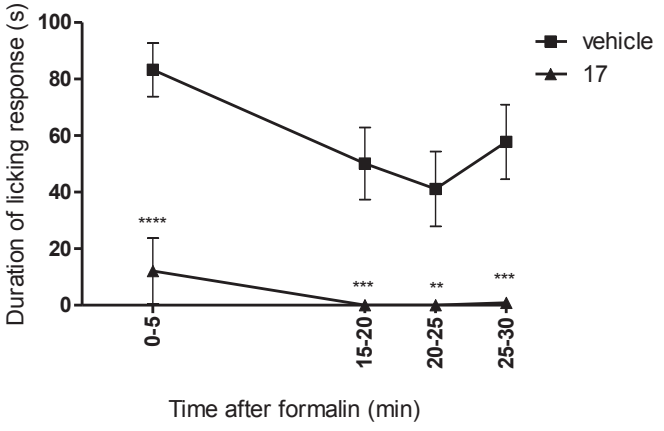


Fig. 5. Time course of the antinociceptive activity of compound **17** in the formalin test. Statistical analysis of the result was conducted using two-way ANOVA, followed by Bonferroni's multiple comparison. Results compared to vehicle-treated mice at the same time points: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$. Drug effect: F[1,42] = 52.65; $p < 0.0001$; time effect: F[3,42] = 7.45; $p < 0.01$; interaction: F[3,42] = 2.10; NS.

Table 5
Effect of tested compounds on motor performance in the chimney test.

No	Dose [mg/kg]	Time [s] ± SEM
Vehicle	–	6.29 ± 2.48
7	114.68	6.50 ± 1.73
8	104.11	6.14 ± 1.14
17	97.51	8.43 ± 1.99

Results are shown as mean time which mice spent in chimney. Statistical analysis: Student's *t*-test: NS. For each compound the dose tested was its median effective dose (ED_{50}) determined in MES test. Each value represents the mean ± SEM obtained from 8 mice. The compounds and the vehicle were administered *i.p.* 30 min (cmpds **7**, **17**) or 2 h (cmpd **8**) before the assay.

rotarod test. Nevertheless, the proposed structural modification yielded molecules with interesting dual anticonvulsant and antinociceptive properties. Thus this chemical space is worth of detailed exploring in future.

2.3. In vitro radioligand binding studies

Intensive studies into the physiological and biochemical events taking place during epileptic seizures have provided insight into the molecular mechanisms by which these might be controlled. The fundamental role in establishing and regulating excitability of CNS neurons as well as suppression of seizures is ascribed to voltage-gated sodium channels (VGSCs) and voltage-dependent calcium channels (VDCC) [25]. Thus the brain ion channels are the molecular targets of a number of chemically diverse antiepileptic drugs from which the most important are phenytoin, lamotrigine, carbamazepine and oxcarbazepine that are known to be a sodium channel blockers [26–28]. It should be stressed here that such mode of action is characteristic for compounds active in the MES test. Taking into consideration the activity of compounds synthesized in the electrically induced convulsions, for 3 compounds **6**, **7**, and **17** with various activity in the preclinical seizure model (see Table 2), their binding to Na^+ and Ca^{2+} (L-type) channels were studied *in vitro* (Table 6).

As it is indicated in Table 6 the most effective binder to sodium and calcium channels was **7**, which showed activity in both MES and *sc*PTZ seizures. However, it should be stressed that this molecule revealed weaker protection comparing to **6** (PTZ test) and **17** (MES test) which showed only moderate interaction with mentioned ion channels. Thus, the results obtained may suggest other mechanism of action responsible for anticonvulsant activity beyond the interaction with Na^+ and Ca^{2+} (L-type) channels.

2.4. Mutagenic and antimutagenic activity

In the present study, 3 structurally and biologically diversified compounds were chosen for the estimation of their anti/mutagenic properties: **13** – 3,4-dichloro-derivative active in the MES test, **17** – 2-OCH_3 analog, the most active in the MES seizures, and **22** – benzylpiperidine-derivative, active in both tests (MES, *sc*PTZ). Firstly, the selected molecules were evaluated *in vitro* using the novel *V. harveyi* assay. Subsequently, anti/mutagenic effects of tested compounds obtained in *V. harveyi* were confirmed in the Ames TA100 assay system. The results of the *V. harveyi* and the Ames mutagenicity assays are presented in Table 7, whereas antimutagenic effects are summarized in Table 8.

The current *in vitro* mutagenicity studies demonstrated that in a concentration of 40 ng/ml the compounds tested exhibited no mutagenic activity in *V. harveyi* BB7 and BB7XM strains. Similarly, as shown in Table 7 the compounds tested were non mutagenic in the Ames TA100 mutagenicity assay. It was further found that in

Table 6
In vitro binding assays (concentration 100 μM).

No	% Inhibition of control specific binding[a]	
	Voltage-sensitive Na^+ channel (site 2)[b]	Voltage-sensitive Ca^{2+} channel (antagonist site)[c]
6	34.2	43.2
7	56.1	83.3
17	44.6	36.4

[a] Results showing an inhibition higher than 50% are considered to represent significant effects of the test compounds; results showing an inhibition between 25% and 50% are indicative of moderate effect; results showing an inhibition lower than 25% are not considered significant and mostly attributable to variability of the signal around the control level.
[b] Compounds were evaluated in synaptoneurosomal preparations from rat cerebral cortex as inhibitors of the specific binding of [^{3}H]BTX to the voltage-sensitive sodium channel.
[c] Compounds were evaluated in synaptoneurosomal preparations from rat cerebral cortex as inhibitors of the specific binding of [^{3}H]nitrendipine to the voltage-sensitive Ca^{2+} channel.

Table 7
Mutagenic activity of compounds **13**, **17** and **22** in the Ames and the *V. harveyi* tests.

No	Ames test		*Vibrio harveyi* test			
	TA100[a]		BB7[a]		BB7XM[a]	
	Mean ± SD	M.I.[c]	Mean ± SD	M.I.[c]	Mean ± SD	M.I.[c]
DMSO[b]	16 ± 5	–	16 ± 3	–	12 ± 4	–
NQNO[b]	33 ± 4	2.0	32 ± 4	2.0	24 ± 3	2.1
13	6 ± 2	0.3	20 ± 3	1.2	8 ± 3	0.7
17	9 ± 3	0.6	19 ± 3	1.2	10 ± 6	0.9
22	8 ± 4	0.5	24 ± 3	1.5	19 ± 4	1.6

[a] Number of revertants.
[b] NQNO (nitroquinoline-*N*-oxide, 40 ng/ml) – positive control; DMSO, H_2O – negative control.
[c] M.I. (mutagenic index): number of induced revertants/number of spontaneous revertants (positive assay when M.I. ≥ 2).

Salmonella typhimurium TA100 mutagenic index values were lower (ranging from 0.3 to 0.6) than in *V. harveyi* strains (M.I. in the range 0.7–1.6) for the tested compounds. This phenomenon could be explained in terms of a higher sensitivity of the *V. harveyi* mutagenicity assay in comparison to the Ames test [29].

The results summarized in Table 8 show that two of the investigated compounds **13** and **17** were strong inhibitors of the mutagenicity induced by a direct acting agent NQNO in all bacterial strains used in the experiment. In *S. typhimurium* TA100 strain the strongest antimutagenic activity (inhibition rate of 42%) was observed for compound **17**. The inhibition percentage for compound **13**, was 36%, which indicates a moderate antimutagenic effect. Only compound **22** exhibited weak antimutagenic effect with inhibition rate of 5%. In the *V. harveyi* antimutagenicity assay compounds **17** and **13** demonstrated strong antimutagenic activity against NQNO induced mutagenicity. The inhibition percentages for compound **17** were 49% (BB7) and 69% (BB7XM), and for compound **13** 47% (BB7) and 63% (BB7XM), respectively. Compound **22** weakly suppressed the mutagenicity of NQNO in all *V. harveyi* strains tested. The inhibition rates for this compound were between 22% (BB7) and 26% (BB7XM). Summing up, all of the tested 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetic acid derivatives displayed a protective effect against the mutagenicity induced by a direct acting mutagen NQNO in the *V. harveyi* and the Ames assays. The most beneficial antimutagenic properties showed compounds **13** and **17** which exhibited strong antimutagenic properties in all tested *V. harveyi* strains. Moreover, antimutagenic effects of **13** and **17** obtained in *V. harveyi* were closely related to the Ames test. It can be concluded that antimutagenicity data are comparable between *V. harveyi* and *Salmonella* assays.

3. Conclusion

In the current research, the library of 18 new amide derivatives of 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetic acid was synthesized and evaluated for anticonvulsant activity in the maximal electroshock (MES) and pentylenetetrazole (*sc*PTZ) seizure tests in mice. Additionally for chosen, active compounds the antinociceptive, mutagenic and antimutagenic activity were assessed. The obtained results showed that the majority of these compounds demonstrated anticonvulsant activity in the MES and/or *sc*PTZ test. It seems to be particularly interesting that compounds **6** and **22** were distinctly more potent in the *sc*PTZ test than model PTZ-active AED ethosuximide. Furthermore, **7** showed higher protection in the MES and PTZ seizures as well as lower neurotoxicity comparing to MES/PTZ active valproic acid. Moreover, for the three most active compounds their probable mechanism of action *in vitro* were established. Several compounds were also active in the psychomotor (6-Hz) seizures test and moreover showed antinociceptive activity in the formalin test. The results of mutagenicity screening suggest that this series of compounds can

Table 8
Antimutagenicity of compounds **13**, **17** and **22** in the Ames and the *V. harveyi* tests.

No	Ames test		*Vibrio harveyi* test			
	TA100[a]		BB7[a]		BB7XM[a]	
	Mean ± SD	Inhib. (%)[c]	Mean ± SD	Inhib. (%)[c]	Mean ± SD	Inhib. (%)[c]
DMSO[b]	6 ± 2	–	17 ± 2	–	15 ± 3	–
NQNO[b]	22 ± 4	–	38 ± 5	–	24 ± 4	–
13	14 ± 4	(36)	20 ± 4	(47)	9 ± 3	(63)
17	13 ± 2	(42)	21 ± 3	(45)	12 ± 3	(51)
22	21 ± 3	(5)	30 ± 4	(22)	18 ± 3	(26)

[a] Number of revertants.
[b] NQNO (nitroquinoline-*N*-oxide, 40 ng/ml – positive control; DMSO – negative control.
[c] The values in parenthesis are the inhibition rates (%) of mutagenicity: 40% or more inhibition – strong antigenotoxicity; 25–40% inhibition – moderate antigenotoxicity; 25% or less inhibition – no antigenotoxicity.

be considered as genotoxically safe. Notably, several compounds tested demonstrated significant antimutagenic properties.

4. Experimental section

4.1. General

All chemicals and solvents were purchased from Sigma–Aldrich (St. Louis, USA) and were used without further purification. Melting points (m.p.) were determined in open capillaries on a Büchi 353 melting point apparatus (Büchi Labortechnik, Flawil, Switzerland) and are uncorrected. The purity of the compounds was confirmed by the thin-layer chromatography (TLC) performed on Merck silica gel 60 F_{254} aluminum sheets (Merck; Darmstadt, Germany), using developing systems consisted of chloroform:isopropanol:ammonia (9:11:2, *v*/*v*) (S_1), chloroform:acetone (9:1, *v*/*v*) (S_2), ethyl acetate:hexane (8:5, *v*/*v*) (S_3). Spots were detected by their absorption under UV light ($\lambda = 254$ nm) and by visualization with 0.05 mol I_2 in 10% HCl. HPLC analyses were run on a HPLC Waters® 2695 Separation Module equipped with photodiode array detector (Waters® 2698). A Chromolith RP-18 SpeedROD column (4.6 × 50 mm) was used. Conditions applied were as follow: eluent A (water/0.1% TFA), eluent B (acetonitrile/0.1% TFA); flow rate of 5 ml/min, gradient of 0–100% B over 3 min were used, injection volume was 10 μL. Standard solutions (1 mg/ml) of each compound were prepared in analytical grade acetonitrile and analyzed at wavelengths 214 nm and 254 nm. Retention times (t_R) are given in minutes. Elemental analysis for C, H, and N were carried out by a micro method using the elemental Vario El III Elemental analyser (Hanau, Germany). The results of elemental analyses were within ±0.4% of the theoretical values. ^{1}H NMR and ^{13}C NMR spectra were obtained in a Varian Mercury spectrometer (Varian Inc., Palo Alto, CA, USA), in $CDCl_3$ or DMSO, operating at 300 MHz or 75 MHz, respectively. Chemical shifts are reported in δ values (ppm) relative to TMS $\delta = 0$ (^{1}H), as internal standard. The *J* values are expressed in Hertz (Hz). Signal multiplicities are represented by the following abbreviations: s (singlet), brs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet). The mass spectra for compounds **6**–**23** were obtained on Waters ACQUITY™ TQD system with the TQ Detector (Waters, Milford, USA). The ACQUITY UPLC BEH C18, 1.7 μm, 2.1 × 50 mm column was used (Waters, Milford, USA).

4.2. Chemical synthesis

4.2.1. General procedure for the preparation of the 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetic acid (**5**)

To 3-methyl-3-phenyl-succinic acid (0.04 mol) was dissolved in 25 ml of water and 2-aminoacetic acid (0.04 mol) was gradually added. The mixture was heated in a term-regulated sand bath (ST 72 Roth, Karlsruhe, Germany) with simultaneous distillation of water. After complete removal of water, the temperature of the reaction mixture rose up to 190 °C and was maintained for 1.5 h. The crude product was recrystallized from isopropanol.

4.2.1.1. 3-Methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetic acid (5). White solid. Yield: 70%; m.p. 155–157 °C; TLC: $R_f = 0.17$ (S_1); $R_f = 0.52$ (S_3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.77 (s, 3H, CH_3), 3.05 (d, 2H, imide, $J = 18.4$ Hz), 4.38 (s, 2H, CH_2), 7.26–7.35 (m, 2H, ArH), 7.36–7.40 (m, 3H, ArH), 11.08 (s, rough, 1H, OH). Anal. calcd for $C_{13}H_{13}N_1O_4$ (247.25): C, 63.22; H, 5.31; N, 5.67. Found: C, 63.58; H, 5.47; N, 5.73.

4.2.2. General procedure for the synthesis of compounds **6**–**23**

The intermediate **5** (0.01 mol) was dissolved in 5 ml of dry DMF and *N*,*N*-carbonyldiimidazole (0.01 mol) was added. Afterward, the appropriate substituted piperazine, piperidine or morpholine (0.01 mol) in dry DMF was added. The reaction mixture was stirred for 24 h at room temperature and then poured out into ice. The precipitated products were filtrated and purified by recrystallization from 2-propanol. Compound **8** was obtained as light oil and was converted into hydrochloride salts.

*4.2.2.1. 3-Methyl-1-[(2-oxo-2-(4-phenylpiperazin-1-yl)-ethyl]-3-phenyl-pyrrolidine-2,5-dione (**6**).* White solid. Yield: 70%; m.p. 175–177 °C; TLC: $R_f = 0.61$ (S_1); $R_f = 0.44$ (S_2); HPLC (t_R 1.435 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.96 (d, 1H, imide, $J = 18.2$ Hz), 3.17 (d, 1H, imide, $J = 18.4$ Hz), 3.25 (brs, 2H, piperazine), 3.33 (brs, 2H, piperazine), 3.78 (brs, 2H, piperazine), 3.89 (brs, 2H, piperazine), 4.42 (s, 2H, CH_2), 7.04–7.11 (m, 3H, ArH), 7.26–7.46 (m, 7H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.3, 39.4, 39.7, 41.7, 45.7, 48.1, 118.0, 125.8, 127.5, 128.9, 129.0, 129.5, 141.7, 163.4, 173.4, 175.3, 180.8. ESI-MS: 392.4 ($C_{23}H_{25}N_3O_3$ $[M + H]^+$). Anal. calcd for $C_{23}H_{25}N_3O_3$ (391.47): C, 70.65; H, 6.44; N, 10.75. Found: C, 70.68; H, 6.47; N, 10.73.

*4.2.2.2. 1-{2-[4-(2-Fluorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**7**).* White solid. Yield: 65%; m.p. 116–118 °C; TLC: $R_f = 0.94$ (S_1); $R_f = 0.70$ (S_2); HPLC (t_R 1.705 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.96 (d, 1H, imide, $J = 18.2$ Hz), 3.09 (t, 2H, piperazine, $J = 5.0$ Hz), 3.14–3.20 (m, 3H, 1H, imide, 2H piperazine), 3.68 (t, 2H, piperazine, $J = 5.0$ Hz), 3.80 (t, 2H, piperazine, $J = 5.0$ Hz), 4.42 (s, 2H, CH_2), 6.91–7.11 (m, 4H, ArH), 7.26–7.49 (m, 5H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.3, 39.8, 42.4, 44.9, 45.7, 48.1, 50.5, 116.4, 119.2, 123.3, 124.5, 124.6, 125.8, 127.4, 128.9, 139.3, 139.4, 141.7, 154.1, 157.3, 163.2, 175.3, 180.8. ESI-MS: 410.3 ($C_{23}H_{24}N_3O_3F_1$ $[M + H]^+$). Anal. calcd for $C_{23}H_{24}N_3O_3F_1$ (409.45): C, 67.54; H, 5.91; N, 10.27. Found: C, 67.50; H, 5.93; N, 10.24.

*4.2.2.3. Monohydrochloride 1-{2-[4-(4-fluorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**8**).* White solid. Yield: 72%; m.p 204-207 °C; TLC: $R_f = 0.75$ (S_2); $R_f = 0.46$ (S_3); HPLC (t_R 1.541 min); ^{1}H NMR (300 MHz, DMSO): δ 1.65 (s, 3H, CH_3), 3.00 (s, 2H, imide), 3.07 (t, 2H, piperazine, $J = 5.0$ Hz), 3.15 (brs, 2H, piperazine), 3.60 (brs, 2H, piperazine), 3.68 (brs, 2H, piperazine), 4.42 (s, 2H, CH_2), 7.00–7.24 (m, 4H, ArH), 7.27–7.46 (m, 5H, ArH). ESI-MS: 410.3 ($C_{23}H_{25}N_3O_3F_1Cl_1$ $[M + H]^+$). Anal. calcd for $C_{23}H_{25}N_3O_3F_1Cl_1$ (445.91): C, 62.08; H, 5.66; N, 9.44. Found: C, 62.09; H, 5.69; N: 9.46.

*4.2.2.4. 1-{2-[4-(2-Chlorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**9**).* White solid. Yield: 86%; m.p. 129–131 °C; TLC: $R_f = 0.75$ (S_2); $R_f = 0.56$ (S_3); HPLC (t_R 1.841 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.96 (d, 1H, imide, $J = 18.2$ Hz), 3.05 (t, 2H, piperazine, $J = 5.0$ Hz), 3.12 (t, 2H, piperazine, $J = 5.3$ Hz), 3.17 (d, 1H, imide, $J = 18.2$ Hz), 3.67 (t, 2H, piperazine, $J = 5.0$ Hz), 3.81 (t, 2H, piperazine, $J = 5.0$ Hz), 4.42 (s, 2H, CH_2), 6.99–7.05 (m, 2H, ArH), 7.21–7.47 (m, 7H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.3, 39.8, 42.6, 45.7, 48.1, 50.8, 51.2, 120.8, 124.4, 125.8, 127.4, 128.9, 128.9, 130.7, 141.8, 148.4, 163.3, 175.3, 180.9. ESI-MS: 426.3 ($C_{23}H_{24}N_3O_3Cl_1$ $[M + H]^+$). Anal. calcd for $C_{23}H_{24}N_3O_3Cl_1$ (425.91): C, 64.85; H, 5.68; N, 9.86. Found: C, 64.86; H, 5.67; N, 9.89.

*4.2.2.5. 1-{2-[4-(3-Chlorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**10**).* White solid. Yield: 68%; m.p. 80–82 °C; TLC: $R_f = 0.81$ (S_1); $R_f = 0.64$ (S_2); $R_f = 0.47$ (S_3); HPLC (t_R 1.839 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.95 (d, 1H, imide, $J = 18.2$ Hz), 3.17 (d, 1H, imide, $J = 18.4$ Hz),

3.22 (brs, 2H, piperazine), 3.27 (t, 2H, piperazine, $J = 5.1$ Hz), 3.65 (t, 2H, piperazine, $J = 5.0$ Hz), 3.78 (t, 2H, piperazine, $J = 5.0$ Hz), 4.41 (s, 2H, CH_2), 6.78–6.90 (m, 3H, ArH), 7.17–7.46 (m, 6H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.3, 39.7, 45.7, 48.1, 48.7, 114.5, 116.5, 120.3, 125.8, 127.5, 128.9, 130.2, 135.0, 141.7, 151.7, 163.3, 175.3, 180.9. ESI-MS: 426.3 ($C_{23}H_{24}N_3O_2Cl_1$ [M + H]$^+$). Anal. calcd for $C_{23}H_{24}N_3O_3Cl_1$ (425.91): C, 64.85; H, 5.68; N, 9.89. Found: C, 64.80; H, 5.71; N: 9.92.

*4.2.2.6. 1-{2-[4-(4-Chlorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**11**).* White solid. Yield: 61%; m.p. 123–125 °C; TLC: $R_f = 0.73$ (S_1); $R_f = 0.60$ (S_2); $R_f = 0.48$ (S_3); HPLC (t_R 1.790 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.95 (d, 1H, imide, $J = 18.4$ Hz), 3.15 (brs, 2H, piperazine), 3.16 (d, 1H, imide, $J = 18.4$ Hz), 3.22 (t, 2H, piperazine, $J = 5$. Hz), 3.65 (t, 2H, piperazine, $J = 5.1$ Hz), 3.78 (t, 2H, piperazine, $J = 5.1$ Hz), 4.41 (s, 2H, CH_2), 6.82–6.88 (m, 2H, ArH), 7.21–7.31 (m, 3H, ArH), 7.34–7.46 (m, 4H, ArH). ESI-MS: 426.3 ($C_{22}H_{24}N_3O_2Cl_1$ [M + H]$^+$). Anal. calcd for $C_{23}H_{24}N_3O_3Cl_1$ (425.91): C, 64.85; H, 5.68; N, 9.89. Found: C, 64.84; H, 5.67; N: 9.85.

*4.2.2.7. 1-{2-[4-(2,3-Dichlorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**12**).* White solid. Yield: 85%; m.p. 119–121 °C; TLC: $R_f = 0.72$ (S_2); $R_f = 0.52$ (S_3). HPLC (t_R 1.97 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 3.04 (t, 2H piperazine, $J = 5.0$ Hz), 2.90 (d, 1H, imide $J = 14.1$ Hz), 3.10 (t, 2H, piperazine, $J = 4.8$ Hz), 3.17 (d, 1H, imide, $J = 18.4$ Hz), 3.67 (t, 2H, piperazine, $J = 4.8$ Hz), 3.81 (t, 2H, piperazine, $J = 4.7$ Hz), 4.42 (s, 2H, CH_2), 6.92–6.95 (d, 1H, ArH, $J = 2.0$ Hz), 7.14–7.47 (m, 7H, ArH). ESI-MS: 460.3 ($C_{23}H_{23}N_3O_3Cl_2$ [M + H]$^+$). Anal. calcd for $C_{23}H_{23}N_3O_3Cl_2$ (460.35): C, 60.01; H, 5.04; N, 9.13. Found: C, 60.03; H, 5.08; N, 9.15.

*4.2.2.8. 1-{2-[4-(3,4-Dichlorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**13**).* White solid. Yield: 80%; m.p. 74–76 °C; TLC: $R_f = 0.68$ (S_2); $R_f = 0.43$ (S_3); HPLC (t_R 1.971 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.80 (s, 3H, CH_3), 2.95 (d, 1H, imide, $J = 18.4$ Hz), 3.16 (d, 1H, imide, $J = 18.4$ Hz), 3.17 (brs, 2H, piperazine), 3.24 (t, 2H, piperazine, $J = 5.0$ Hz), 3.65 (t, 2H, piperazine, $J = 5.0$ Hz), 3.77 (t, 2H, piperazine, $J = 5.1$ Hz), 4.41 (s, 2H, CH_2), 6.73–6.77 (dd, 1H, ArH, $J = 2.8$ Hz), 6.97 (d, 1H, ArH, $J = 2.8$ Hz), 7.26–7.46 (m, 6H, ArH). ESI-MS: 460.3 ($C_{23}H_{23}N_3O_3Cl_2$ [M + H]$^+$). Anal. calcd for $C_{23}H_{23}N_3O_3Cl_2$ (460.35): C, 60.01; H, 5.04; N, 9.13. Found: C, 60.03; H, 5.08; N, 9.15.

*4.2.2.9. 1-{2-[4-(3-Trifluoromethylphenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**14**).* White solid. Yield: 86%; m.p. 92–94 °C; TLC: $R_f = 0.63$ (S_2); $R_f = 0.41$ (S_3); HPLC (t_R 1.916 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.96 (d, 1H, imide, $J = 18.4$ Hz), 3.17 (d, 1H, imide, $J = 18.2$ Hz), 3.25 (t, 2H, piperazine, $J = 5.1$ Hz), 3.31 (t, 2H, piperazine, $J = 5.0$ Hz), 3.67 (t, 2H, piperazine, $J = 5.0$ Hz), 3.80 (t, 2H, piperazine, $J = 5.0$ Hz), 4.42 (s, 2H, CH_2), 7.06–7.16 (m, 3H, ArH), 7.26–7.31 (m, 1H, ArH), 7.35–7.46 (m, 5H, ArH). ESI-MS: 460.3 ($C_{24}H_{24}N_3O_3F_3$ [M + H]$^+$). Anal. calcd for $C_{24}H_{24}N_3O_3F_3$ (459.46): C, 62.74; H, 5.26; N, 9.15. Found: C, 62.70; H, 5.31; N, 9.13.

*4.2.2.10. 1-{2-[4-(2-Methylphenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**15**).* White solid. Yield: 50%; m.p. 200–202 °C; TLC: $R_f = 0.66$ (S_2); $R_f = 0.67$ (S_3); HPLC (t_R 1.764 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.91 (brs, 2H, piperazine), 2.96 (d, 1H imide, $J = 18.2$ Hz), 2.97 (brs, 2H, piperazine), 3.17 (d, 1H, imide, $J = 18.2$ Hz), 3.64 (t, 2H, piperazine, $J = 4.8$ Hz), 3.77 (t, 2H, piperazine, $J = 4.8$ Hz), 4.42 (s, 2H, CH_2), 6.99–7.05 (m, 2H, ArH), 7.19 (t, 2H, ArH, $J = 7.8$ Hz), 7.26–7.29 (m, 2H, ArH), 7.31 (t, 1H, ArH, $J = 1.28$ Hz), 7.36–7.47 (m, 2H, ArH). ESI-MS: 406.3 ($C_{24}H_{27}N_3O_3$ [M + H]$^+$). Anal. calcd for $C_{24}H_{27}N_3O_3$ (405.50): C, 71.09; H, 6.71; N, 10.36. Found: C, 71.14; H, 6.75; N, 10.38.

*4.2.2.11. 1-{2-[4-(3-Methylphenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**16**).* White solid. Yield: 45%; m.p. 54–56 °C; TLC: $R_f = 0.61$ (S_1); $R_f = 0.52$ (S_3); HPLC (t_R 1.513 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.95 (d, 1H, imide, $J = 18.5$ Hz), 3.13–3.19 (m, 3H, 1H imide, 2H piperazine), 3.24 (t, 2H, piperazine, $J = 5.0$ Hz), 3.64 (t, 2H, piperazine, $J = 4.8$ Hz), 3.77 (t, 2H, piperazine, $J = 5.1$ Hz),4.42 (s, 2H, CH_2), 6.73 (d, 3H, ArH, $J = 1.5$ Hz), 7.18 (t, 1H, ArH, $J = 7.9$ Hz), 7.26–7.31 (m 2H, ArH), 7.35–7.47 (m, 3H, ArH). ESI-MS: 406.3 ($C_{24}H_{27}N_3O_3$ [M + H]$^+$). Anal. calcd for $C_{24}H_{27}N_3O_3$ (405.50): C, 71.09; H, 6.71; N, 10.36. Found: C, 71.10; H, 6.76; N, 10.40.

*4.2.2.12. 1-{2-[4-(2-Methoxyphenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**17**).* White solid. Yield: 62%; m.p. 110–112 °C; TLC: $R_f = 0.58$ (S_2), $R_f = 0.28$ (S_3); HPLC (t_R 1.326 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.96 (d, 1H, imide, $J = 18.4$ Hz), 3.06 (t, 2H, piperazine, $J = 5.0$ Hz), 3.15–3.20 (m, 3H, 2H piperazine, 1H imide), 3.67 (t, 2H, piperazine, $J = 4.8$ Hz), 3.81 (t, 2H, piperazine, $J = 4.8$ Hz), 3.89 (s, 3H, OCH_3), 4.42 (s, 2H CH_2), 6.89–6.96 (m, 3H, ArH), 7.02–7.08 (m, 1H, ArH), 7.27–7.47 (m, 5H, ArH).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.3, 39.8, 42.5, 45.0, 45.7, 48.15, 50.3, 50.6, 55.4, 111.3, 118.5, 121.0, 123.7, 125.8, 127.4, 128.9, 140.4, 141.8, 152.2, 163.1, 175.3, 180.9. ESI-MS: 422.4 ($C_{24}H_{27}N_3O_4$ [M + H]$^+$). Anal. calcd for $C_{24}H_{27}N_3O_4$ (421.49): C, 68.39; H, 6.46; N, 9.97. Found: C, 68.41; H, 6.50; N: 9.92.

*4.2.2.13. 1-{2-[4-(3-Methoxyphenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**18**).* White solid. Yield: 46%; m.p. 65–67 °C; TLC: $R_f = 0.40$ (S_2); $R_f = 0.43$ (S_3); HPLC (t_R 1.546 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.95 (d, 1H, imide, $J = 18.2$ Hz), 3.13–3.21 (m, 3H, 2H piperazine, 1H imide), 3.25 (t, 2H, piperazine, $J = 5.1$ Hz), 3.64 (t, 2H, piperazine, $J = 5.1$ Hz), 3.76 (d, 2H, piperazine, $J = 5.1$ Hz), 3.80 (s, 3H, OCH_3), 4.41 (s, 2H, CH_2), 6.46–6.57 (m, 3H, ArH), 7.17–7.23 (m, 1H, ArH), 7.26–7.37 (m, 1H, ArH) 7.38–7.46 (m, 4H, ArH). ESI-MS: 422.4 ($C_{24}H_{27}N_3O_4$ [M + H]$^+$). Anal. calcd for $C_{24}H_{27}N_3O_4$ (421.49): C, 68.39; H, 6.46; N, 9.97. Found: C, 68.43; H, 6.49; N, 9.96.

*4.2.2.14. 1-[2-(4-Benzyl-piperazin-1-yl)-2-oxo-ethyl]-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**19**).* White solid. Yield: 60%; m.p. 122–125 °C; TLC: $R_f = 0.93$ (S_1); $R_f = 0.26$ (S_3); HPLC (t_R 1.204 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.79 (s, 3H, CH_3), 2.46 (t, 2H, piperazine, $J = 5.1$ Hz), 2.50 (t, 2H, piperazine, $J = 5.1$ Hz), 2.94 (d, 1H, imide, $J = 18.1$ Hz), 3.14 (d, 1H, imide, $J = 18.2$ Hz), 3.48 (t, 2H, piperazine, $J = 5.0$ Hz), 3.54 (s, 2H, CH_2, benzyl), 3.63 (t, 2H, piperazine, $J = 5.0$ Hz), 4.35 (s, 2H, CH_2), 7.26–7.45 (m, 10H, ArH). ESI-MS: 406.3 ($C_{24}H_{27}N_3O_3$ [M + H]$^+$). Anal. calcd for $C_{24}H_{27}N_3O_3$ (405.50): C, 71.09; H, 6.71; N, 10.36. Found: C, 71.11; H, 6.75; N, 10.38.

*4.2.2.15. 3-Methyl-1-[2-oxo-2-(4-pyrimidin-2-yl-piperazin-1-yl)-ethyl-3-phenyl-pyrrolidine-2,5-dione (**20**).* White solid. Yield: 60%; m.p. 118–120 °C; TLC: $R_f = 0.90$ (S_1); $R_f = 0.26$ (S_3); HPLC (t_R 1.207 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.81 (s, 3H, CH_3), 2.95 (d, 1H, imide, $J = 18.2$ Hz), 3.16 (d, 1H, imide, $J = 18.4$ Hz), 3.56 (t, 2H, piperazine, $J = 5.1$ Hz), 3.70 (t, 2H, piperazine, $J = 5.1$ Hz), 3.87 (t, 2H, piperazine, $J = 5.1$ Hz), 4.95 (t, 2H, piperazine, $J = 5.1$ Hz), 4.42 (s, 2H, $-CH_2$), 6.56 (t, 1H, ArH, $J = 4.7$ Hz), 7.27–7.46 (m, 5H, ArH), 8.33 (d, 2H, ArH, $J = 4.8$ Hz). ESI-MS: 394.3 ($C_{21}H_{23}N_5O_3$ [M + H]$^+$).

Anal. calcd for $C_{21}H_{23}N_5O_3$ (393.45): C, 64.11; H, 5.89; N, 17.80. Found: C, 64.15; H, 5.91; N, 17.84.

*4.2.2.16. 1-[2-(4-Cyclohexyl-piperazin-1-yl)-2-oxo-ethyl]-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**21**).* White solid. Yield: 51%; m.p. 110–112 °C; TLC: $R_f = 0.88$ (S_1); $R_f = 0.15$ (S_2); HPLC (t_R 1.174 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.10–1.25 (m, 5H, cyclohexane), 1.55–1.65 (m, 5H, cyclohexane), 1.80 (s, 3H, CH_3), 2.30 (brs, 1H, cyclohexane), 2.55 (t, 2H, piperazine, J = 5.0 Hz), 2.62 (t, 2H, piperazine, J = 5.1 Hz), 2.94 (d, 1H, imide, J = 18.4 Hz), 3.15 (d, 1H, imide, J = 18.2 Hz), 3.47 (t, 2H, piperazine, J = 5.0 Hz), 3.61 (t, 2H, piperazine, J = 5.0 Hz), 4.36 (s, 2H, CH_2), 7.26–7.46 (m, 5H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.3, 25.7, 26.2, 28.8, 39.8, 42.8, 45.2, 45.7, 48.1, 48.3, 48.8, 63.5, 125.8, 127.4, 128.9, 141.8, 162.9, 175.3, 180.9. ESI-MS: 398.4 ($C_{23}H_{31}N_3O_3$ [M + H]$^+$). Anal. calcd for $C_{23}H_{31}N_3O_3$ (397.52): C, 69.49; H, 7.86; N, 10.57. Found: C, 69.47; H, 7.83: N, 10.59.

*4.2.2.17. 1-[2-(4-Benzyl-piperidin-1-yl)-2-oxo-ethyl]-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**22**).* White solid. Yield: 69%; m.p. 111–113 °C; TLC: $R_f = 0.68$ (S_2); $R_f = 0.69$ (S_3); HPLC (t_R 1.913 min); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.11–1.35 (m, 2H, piperidine), 1.55 (s, 3H, CH_3), 1.65–1.78 (m, 2H, piperidine) 1.80 (m, 4H, piperidine), 2.56 (dd, 2H, CH_2, J = 7.0, 3.9 Hz), 2.86–3.25 (m, 3H, 2H imide, 1H piperidine), 4.35 (s, 2H, CH_2), 7.10–7.18 (m, 2H, ArH), 7.19–7.26 (m, 2H, ArH), 7.27–7.36 (m, 3H, ArH),7.36–7.47 (m, 3H, ArH). ESI-MS: 405.3 ($C_{25}H_{28}N_2O_3$ [M + H]$^+$). Anal. calcd for $C_{25}H_{28}N_2O_3$ (404.50): C, 74.23; H, 6.98; N, 6.93. Found: C, 74.23; H, 6.98; N, 6.95.

*4.2.2.18. 3-Methyl-1-(2-morpholin-4-yl-2-oxo-ethyl)-3-phenyl-pyrrolidine-2,5-dione (**23**).* White solid. Yield: 50%; m.p. 103–105 °C; TLC: $R_f = 0.89$ (S_1); $R_f = 0.38$ (S_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.79 (s, 3H, CH_3), 2.94 (d, 1H, imide, J = 18.4 Hz), 3.15 (d, 1H, imide, J = 18.2 Hz), 3.49 (t, 2H, morpholine, J = 4.7 Hz), 3.61 (t, 2H, morpholine, J = 5.3 Hz), 3.67–3.75 (m, 4H, morpholine), 4.35 (s, 2H, CH_2), 7.27–7.33 (m, 1H, ArH), 7.40–7.45 (m, 4H, ArH). ESI-MS: 317.3 ($C_{17}H_{20}N_2O_4$ [M + H]$^+$). Anal. calcd for $C_{17}H_{20}N_2O_4$ (316.36): C, 64.54; H, 6.37; N, 8.86. Found C, 64.59; H, 6.38; N, 8.90.

4.3. Pharmacology

Male albino mice (CD-1 strain) weighing 16–26 g were used as experimental animals. The animals were housed in cages at room temperature of 22 ± 2 °C, under a light/dark (12/12) cycle and had free access to food and water. Ambient temperature of the room and humidity were kept consistent throughout all tests. For the experiments, the animals were randomly selected. Each group consisted of four animals (anticonvulsant screening) and each mouse was used only once. The compounds were suspended in 0.5% methylcellulose/water mixture. All the compounds were injected intraperitoneally into mice at the dose levels of 30, 100, and 300 mg/kg with anticonvulsant activity and neurotoxicity assessment at 0.5 and 2 h after administration.

Compounds **8**, **14**, **15**, **17**, **20**, and **23** were pharmacologically pre-evaluated within the Antiepileptic Drug Development (ADD) Program, realized in NINDS/NIH, Rockville, USA, using procedures described elsewhere [30,31]. Compounds **6**, **7**, **9**–**13**, **16**, **18**, **19**, **21**, and **22** were tested at the Department of Pharmacodynamics, Jagiellonian University Medical College. All the procedures were approved by the Local Ethics Committee of the Jagiellonian University in Kraków.

4.3.1. The maximal electroshock test (MES)

In the MES screen, electroconvulsions were produced by an electrical stimulus of 0.2 s in duration at a frequency of 50 Hz delivered via standard auricular electrodes by an electroshock generator (Hugo Sachs rodent shocker, Germany). The tonic hind-limb extension was taken as the end-point. Mice not displaying hind-limb tonic extension were considered protected from seizure.

4.3.2. Subcutaneous pentylenetetrazole seizure test (scPTZ)

*sc*PTZ-induced seizure was performed by subcutaneously injection of pentylenetetrazole (Sigma Aldrich, Poland) at a dose of 85 mg/kg. This produced clonic convulsions lasting for at least 5 s in 97% of animal tested. The absence of clonic convulsions in the observed time period of 30 min was interpreted as the compound's ability to protect against PTZ-induced seizure.

4.3.3. The neurological toxicity (NT)

Neurological toxicity induced by compound was detected in mice using standardized rotarod test. Untreated control mice when placed on the rod, can maintain their equilibrium for a prolonged time period. The acute motor impairment can be demonstrated by the inability of animal to maintain equilibrium on the rod revolting at 10 rpm for at least 1 min.

4.3.4. The 6-Hz model

This screen was carried out according to the protocol originally described by Brown et al. [32] and more recently by Barton et al. [24] and Kaminski et al. [33]. It is an alternative electroshock paradigm that uses low–frequency (6 Hz), long–duration (3 s) electrical stimulation. Corneal stimulation (0.2 ms-duration monopolar rectangular pulses at 6-Hz for 3 s) was delivered by a constant–current device. During the stimulation, mice were manually restrained and released into the observation cage immediately after the current application. The seizures manifest in "stunned" posture associated with rearing, forelimb, automatic movements and clonus, twitching of the vibrissae and Straub–tail. The duration of the seizure activity ranges from 60 to 120 s in untreated animals. At the end of the seizure, animals resume their normal exploratory behavior. The experimental end point is protection against the seizure. The animal is considered to be protected if it resumes its normal exploratory behavior within 10 s from the stimulation [33].

4.3.5. Quantification studies

The ED_{50} is defined as the dose of a drug protecting 50% of animals against the MES and PTZ test. Groups of six animals received various doses of the compound until at least three points were established in the range of 10–90% seizure protection or minimal motor impairment. Both ED_{50} and TD_{50} values with 95% confidence limits were calculated by probit analysis [34]. The PI (protective index) value was calculated as the ratio of TD_{50} to ED_{50} (TD_{50}/ED_{50} = PI). The results are shown in Table 2.

4.3.6. Antinociceptive activity – the formalin test

Antinociceptive activity in the formalin hind paw test was examined according to the method previously described [35]. In mice intraplantarly injection of diluted formalin produces a biphasic nocifensive behavioral response (i.e., licking or biting the injected hind paw). The acute nociceptive phase lasts for the first 5 min, whereas the second inflammatory phase occurs between 15 and 30 min after formalin injection. The mice were pretreated with the test compounds or the vehicle 30 min (**7**, **17**) or 2 h (**8**) before the experiment at the doses equal to the respective ED_{50} values obtained in MES test. Then, 20 μL of a 5% formalin solution was injected intraplantarly into the right hind paw of the mouse. Immediately after formalin injection, the animals were placed individually into transparent Plexiglas cages and were observed during the next 30 min. Time (in seconds) spent on licking or biting

Table 9
Experimental conditions of binding assays.

Assay	Source	Ligand	Concentration	K_d	Nonspecific binding	Incubation	Detection method
Na^+ channel (site 2)	Rat cerebral cortex	[^{3}H]Batrachotoxinin	10 nM	91 nM	Veratridine (300 μM)	60 min 37 °C	Scintillation counting
Ca^{2+} channel L, dihydro-pyridine site) (antagonist radioligand)	Rat cerebral cortex	[^{3}H]Nitrendipine	0.2 nM	0.4 nM	Nifedipine (1 μM)	120 min Room temp.	Scintillation counting

Table 10
Bacterial strains used in the present study with their genotypes.

Strain	Genotype and/or description[a]	References
Salmonella typhimurium		
TA100	*hisG46 rfa* Δ(*bio chlD uvrB*), bearing plasmid pKM101	Mortelmans and Zeiger, 2000 [42]
Vibrio harveyi		
BB7	Wild-type	Belas et al. 1982 [45]
BB7XM	UV-hypersensitive strain bearing plasmid pAB91273, that contains *muc*A and *muc*B genes, responsible for enhanced error-prone DNA repair	Czyż et al. 2000 [40]

[a] (*his*), histidine-auxotrophic point mutations; (Δ *uvrB*), deletion mutants derived from wild-type strain LT-2; (*bio*), biotin requirement for growth on minimal medium; (*chlD*), chlorate resistance; (*rfa*), increased permeability to large molecules; (pKM101), plasmid carrying the umuDC genes that encode the error-prone DNA-repair system and confers resistance to ampicillin.

the injected hind paw in selected intervals, 0–5, 15–20, 20–25, and 25–30 min, was measured in each experimental group and was indicator of nociceptive behavior.

4.3.7. Influence on motor coordination in the chimney test

The chimney test was performed according to a method described by Boissier et al. [36]. The animals were placed in a 25 cm long and 2.5 cm in diameter horizontally located tube, which was reversed in such a way that the mice were able to leave it only climbing backward up until they reached another end. Before test the animals were trained and those which were able to leave the chimney without much problem (up to 15 s) were selected. Motor impairment was indicated by the inability of mice to perform the test within 60 s.

4.4. In vitro pharmacology

4.4.1. Sodium and calcium channels binding assay

The radioligand binding studies were performed commercially in Cerep Laboratories (Poitiers, France) using testing procedures described elsewhere [37,38]. The general information is listed in Table 9.

4.4.2. In vitro mutagenicity and antimutagenicity studies

The bacterial strains used in the experiments are listed in Table 10.

In the Ames test the minimal medium described previously by Maron and Ames [39] was used. In case of the *V. harveyi* assay BOSS medium was employed [40]. *S. typhimurium* strains were cultivated at 37 °C and *V. harveyi* strains were grown at 30 °C. The standard mutagen used as a positive control was 4-nitroquinoline-*N*-oxide (NQNO). The final concentration of NQNO solution used in the tests was 40 ng/ml.

4.4.3. Mutagenicity assays

Mutagenicity of the tested chemicals was assessed by the Ames test (liquid pre-incubation method) and the *V. harveyi* assay as reported by Maron and Ames [39] and Czyż et al. [40]. All the assays were carried out in triplicate and the results were expressed as mutagenic index (M.I. = the number of revertant colonies induced in the tested sample/the number of spontaneous revertants in the negative control) [41]. A compound was considered mutagenic when the M.I. was equal or greater than 2.

4.4.4. Antimutagenicity assays

Antimutagenicity assays were performed according to previously described procedures [20,40,42]. All experiments were analyzed in three independent repetitions. The inhibition of mutagenicity was expressed as percentage decrease of reverse mutation and calculated using the following equation: Percent Inhibition = 100 − [(R1/R2) × 100], where R1 is the number of revertants per plate induced by test compound plus mutagen and R2 is the number of revertants per plate induced by mutagen alone [43]. A 25–40% inhibition was defined as moderate antigenotoxicity, 40% or higher inhibition as strong antigenotoxicity, and 25% or less inhibition as no antigenotoxicity [41].

Acknowledgments

The authors wish to thank the Antiepileptic Drug Development Program (Epilepsy Branch, National Institute of Neurological Disorders and Stroke, National Institute of Health, Rockville, MD, USA) for pharmacological data of compounds **8**, **14**, **15**, **17**, **23**. We are also grateful to Professor Katarzyna Kieć-Kononowicz (Department of Technology and Biotechnology of Drugs, Jagiellonian University Medical College, Kraków, Poland) the coordinator of the ADD Project from Polish site and Ms Karolina Szczerba for synthesis of several compounds. We thank Prof. G. Węgrzyn and Dr. T. Nohmi for providing bacterial strains for the experiment.

We are pleased to acknowledge the generous financial support of this work by the grant of the Polish National Scientific Centre, Poland (Grant No. DEC-2013/11/B/NZ7/02081).

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