Full Paper

Synthesis, Physicochemical, and Anticonvulsant Properties of New *N*-Mannich Bases Derived from Pyrrolidine-2,5-dione and Its 3-Methyl Analog

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A series of 22 new *N*-[(4-phenylpiperazin-1-yl)-methyl]-3-methyl-pyrrolidine-2,5-dione and pyrrolidine-2,5-dione derivatives were synthesized and evaluated for their anticonvulsant activities in the maximum electroshock (MES) and subcutaneous pentylenetetrazole (*sc*PTZ) seizure tests after intraperitoneal injection into mice. The neurotoxicity was determined applying the rotarod test. The *in vivo* results in mice showed that seven compounds were effective in the MES or/and *sc*PTZ seizure tests. The quantitative evaluation in both tests after i.p. administration into mice revealed that the most active compounds were *N*-[{4-(3,4-dichlorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**12**) with $ED_{50} = 16.13$ mg/kg (MES), $ED_{50} = 133.99$ mg/kg (*sc*PTZ) and *N*-[{4-(3,4-dichlorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**23**) with $ED_{50} = 37.79$ mg/kg (MES), $ED_{50} = 128.82$ mg/kg (*sc*PTZ), whereas *N*-[{4-(3-trifluoromethylphenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**24**) was effective only in the MES test with $ED_{50} = 16.37$ mg/kg. These molecules showed higher potency and also lower neurotoxicity than the reference antiepileptic drugs such as ethosuximide and valproic acid.

Keywords: Anticonvulsant activity / *In vivo* studies / Mannich bases

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Introduction

Epilepsy is a chronic disorder characterized by recurrent unprovoked seizures, which are defined as a discrete clinical event arising from transient, hypersynchronous, and abnormal neuronal behavior. Epilepsy then is not a disease but rather a syndrome arising from a group of non-specific, dysfunctional events in the brain. In spite of over 40 antiepileptic drugs (AEDs) currently in clinical use, 30% of convulsions are inadequately controlled by standard drug therapy. Furthermore, compliance is often limited by adverse side effects most notably related to CNS exposure such as like diminished attention, executive function, intelligence, language skills, memory, and processing speed [1–3]. Thus, there is a need for more efficient and less toxic antiepileptic drugs.

The complex mechanism of action of the majority of antiepileptic drugs makes it difficult to use rational methodologies to discover the pathogenesis of epilepsy. It has not been completely explained up to now. Therefore, conceptually, there are two most important methods of obtaining new anticonvulsants, namely ligand-based and screening approaches [4]. The ligand-based approach relies on the use of different pharmacophores that were established through the analysis of structural characteristics of clinically effective AEDs and other anticonvulsant active compounds. The serendipitous approach involves a comprehensive screening process that utilizes rodent models. Traditionally, most screening programs employ mice and these examinations assess anticonvulsant activity. Seizures can be induced both ways, electrically (e.g., maximal electroshock (MES)) and chemically (e.g., pentylenetetrazol, bicuculline, or picrotoxin). The number of new currently available AEDs,

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or in development, for the management of epilepsy certainly attests to the success of this approach [5–7].

Over the past decades many attempts have been made to identify the structural features crucial for anticonvulsant activity of different compounds. As a result, it was proved that one of the important core fragments is defined by the nitrogen heteroatomic system (imide or lactam) with at least one carbonyl group and phenyl or alkyl groups attached to the heterocyclic system. The examples of the above-mentioned structures are pyrrolidine-2,5-dione, pyrrolidin-2-one, or imidazolidine-2,4-dione. They are present in the molecules of the currently available antiepileptic drugs, e.g., ethosuximide, phenytoin, and also in newer drugs, e.g., levetiracetam, brivaracetam, or seletracetam (Fig. 1) [8–10].

Taking into consideration all the above our researches have been focused on a group of 1,3-substituted pyrrolidine-2,5-diones as targets for new antiepileptic drugs [11–20]. Many of these compounds were effective in the maximal electroshock (MES) or/and subcutaneous pentylenetetrazole (scPTZ) screens that are still recognized as the most popular seizure models in the early stages of the search for new anticonvulsants [6, 21–24]. Moreover, the *in vitro* electrophysiological studies showed that anticonvulsant effect is connected with the inhibition of $Na_V1.2$ sodium channel currents [12, 13]. The structure–activity relationship (SAR) analysis demonstrated that the highest activity showed *N*-Mannich bases derived from pyrrolidine-2,5-dione with an aromatic and alkyl groups at the position-3 and the phenylpiperazine moiety with different electron-withdrawing substituents as an amine function. The structures of the chosen, active compounds obtained in the previous studies are shown in Fig. 2.

As a continuation of systematic SAR discussion in the current work, we have synthesized a small library consisting of 22 new *N*-Mannich bases of pyrrolidine-2,5-diones with a small electron-donor methyl group at the position-3 of imide ring (**5**–**15**) as well as their unsubstituted analogs (**16**–**26**). Based on the previous results for 3-arylpyrrolidine-25-diones, the structural modifications proposed herein enabled to assess the influence of aryl or alkyl substituents at position-3 of imide ring on anticonvulsant activity.

Results and discussion

Chemistry

Compounds **5**–**26** were synthesized according to Scheme 1. The starting materials 2-methylsuccinic acid (**1**) and succinic acid (**2**) were purchased from Sigma–Aldrich (USA). The 3-methylpyrrolidine-2,5-dione (**3**) and pyrrolidine-2,5-dione (**4**) were obtained in the cyclization reaction of **1** or **2** with 25% ammonia. The final compounds **5**–**26** were synthesized in the aminoalkylation – Mannich-type reaction from the appropriately substituted intermediates **3** or **4**, formaldehyde and corresponding 4-substituted piperazines. The reaction was carried out in ethanol at room temperature for 12 h. The crude products were crystallized from 96% ethanol. The final compounds were obtained as racemic mixtures in good yields (58–82%). Their purity and homogeneity were assessed by TLC chromatography. The chemical structures were confirmed by spectral (^{1}H NMR, ^{13}C NMR, ^{19}F NMR, and LC/MS) and elemental (C, H, and N) analyses. The detailed physical and analytical data are listed in the Experimental section.

Anticonvulsant activity

The pre-clinical discovery and development of new chemical agents potentially useful for the treatment of epilepsy are based mainly on the use of predictable animal seizure models. At present, there are two *in vivo* screens used routinely – the maximal electroshock seizure test (MES) and the *sc*PTZ test. These models are still recognized as the "gold standard" for the screening tests of new anticonvulsants. Furthermore, they enable the detection of compounds that protect against generalized tonic–clonic seizures and generalized absence seizures, respectively [6].

The profile of anticonvulsant activity of **5**–**26** was established in the maximal electroshock (MES) and *sc*PTZ tests, after intraperitoneal (i.p.) injection in mice at doses of 30, 100, and 300 mg/kg. Observation was carried out at two different time intervals – 0.5 and 4 h. The acute neurological toxicity (NT) was determined by use of the minimal motor impairment – rotarod screen (NT). The results are shown in Table 1.

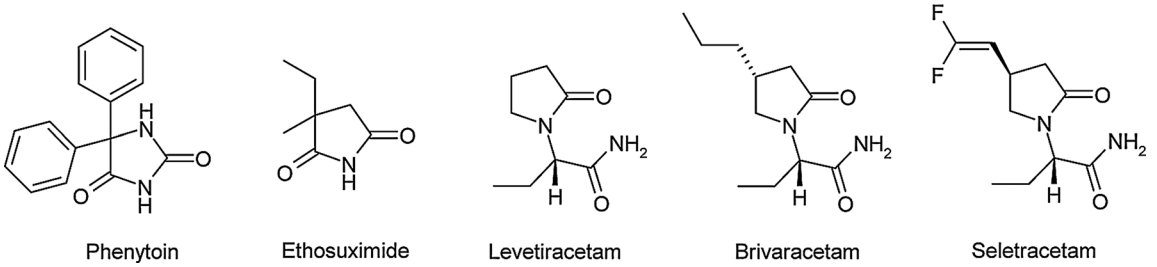
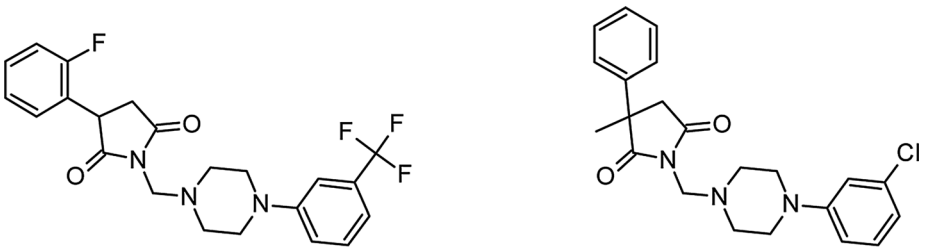


Figure 1. Structures of model AEDs based on the 5-membered heterocyclic ring.



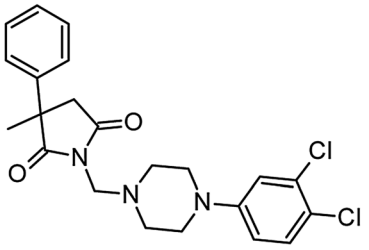
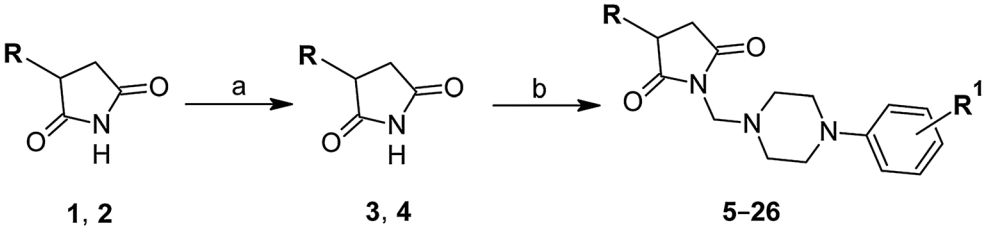


Figure 2. Structures of active compounds obtained in the previous studies (MES test in mice after i.p. administration).

The data obtained revealed an inconsiderable increase of anticonvulsant activity for derivatives with methyl group at the position-3 of pyrrolidine-2,5-dione ring (**6**, **9**, **12**, **13**, and **15**), in comparison with their unsubstituted analogs (**17**, **23**, and **24**). In this series, the highest anticonvulsant activity in the MES screen revealed compounds **12**, **23**, and **24**, which showed protection at dose of 30 mg/kg. It was equivalent to phenytoin, which is still recognized as the model antiepileptic drug active in the MES test. Additionally, among these molecules **12** and **23** showed effectiveness at the dose 300 mg/kg after 0.5 h. The other compounds **9**, **13**, and **15** revealed protection at a dose of 100 mg/kg. It should be stressed here



R = H; CH_3
R^1 = H; 2-F; 4-F; 2-Cl; 3-Cl; 4-Cl; 2,3-diCl; 3,4-diCl; 3-CF_3; 2-CH_3; 3-CH_3

Scheme 1. Synthetic procedures of intermediates **3**, **4**, and target compounds **5**–**26**. Reagents and conditions: (a) 25% NH_4OH, 190°C, 1.5 h, (b) 4 phenylpiperazine derivatives, formaldehyde, 96% EtOH, 12 h, room temperature.

Table 1. The results after i.p. administration into mice.

			MES[a]		*sc*PTZ[b]		NT[c]	
Compd.	**R**	**R[1]**	**0.5 h**	**4 h**	**0.5 h**	**4 h**	**0.5 h**	**4 h**
5	CH_3	H	–	–	–	–	300	300
6[g]	CH_3	2-F	–	–	–	100	–	–
7[g]	CH_3	4-F	–	–	–	–	–	–
8[f]	CH_3	2-Cl	–	–	–	–	–	–
9	CH_3	3-Cl	–	100	–	–	100	–
10[g]	CH_3	4-Cl	–	–	–	–	–	–
11[f]	CH_3	2,3-Cl	–	–	–	–	–	–
12	CH_3	3,4-Cl	300	30	300	100	–	–
13	CH_3	3-CF_3	100	100	–	–	–	–
14[f]	CH_3	2-CH_3	–	–	–	–	–	–
15	CH_3	3-CH_3	–	100	–	100	300	300
16[f]	H	H	–	–	–	–	–	–
17	H	2-F	–	–	–	100	100	300
18	H	4-F	–	–	–	–	100	300
19[f]	H	2-Cl	–	–	–	–	–	–
20	H	3-Cl	–	–	–	–	100	–
21	H	4-Cl	–	–	–	–	300	–
22[f]	H	2,3-Cl	–	–	–	–	–	–
23	H	3,4-Cl	300	30	300	100	–	–
24	H	3-CF_3	–	30	–	–	–	–
25[f]	H	2-CH_3	–	–	–	–	–	–
26[f]	H	3-CH_3	–	–	–	–	–	–
Phenytoin[d]			30	30	–	–	100	100
Ethosuximide[e]			–	–	100	300	–	–
Valproic acid[e]			–	–	300	–	–	–

Doses of 30, 100, and 300 mg/kg were administered. The data indicate the minimum dose whereby anticonvulsant activity or neurotoxicity was demonstrated in half or more animals (out of four mice). 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] Reference drugs, data from [23].
[e] Reference drugs, data from [24].
[f] Rapid death of animals after compounds administration at dose of 100 mg/kg.
[g] Rapid death of animals after compounds administration at dose of 300 mg/kg.

that MES test permits evaluation of the ability of a substance to prevent seizure spread through neural tissue. The further analysis showed that compounds **12**, **13**, and **23** were active in both time intervals (0.5 and 4 h after i.p. administration) whereas the other derivatives showed activity only at 4 h (**9**, **15**, and **24**). Apart from anti-MES protection, compounds **12**, **15**, and **23** were found to be active in the *sc*PTZ test, which identifies substances elevating seizure threshold. Among these molecules **12** and **23** showed anti-*sc*PTZ activity at the dose of 100 mg/kg after 4 h and at the dose 300 mg/kg after 0.5 h. Furthermore, two compounds **6** and **17** were active only in the *sc*PTZ test at the dose of 100 mg/kg after 4 h. It should be emphasized that **6**, **12**, **15**, and **17** revealed better protection at time point 4 h than ethosuximide, which is recognized as the model antiepileptic drug effective in the *sc*PTZ test. In the rotarod test (NT) for acute neurological toxicity **6**, **12**, **13**, **23**, and **24** did not show neurotoxicity in the maximum dose administered (300 mg/kg). Other derivatives **9**, **17**, **18**, and **20** showed neurotoxicity at a dose of 100 mg/kg or 300 mg/kg – **5**, **15**, and **21** after 0.5 or/and 4 h. It was not possible to perform MES, *sc*PTZ, and NT tests for the compounds **8**, **11**, **14**, **16**, **19**, **22**, **25**, and **26** due to rapid death following administration of dose 100 mg/kg. Similar situation took place for **6**, **7**, and **10** at dose of 300 mg/kg.

Based on the above preliminary screening data in mice, the most active molecules, namely **9**, **12**, **13**, **15**, **23**, and **24**, were chosen for quantification of the pharmacological parameters (ED_{50} and TD_{50}) in the MES and *sc*PTZ tests after i.p. administration in mice. The quantitative evaluation of the MES and *sc*PTZ median effective doses (ED_{50}) and neurotoxic dose (TD_{50}) were performed at previously estimated time to peak effect (TPE). Results of the quantitative tests along with the data for the standard drugs phenytoin, ethosuximide, and valproic acid are shown in Table 2.

The quantitative data revealed that **12** and **23** showed higher potency in the MES and *sc*PTZ tests in comparison with reference drugs – ethosuximide and valproic acid. Such *in vivo* profile may indicate potential effectiveness of **12** and **23** in the treatment of different types of seizures, including human generalized tonic–clonic seizures and absence seizures. It should be stressed also that both compounds revealed lower rotarod toxicity than phenytoin, ethosuximide, and valproic acid that resulted in favorable protection indexes. Based on the screening data for compounds **9**, **13**, **15**, and **24** quantitative data only in the MES test were designated. All of these compounds had better ED_{50} in the MES seizures than valproic acid but showed higher neurotoxicity. It should be noted that **24** was three times less effective and less neurotoxic than phenytoin. The data obtained will enable to design new molecules, effective in controlling different types of seizures, in future studies.

Structure–activity relationships

The preliminary pharmacological screening revealed that anticonvulsant activity depended strongly on the substituents at position-3 of the imide ring. In general, the higher anticonvulsant protection was observed for 3-methylpyrrolidine-2,5-diones (**5**–**15**), whereas removal of the 3-methyl group resulted in less active connections (**16**–**26**). The second factor influencing on anticonvulsant activity was the presence of electron-withdrawing atoms, especially at the position-3 or 4 of phenylpiperazine moiety. Thus, in both series of compounds the most active in the MES test were with 3-trifluoromethyl- (**13**, **24**) and 3,4-dichloro-phenylpiperazine

Table 2. Quantitative anticonvulsant data in mice after i.p. administration.

Compd.	TPE (h)[a)]	ED_{50} MES (mg/kg)[b)]	ED_{50} *sc*PTZ (mg/kg)[b)]	TD_{50} (mg/kg)[b)]	PI (TD_{50}/ED_{50})[c)]
9	4	91.87 (68.88–122.55)	ND	98.45 (63.03–153.79)	1.07 (MES)
12	4	16.13 (7.71–33.75)	133.99 (81.42–220.49)	>500	>31 (MES) >3.73 (*sc*PTZ)
13	4	42.71 (32.61–55.93)	ND	133.20 (91.93–192.99)	3.11 (MES)
15	4	46.07 (23.24–91.30)	ND	166.21 (124.21–222.45)	3.61 (MES)
23	4	37.79 (15.99–89.33)	128.82 (103.59–160.21)	>500	>13 (MES) >3.88 (*sc*PTZ)
24	4	16.37 (7.27–36.85)	ND	110.01 (82.43–146.80)	6.71 (MES)
Phenytoin[d)]	1.0	5.32 (5.44–7.23)	>500	41.2 (36.9–46.1)	7.74 (MES)
Etosuximide[d)]	1.0	>500	136 (101–184)	341 (209–384)	2.50 (*sc*PTZ)
Valproic acid[d)]	1.0	263 (237–282)	220 (177–268)	398 (356–445)	1.5 (MES) 1.8 (*sc*PTZ)

ND, no data.
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; neurotoxicity-rotarod screen).
c) Protection index (TD_{50}/ED_{50}).
d) Reference drug, data from [25].

(**12** and **23**) derivatives. Furthermore, **12** and **23** showed also activity in the *sc*PTZ screen. Compounds **6** and **17** with fluorine atom at the position-2 of phenylpiperazine were active only in the *sc*PTZ test. Among the chloro-derivatives **8–10** and **19–21**, only compound **9** with chlorine atom at position-3 demonstrated anticonvulsant protection, while all *ortho* or *para* analogs as well as compounds with two chlorine atoms at position-2, 3 were devoid of activity. Likewise, the absence of activity was observed for unsubstituted compounds (**5** and **16**) and molecules with electron-donating methyl group (**14**, **25**, and **26**). An exception was observed for **15**, which was active in both MES and *sc*PTZ tests. The comparison of the pharmacological data for 3-phenyl-pyrrolidine-2,5-diones described in advance [18–20] and compounds obtained in the current studies proved that the exchange of aromatic substituents into methyl group did not influence the anticonvulsant activity distinctly. However, removal of both aryl or alkyl group from position-3 of succinimide ring decreased the anticonvulsant properties.

Conclusion

In the current studies, the focused library of 22 new *N*-Mannich bases of 3-methyl-pyrrolidine-2,5-dione and pyrrolidine-2,5-dione was synthesized and evaluated for anticonvulsant activity in the maximal electroshock (MES) and pentylenetetrazole (*sc*PTZ) seizures tests in mice. The results obtained revealed that several compounds exhibited satisfying protection in the MES and/or *sc*PTZ screens. The SAR analysis proved that the presence of aromatic ring or small methyl group at position-3 of pyrrloidine-2,5-dione is exclusively preferred for anticonvulsant activity. Apart from the structure of imide fragment, the second crucial factor influencing on biological properties was the substitution mode of phenylpiperazine moiety. Due to favorable pharmacological properties of compounds described in the current work, the further chemical, pharmacological (*in vivo* and *in vitro* assays) and pharmacokinetic studies are planned.

Experimental

Chemistry

All the 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 and homogeneity of the compounds were assessed with thin-layer chromatography (TLC). The TLC was performed on Merck silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany), using developing systems consisting of S_1 chloroform/acetone (9:1 v/v). Spots were detected by their absorption under UV light ($\lambda = 254$ nm). Elemental analysis for C, H, and N were carried out by a micro-method using the Vario EI III elemental analyzer (Hanau, Germany). The results of elemental analyses were within ±0.4% of the theoretical values.

^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra were obtained in a Varian Mercury spectrometer (Varian, Inc., Palo Alto, CA, USA), in $CDCl_3$ or DMSO, operating at 300 MHz. 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), t (triplet), and m (multiplet). The mass spectra for compounds **5–26** were obtained on Waters ACQUITY^{TM} TQD system with the TQ detector (Waters,

Milford, USA). The ACQUITY UPLC BEH C18, 1.7 μm, 2.1 mm × 50 mm column was used (Waters). Preparative column chromatography was performed using silica gel 60 (particle size 0.063–0.200; 70–230 Mesh ATM) purchased from Merck.

*General procedure for the synthesis of pyrrolidine-2,5-diones or 3-methylpyrrolidine-2,5-diones (**3** and **4**)*

A total of 0.05 mol of the succinic acid or 2-methyl-succinic acid was suspended in 20 mL of water; afterwards 0.05 mol of 25% ammonia 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 was raised up to 180°C and was maintained for 1.5 h. The crude products were purified by column chromatography (dichloromethane/methanol, 9:1 v/v) to afford intermediates **3** and **4** as yellow oils.

*General procedure for the synthesis of the final compounds (**5**–**26**)*

The mixture of 3-methylpyrrolidine-2,5-dione (**3**) or pyrrolidine-2,5-dione (**4**) (0.01 mol), 40% formaldehyde solution (0.01 mol), and corresponding 4-phenylpiperazines (0.01 mol) were dissolved in 96% ethanol. The mixture was left for ca. 12 h at room temperature and then refrigerated at ca. −10°C for 24 h. The precipitated crude products were washed with cold ethanol, separated by filtration, and recrystallized from 96% ethanol. Compound **8** was obtained as light oil and was converted into solid hydrochloride salt in anhydrous ethanol saturated with HCl gas. The obtained precipitate was crystallized from anhydrous ethanol.

*N-[4-(Phenylpiperazin-1-yl)-methyl]-3-methylpyrrolidine-2,5-dione (**5**)*

White solid. Yield: 64%; m.p. 80–82°C; $R_f = 0.58$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.35 (d, 3H, CH_3, $J = 2.05$ Hz), 2.37 (d, 1H, imide, $J = 13.33$ Hz), 2.89–2.91 (m, 4H, piperazine), 2.96 (d, 2H, imide, $J = 8.98$ Hz), 3.13–3.16 (m, 4H, piperazine), 4.52 (s, 2H, CH_2), 6.82–6.91 (m, 3H, ArH), 7.21–7.28 (m, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.00, 34.72, 36.33, 49.38, 50.64, 59.80, 116.31, 119.98, 129.10, 151.21, 177.32, 181.56; ESI-MS: 288.34 ($C_{16}H_{21}N_3O_2$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{21}N_3O_2$ (287.36): C, 66.88; H, 7.37; N, 14.62; Found: C, 66.91; H, 7.39; N, 14.63.

*N-[{4-(2-Fluorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**6**)*

White solid. Yield: 77%; m.p. 133–135°C; $R_f = 0.53$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.36 (d, 3H, CH_3, $J = 2.31$ Hz), 2.34 (d, 1H, imide, $J = 13.33$ Hz), 2.76–2.79 (m, 4H, piperazine), 2.97 (d, 2H, imide, $J = 8.98$ Hz), 3.04 (t, 4H, piperazine, $J = 9.74$ Hz), 4.52 (s, 2H, CH_2), 6.90–6.97 (m, 2H, ArH), 7.01–7.05 (m, 2H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ: −122.96 (s, 1F); ESI-MS: 306.29 ($C_{16}H_{20}N_3O_2$ F $[M+H]^+$). Anal. calcd. for $C_{16}H_{20}N_3O_2$ F (305.35): C, 62.94; H, 6.60; N, 13.76; Found: C, 62.96; H, 6.64; N, 13.80.

*N-[{4-(4-Fluorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**7**)*

White solid. Yield: 48%; m.p. 82–84°C; $R_f = 0.54$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.38 (d, 3H, CH_3, $J = 2.05$ Hz), 2.33 (d, 1H, imide, $J = 13.59$), 2.73–2.76 (m, 4H, piperazine), 2.96 (d, 2H, imide, $J = 9.24$ Hz), 3.05–3.08 (m, 4H, piperazine), 4.51 (s, 2H, CH_2), 6.83–6.85 (m, 2H, ArH), 6.91–6.97 (m, 2H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ: −124.20 (s, 1F); ESI-MS: 306.29 ($C_{16}H_{20}N_3O_3F$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{20}N_3O_2F$ (305.36): C, 62.94; H, 6.60; N, 13.76; Found: C, 62.89; H, 6.59; N, 13.71.

*N-[{4-(2-Chlorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione monohydrochloride (**8**)*

White solid. Yield: 80%; m.p. 165–167°C; $R_f = 0.31$ (S_1), ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.25 (d, 3H, CH_3, $J = 2.05$ Hz), 2.33 (d, 1H, imide, $J = 13.34$ Hz), 2.69–2.74 (m, 4H, piperazine), 2.90 (d, 2H, imide, $J = 9.25$ Hz), 3.43–3.48 (m, 4H, piperazine), 4.57 (s, 2H, CH_2), 7.07–7.19 (m, 2H, ArH), 7.27–7.35 (m, 1H, ArH), 7.39–7.45 (m, 1H, ArH), 11.00 (brs, 1H, +NH); ESI-MS: 322.25 ($C_{16}H_{20}N_3O_2Cl$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{20}N_3O_2 \cdot HCl$ (357.25): C, 53.79; H, 5.64; N, 11.76; Found: C, 53.83; H, 5.60; N,11.77.

*N-[{4-(3-Chlorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**9**)*

White solid. Yield: 72%; m.p. 82–84°C; $R_f = 0.53$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.38 (d, 3H, CH_3, $J = 2.05$ Hz), 2.32 (d, 1H, imide, $J = 13.59$ Hz), 2.71–2.74 (m, 4H, piperazine), 2.96 (d, 2H, imide, $J = 9.23$ Hz), 3.12–3.16 (m, 4H, piperazine), 4.51 (s, 2H, CH_2), 6.76–6.83 (m, 3H, ArH), 7.11–7.17 (m, 1H, ArH); ESI-MS: 322.31 ($C_{16}H_{20}N_3O_2Cl$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{20}N_3O_2Cl$ (321.80): C, 59.72; H, 6.26; N, 13.06; Found: C, 59.78; H, 6.28; N, 13.09.

*N-[{4-(4-Chlorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**10**)*

White solid. Yield: 76%; m.p. 89–91°C; $R_f = 0.55$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.36 (d, 3H, CH_3, $J = 2.05$ Hz), 2.34 (d, 1H, imide, $J = 13.33$ Hz), 2.72–2.75 (m, 4H, piperazine), 2.92 (d, 2H, imide, $J = 9.23$ Hz), 3.15–3.18 (m, 4H, piperazine), 4.51 (s, 2H, CH_2), 6.90 (d, 2H, ArH, $J = 8.98$ Hz), 7.16–7.22 (m, 2H, ArH); ESI-MS: 322.31 ($C_{16}H_{20}N_3O_2Cl$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{20}N_3O_2Cl$ (321.80): C, 59.72; H, 6.26; N, 13.06; Found: C, 59.75; H, 6.22; N,13.01.

*N-[{4-(2,3-Dichlorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**11**)*

White solid. Yield: 43%; m.p. 80–82°C; $R_f = 0.59$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.37 (d, 3H, CH_3, $J = 2.05$ Hz), 2.36 (d, 1H, imide, $J = 13.33$ Hz), 2.70 (t, 4H, piperazine, $J = 4.61$ Hz), 2.94–3.03 (m, 2H, imide, 4H, piperazine), 4.52 (s, 2H, CH_2), 6.91 (d, 1H, ArH, $J = 3.06$), 7.12–7.15 (m, 2H, ArH); ESI-MS: 356.28 ($C_{16}H_{19}N_3O_2Cl_2$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{19}N_3O_2Cl_2$ (356.28): C, 53.94; H, 5.38; N, 11.80; Found: C, 53.98; H, 5.41; N, 11.81.

*N-[{4-(3,4-Dichlorophenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**12**)*

White solid. Yield: 70%; m.p. 92–94°C; $R_f = 0.53$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.36 (d, 3H, CH_3, $J = 2.05$ Hz), 2.36 (d, 1H, imide, $J = 13.33$ Hz), 2.70–2.73 (m, 4H, piperazine), 2.97 (d, 2H, imide, $J = 9.23$), 3.10–3.13 (m, 4H, piperazine), 4.50 (s, 2H, CH_2), 6.71 (d, 1H, ArH, $J = 2.82$), 6.91 (d, 1H, ArH, $J = 2.82$ Hz), 7.23 (d, 1H, ArH, $J = 2.82$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.03, 34.73, 36.32, 48.76, 50.31, 59.72, 115.54, 117.41, 122.38, 130.42, 132.77, 150.55, 177.27, 181.51; ESI-MS: 356.28 ($C_{16}H_{19}N_3O_2Cl_2$ $[M+H]^+$). Anal.

calcd. for $C_{16}H_{19}N_3O_2Cl_2$ (356.25): C, 53.94; H, 5.38; N, 11.80; Found: C, 53.97; H, 5.42; N, 11.82.

*N-[{4-(3-Trifluoromethylphenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**13**)*

White solid. Yield: 64%; m.p. 103–105°C; $R_f = 0.50$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.35 (d, 3H, CH_3, $J = 2.05$ Hz), 2.32 (d, 1H, imide, $J = 13.33$ Hz), 2.73–2.76 (m, 4H, piperazine), 2.96 (d, 2H, imide, $J = 8.97$ Hz), 3.17 (t, 4H, piperazine, $J = 5.13$ Hz), 4.51 (s, 2H, CH_2), 7.00–7.07 (m, 3H, ArH), 7.29–7.35 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.03, 34.73, 36.32, 48.78, 50.44, 59.74, 112.37, 116.09, 118.95, 129.54, 131.20, 151.28, 177.28, 181.53; ^{19}F NMR (282 MHz, $CDCl_3$) δ: −62.78 (s, 3F); ESI-MS: 356.48 ($C_{17}H_{20}N_3O_2F_3$ $[M+H]^+$). Anal. calcd. for $C_{17}H_{20}N_3O_2F_3$ (355.35): C, 57.46; H, 5.67; N, 11.82; Found: C, 57.48; H, 5.68; N, 11.86.

*N-[{4-(2-Methylphenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**14**)*

White solid. Yield: 43%; m.p. 92–94°C; $R_f = 0.47$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.35 (d, 3H, CH_3, $J = 2.05$ Hz), 2.26 (s, 3H, CH_3) 2.35 (d, 1H, imide, $J = 13.33$ Hz), 2.71–2.74 (m, 4H, piperazine), 2.87 (m, 4H, piperazine), 3.00 (d, 2H, imide, $J = 9.0$ Hz), 4.51 (s, 2H, CH_2), 6.94–7.01 (m, 2H, ArH), 7.13–7.17 (m, 2H, ArH); ESI-MS: 302.30 ($C_{17}H_{23}N_3O_2$ $[M+H]^+$). Anal. calcd. for $C_{17}H_{23}N_3O_2$ (301.38): C, 67.75; H, 7.69; N, 13.94; Found: C, 67.69; H, 7.67; N, 13.90.

*N-[{4-(3-Methylphenyl)-piperazin-1-yl}-methyl]-3-methylpyrrolidine-2,5-dione (**15**)*

White solid. Yield: 78%; m.p. 90–92°C; $R_f = 0.44$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.35 (d, 3H, CH_3, $J = 2.31$ Hz), 2.30 (s, 3H, CH_3), 2.32 (d, 1H, imide, $J = 13.33$ Hz), 2.72–2.75 (m, 4H, piperazine), 2.95 (d, 2H, imide, $J = 8.98$ Hz), 3.12–3.15 (m, 4H, piperazine), 4.51 (s, 2H, CH_2), 6.69–6.71 (m, 3H, ArH), 7.13–7.15 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.01, 21.74, 34.72, 36.33, 49.38, 50.66, 59.80, 113.48, 117.16, 120.91, 128.94, 138.81, 151.27, 177.35, 181.58; ESI-MS: 302.37 ($C_{17}H_{23}N_3O_2$ $[M+H]^+$). Anal. calcd. for $C_{17}H_{23}N_3O_2$ (301.38): C, 67.75; H, 7.69; N, 13.94; Found: C, 67.78; H, 7.71; N, 13.95.

*N-[4-(Phenylpiperazin-1-yl)-methyl]-pyrrolidine-2,5-dione (**16**)*

White solid. Yield: 77%; m.p. 140–142°C; $R_f = 0.47$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.74 (s, 4H, CH_2, imide), 2.75 (t, 4H, piperazine, $J = 5.13$ Hz), 3.13 (t, 4H, piperazine, $J = 5.13$ Hz), 4.52 (s, 2H, CH_2), 6.82–6.90 (m, 3H, ArH), 7.22–7.27 (m, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 28.12, 49.30, 50.66, 59.84, 116.36, 119.99, 129.11, 151.20, 178.1; ESI-MS: 274.32 ($C_{15}H_{19}N_3O_2$ $[M+H]^+$). Anal. calcd. for $C_{15}H_{19}N_3O_2$ (273.33): C, 65.91; H, 7.01; N, 15.37. Found: C, 65.96; H, 7.03; N, 15.37.

*N-[{4-(2-Fluorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**17**)*

White solid. Yield: 78%; m.p. 148–150°C; $R_f = 0.47$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.75 (s, 4H, CH_2, imide), 2.76 (t, 4H, piperazine, $J = 5.13$ Hz), 3.04 (t, 4H, piperazine, $J = 5.13$ Hz), 4.52 (s, 2H, CH_2), 6.90–6.97 (m, 3H, ArH), 7.02–7.05 (m, 1H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ: −123.06 (s, 1F); ESI-MS: 292.27 ($C_{15}H_{18}N_3O_2$ F $[M+H]^+$). Anal. calcd. for $C_{15}H_{18}N_3O_2F$ (291.32): C, 61.84; H, 6.23; N, 14.42; Found: C, 61.88; H, 6.23; N, 14.41.

*N-[{4-(4-Fluorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**18**)*

White solid. Yield: 74%; m.p. 146–148°C; $R_f = 0.44$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.73–2.77 (m, 4H, CH_2, imide, 4H, piperazine), 3.05–3.08 (m, 4H, piperazine), 4.52 (s, 2H, CH_2), 6.82–6.86 (m, 2H, ArH), 6.91–6.95 (m, 2H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ: −124.18 (s, 1F); ESI-MS: 292.27 ($C_{15}H_{18}N_3O_2$ F $[M+H]^+$). Anal. calcd. for $C_{15}H_{18}N_3O_2F$ (291.32): C, 61.84; H, 6.23; N, 14.42; Found: C, 61.80; H, 6.20; N, 14.40.

*N-[{4-(2-Chlorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**19**)*

White solid. Yield: 51%; m.p. 68–70°C; $R_f = 0.44$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.76 (s, 4H, CH_2, imide), 2.78 (brs, 4H, piperazine), 3.01 (brs, 4H, piperazine), 4.52 (s, 2H, CH_2), 6.93 (m, 2H, ArH), 7.18 (d, 1H, ArH, $J = 1.54$ Hz), 7.32 (d, 1H, ArH, $J = 1.54$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 28.17, 50.84, 51.16, 59.93, 120.36, 123.79, 127.59, 128.65, 130.63, 149.09 178.23; ESI-MS: 308.29 ($C_{15}H_{18}N_3O_2Cl$ $[M+H]^+$). Anal. calcd. for $C_{15}H_{18}N_3O_2Cl$ (307.78): C, 58.54; H, 5.89; N, 13.65; Found: C, 58.50; H, 5.85; N, 13.65.

*N-[{4-(3-Chlorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**20**)*

White solid. Yield: 71%; m.p. 98–100°C; $R_f = 0.53$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.72–2.75 (m, 4H, CH_2, imide, 4H, piperazine), 3.13–3.17 (m, 4H, piperazine), 4.51 (s, 2H, CH_2), 6.75–6.84 (m, 3H, ArH), 7.11–7.17 (m, 1H, ArH); ESI-MS: 308.29 ($C_{15}H_{18}N_3O_2Cl$ $[M+H]^+$). Anal. calcd. for $C_{15}H_{18}N_3O_2Cl$ (307.78): C, 58.54; H, 5.89; N, 13.65; Found: C, 58.50; H, 5.86; N, 13.63.

*N-[{4-(4-Chlorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**21**)*

White solid. Yield: 77%; m.p. 175–177°C; $R_f = 0.46$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.72–2.75 (m, 4H, CH_2, imide, 4H, piperazine), 3.10 (t, 4H, piperazine, $J = 5.13$ Hz), 4.51 (s, 2H, CH_2), 6.82–6.85 (m, 2H, ArH), 7.17–7.20 (m, 2H, ArH); ESI-MS: 308.22 ($C_{15}H_{17}N_3O_2Cl$ $[M+H]^+$). Anal. calcd. for $C_{15}H_{17}N_3O_2Cl$ (307.78): C, 58.54; H, 5.89; N, 13.65; Found: C, 58.57; H, 5.93; N, 13.69.

*N-[{4-(2,3-Dichlorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**22**)*

White solid. Yield: 67%; m.p. 143–145°C; $R_f = 0.52$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.76–2.79 (m, 4H, CH_2, imide, 4H, piperazine) 2.99 (brs, 4H, piperazine), 4.52 (s, 2H, CH_2), 6.91 (d, 1H, ArH, $J = 3.06$ Hz), 7.12–7.15 (m, 2H, ArH); ESI-MS: 342.26 ($C_{15}H_{17}N_3O_2Cl_2$ $[M+H]^+$). Anal. calcd. for $C_{15}H_{17}N_3O_2Cl_2$ (342.22): C, 52.64; H, 5.01; N, 12.28; Found: C, 52.62; H, 4.98; N, 12.24.

*N-[{4-(3,4-Dichlorophenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**23**)*

White solid. Yield: 80%; m.p. 185–187°C; $R_f = 0.57$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.72–2.75 (m, 4H, CH_2, imide, 4H, piperazine), 3.10–3.13 (m, 4H, piperazine), 4.49 (s, 2H, CH_2), 6.67 (d, 1H, ArH, $J = 2.82$ Hz), 6.90 (d, 1H, ArH, $J = 2.82$ Hz), 7.23 (d, 1H, ArH, $J = 2.82$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 28.12, 48.76, 51.16, 59.93, 115.55, 117.39, 122.33, 130.43, 132.76, 150.52, 176.31; ESI-MS:

342.26 ($C_{15}H_{17}N_3O_2Cl_2$ $[M+H]^+$). Anal. calcd. for $C_{15}H_{17}N_3O_2Cl_2$ (341.07): C, 52.64; H, 5.01; N, 12.28; Found: C, 52.68; H, 5.05; N, 12.31.

*N-[{4-(3-Trifluoromethylphenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**24**)*

White solid. Yield: 63%; m.p. 120–122°C; $R_f = 0.53$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.74–2.77 (m, 4H, CH_2, imide, 4H, piperazine), 3.17–3.20 (m, 4H, piperazine), 4.51 (s, 2H, CH_2), 7.03–7.07 (m, 3H, ArH), 7.30–7.34 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 28.12, 48.78, 50.48, 59.79, 112.42, 116.07, 118.94, 129.55, 151.27, 178.04; ^{19}F NMR (282 MHz, $CDCl_3$) δ: −62.78 (s, 3F); ESI-MS: 342.32 ($C_{16}H_{18}N_3O_2F_3$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{18}N_3O_2F_3$ (341.33): C, 56.30; H, 5.32; N, 12.31; Found: C, 56.34; H, 5.34; N, 12.35.

*N-[{4-(2-Methylphenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**25**)*

White solid. Yield: 76%; m.p. 89–91°C; $R_f = 0.49$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.26 (s, 3H, CH_3), 2.74 (brs, 4H, piperazine), 2.78 (s, 4H, CH_2, imide), 2.87–2.90 (m, 4H, piperazine), 4.52 (s, 2H, CH_2), 6.94–6.99 (m, 2H, ArH), 7.12–7.17 (m, 2H, ArH); ESI-MS: 288.28 ($C_{16}H_{21}N_3O_2$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{21}N_3O_2$ (287.36): C, 66.88; H, 7.37; N, 14.62; Found: C, 66.93; H, 7.39; N, 14.66.

*N-[{4-(3-Methylphenyl)-piperazin-1-yl}-methyl]-pyrrolidine-2,5-dione (**26**)*

White solid. Yield: 71%; m.p. 99–101°C; $R_f = 0.45$ (S_1); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.30 (s, 3H, CH_3), 2.73–2.76 (m, 4H, CH_2, imide, 4H, piperazine), 3.12–3.15 (m, 4H, piperazine), 4.52 (s, 2H, CH_2), 6.67–6.71 (m, 3H, ArH), 7.11–7.16 (m, 1H, ArH); ESI-MS: 288.28 ($C_{16}H_{21}N_3O_2$ $[M+H]^+$). Anal. calcd. for $C_{16}H_{21}N_3O_2$ (287.36): C, 66.88; H, 7.37; N, 14.62; Found: C, 66.86; H, 7.33; N, 14.59.

Pharmacology

The initial anticonvulsant evaluations were performed at the Department of Pharmacodynamics, Faculty of Pharmacy, Jagiellonian University Medical College. Phase I studies involved three tests: maximal electroshock (MES), *sc*PTZ, and rotorod test for acute neurological toxicity (NT). The compounds were injected intraperitoneally into mice as a suspension in 0.5% methylcellulose/water mixture at doses of 30, 100, and 300 mg/kg with anticonvulsant activity and neurotoxicity assessment at 0.5 and 4 h intervals after administration. For the most promising derivatives, the ED_{50} and TD_{50} values were designated in mice.

Chemicals

Pentylenetetrazole (PTZ) was purchased from Sigma–Aldrich (Poland). PTZ was dissolved in saline solution and administered subcutaneously. The compounds were suspended in 0.5% methylcellulose/water mixture (Loba Chemie, Germany). All the compounds were administered intraperitoneally into mice in volumes of 0.1 mL per 10 g body weight. Control animals were given appropriate amounts of vehicle (methylcellulose). Anticonvulsant activity and neurotoxicity assessment were done at 0.5 and 4 h intervals after administration.

Animals

For the experiments, adult male Albino Swiss (CD-1) mice weighing 16–26 g were used. The animals were kept in cages at room temperature of 22 ± 2°C, under a light/dark (12/12) cycle with access to food and water before experiments. The 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 and each mouse was used only once. The experiments were performed between 8 a.m. and 3 p.m. All the procedures were approved by the Local Ethics Committee of the Jagiellonian University in Cracow.

Maximal electroshock seizure test (MES)

The maximal electroshock test was performed according to procedure originally described by Toman et al. [26]. Briefly, the mice received a stimulus of sufficient intensity (50 mA, 60 Hz) delivered by an electroshock generator (rodent shocker, type GE; COMT, Bialystok, Poland) to induce maximal seizures. Electroconvulsions were produced with the use of auricular electrodes and the stimulus duration was 0.2 s. The endpoint was the tonic extension of the hind limbs. In the control groups, the procedures caused immediate hindlimb tonic extension. Mice not displaying hind-limb tonic extension were considered to be protected from seizure. The maximal electroshock test was done at 0.5 and 4 h intervals after administration of tested compounds.

Subcutaneous pentylenetetrazole seizure test (scPTZ)

*sc*PTZ-induced seizure was performed by subcutaneously injection of PTZ (85 mg/kg). This produced clonic convulsions lasting for at least 5 s in 97% of animal tested. PTZ was administered 0.5 and 4 h after injections of tested compounds and observation were carried out for 30 min. In the control groups, the first episode of clonic convulsions was observed between 6 and 15 min of observation. The absence of clonic convulsions in the observed time period of 0.5 and 4 h was interpreted as the compound's ability to protect against PTZ-induced seizure [27].

Neurotoxicity screening

Minimal motor impairment was established in mice by standard rotarod procedure [28]. Mice were trained to balance on an accelerating rotarod that rotated at 10 revolutions per minute (rotarod apparatus, May Commat RR0711, Turkey; rod diameter: 2 cm). During the training session, the animals were placed on a rotating rod for 3 min with an unlimited number of trials. Proper experimentation was conducted at least 24 h after the training trial. On the test day, trained mice were intraperitoneally pretreated with the test compound and after 0.5 and 4 h were tested on the rotarod revolving at 10 rpm. Neurotoxicity was indicated by the inability of the animal to maintain equilibration on the rod for at least 1 min.

Median effective dose (ED_{50}), median toxic dose (TD_{50}), and protective index (PI)

The ED_{50} is defined as the dose of a drug protecting 50% of animals against the MES and PTZ seizures. To evaluate the ED_{50}, at least four groups of animals were injected with various doses of tested compounds. Each group consisted of six animals. The neurotoxic effect was expressed as a TD_{50} value, representing the doses at which the compound resulted in minimal motor

impairment in 50% of the animals in the rotarod test. Similarly, to evaluate the TD_{50}, four groups of animals were injected with various doses of compound. Both ED_{50} and TD_{50} values with 95% confidence limits were calculated by probit analysis [29]. The protective index (PI) value was calculated as the ratio of TD_{50} to ED_{50} ($TD_{50}/ED_{50} = PI$).

The authors have declared no conflict of interest.

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