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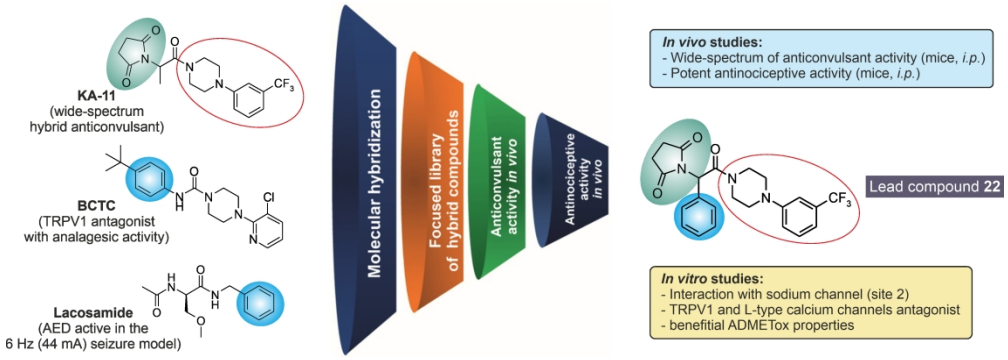
KA-11
(wide-spectrum
hybrid anticonvulsant)
BCTC
(TRPV1 antagonist
with analagesic activity)
Lacosamide
(AED active in the
6 Hz (44 mA) seizure model)
Molecular hybridization
Focused library of hybrid compounds
Anticonvulsant activity in vivo
Antinociceptive activity in vivo
In vivo studies:
- Wide-spectrum of anticonvulsant activity (mice, i.p.)
- Potent antinociceptive activity (mice, i.p.)
Lead compound 22
In vitro studies:
- Interaction with sodium channel (site 2)
- TRPV1 and L-type calcium channels antagonist
- benefitial ADMETox properties

Multitargeted Compounds Derived from (2,5-Dioxopyrrolidin-1-yl)(phenyl)-Acetamides as Candidates for Effective Anticonvulsant and Antinociceptive Agents

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ABSTRACT: We developed a focused set of original hybrid pyrrolidine-2,5-dione derivatives with potent anticonvulsant and antinociceptive properties. These hybrid compounds demonstrated broad-spectrum protective activity in a range of mouse models, such as the maximal electroshock (MES) test, the pentylenetetrazole-induced seizures (*sc*PTZ), and the 6 Hz (32 mA) seizures. Compound **22** showed the most potent anticonvulsant activity (ED_{50} MES = 23.7 mg/kg, ED_{50} 6 Hz (32 mA) = 22.4 mg/kg, ED_{50} *sc*PTZ = 59.4 mg/kg). In addition, **22** revealed potent efficacy in the formalin-induced tonic pain. These *in vivo* activities of **22** are likely mediated by several targets and may result from inhibition of central sodium/calcium currents and TRPV1 receptor antagonism. Finally, the lead compound **22** revealed drug-like ADME-Tox properties in the *in vitro* assays making it a potential candidate for further development in epilepsy and neuropathic pain indications.

KEYWORDS: Hybrid compounds, multi-targeted drugs, epilepsy, neuropathic pain, anticonvulsants, antinociceptive activity.

INTRODUCTION

A considerable interest in designing new multi-targeted compounds has been observed in recent years. Such compounds have been proven to be advantageous in the treatment of multifactorial diseases (characterized by complex pathomechanisms) often linked with drug resistance.[1–3] Epilepsy, which is recognized as a common neurological disorder clearly falls into this category. In fact, about one-third of the patients with epilepsy do not respond to current pharmacological treatments and are diagnosed with drug-resistant epilepsy.[4] Multifunctional (or multi-targeted) antiepileptic drugs (AEDs), such as valproic acid (VPA), belong to the most frequently prescribed and most effective compounds in different types of epilepsies.[5,6] The combination of different mechanisms of action tends to be also more beneficial in case of drug-

resistant epilepsy.[7,8] Therefore multi-targeted AEDs, which involve synergistic or additive mechanisms, may provide a more comprehensive spectrum of activity compared to single-targeted drugs as well as may be characterized by a better therapeutic window. This is exemplified by padsevonil, a dual acting AED candidate that integrates pharmacophoric features of both $GABA_A$ (benzodiazepine receptor, BZD) and SV2A (synaptic vesicle glycoprotein 2A) ligands.[9] Consequently, padsevonil displays both a broad profile of anticonvulsant activity and an improved therapeutic window versus many other AEDs.[10]

In keeping with the above-described concepts of multi-targeted drugs and with the aim of obtaining new highly effective broad-spectrum anticonvulsants, we have developed a series of integrated hybrid molecules derived from the pyrrolidine-2,5-dione ring.[11–13] These compounds were designed by applying the fragment-based approach[14] incorporating common structural features found in three chemically and pharmacologically diverse AEDs, such as ethosuximide (ETX, active primarily in the pentylenetetrazole-induced seizure model (*sc*PTZ)), levetiracetam (LEV, active in the 6 Hz (32 mA) model), and lacosamide (LCS, active in both maximal electroshock (MES) and 6 Hz (32/44 mA) seizure models). As a result, we obtained hybrid compounds with potent and broad-spectrum anticonvulsant activity sharing the pharmacological properties of all aforementioned AEDs. The most beneficial anticonvulsant and safety profile was observed for compound **KA-11** (Figure 1). In addition to extensive anticonvulsant activity of **KA-11** (MES, *sc*PTZ, and 6 Hz seizure models), this substance was also found to effectively decrease pain responses in the formalin-induced tonic pain, the capsaicin-induced neurogenic pain, and notably, in the oxaliplatin-induced neuropathic pain in mice.[10,11] Aiming to further improve the anticonvulsant activity of **KA-11**, particularly in the MES and 6 Hz (32 mA), we now developed a new series of (2,5-dioxopyrrolidin-1-yl)(phenyl)acetamides (Figure 1A). We hypothesized that the presence of an additional aromatic ring (marked in blue) in the vicinity of the amide moiety may provide an orientation

that is more comparable to that observed in the structure of lacosamide, and thus will improve protection in the MES and 6 Hz (32 mA) models in mice.

The transient receptor potential vanilloid 1 (TRPV1) is a nonselective cation channel, expressed mainly in the afferent sensory neurons, where it plays a key role in the detection of noxious painful stimuli. Thus, TRPV1 antagonists are recognized primarily as potential drugs useful in the treatment of different pain syndromes, including neuropathic pain, pain associated with inflammation and migraine, but also epilepsy, diabetes, cough, bladder disorders, and hearing loss.[15,16] Bearing in mind the aforementioned facts, the introduction of phenyl substituent (marked in blue) also brought the structure of these new compounds closer to that observed in known TRPV1 antagonists e.g. piperazine carboxamide derivative – BCTC (Figure 1), and in consequence may lead to an improvement in their antinociceptive activity. We also aimed to assess the influence of certain structural modifications on anticonvulsant activity by further expansion of structural diversity, i.e. the distance between aromatic side ring and the acetamide linker (Figure 1B, marked in orange), the kind and size of the heterocyclic amine (e.g. piperazine, piperidine or diazepine, Figure 1C, marked in yellow), and finally the presence of the benzene ring as rigidified fragment of the molecule (Figure 1D, marked in green). In addition, we have assessed several ADME-Tox parameters in the *in vitro* assays as well as we attempted to elucidate the possible mechanism of action.

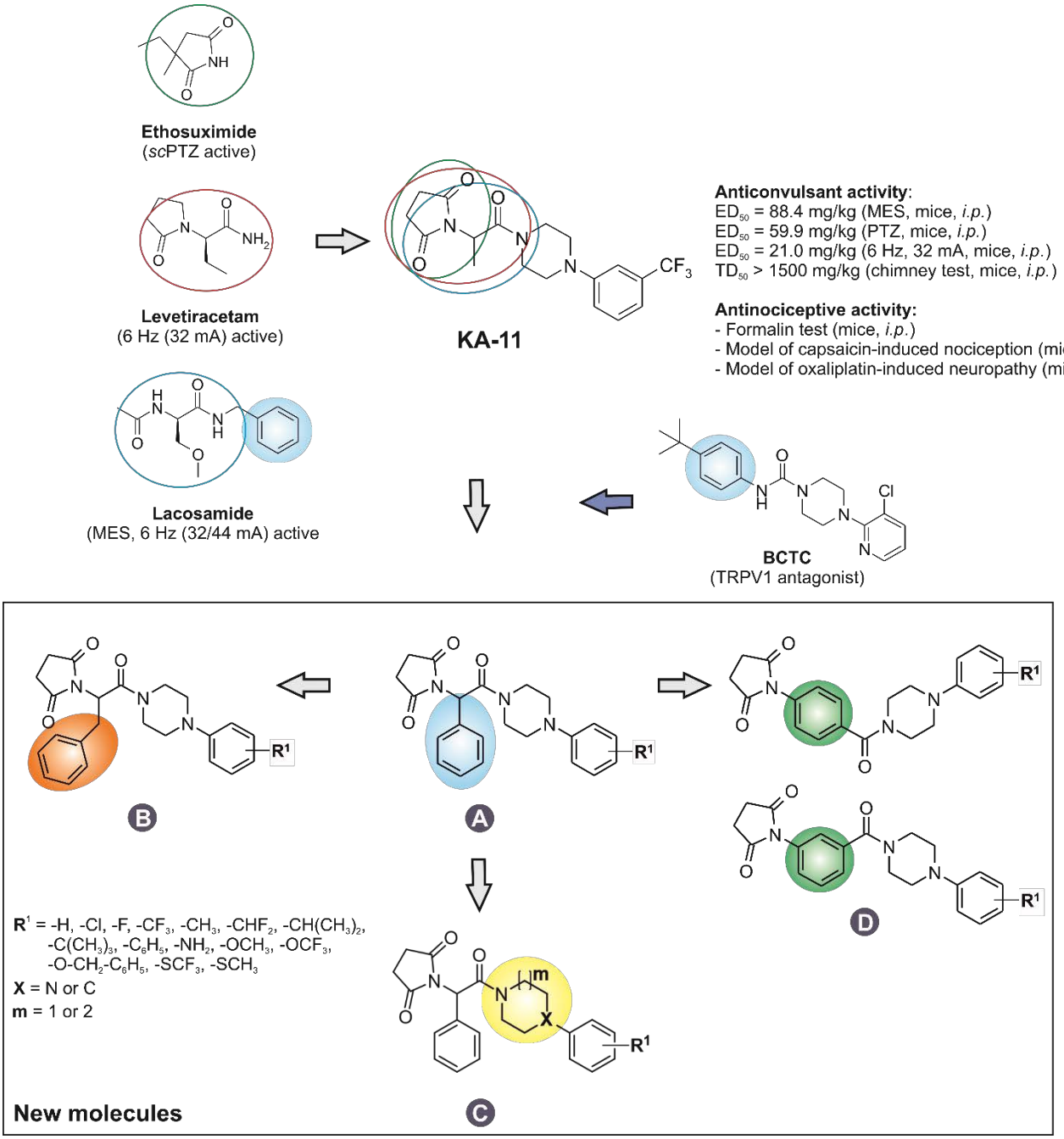
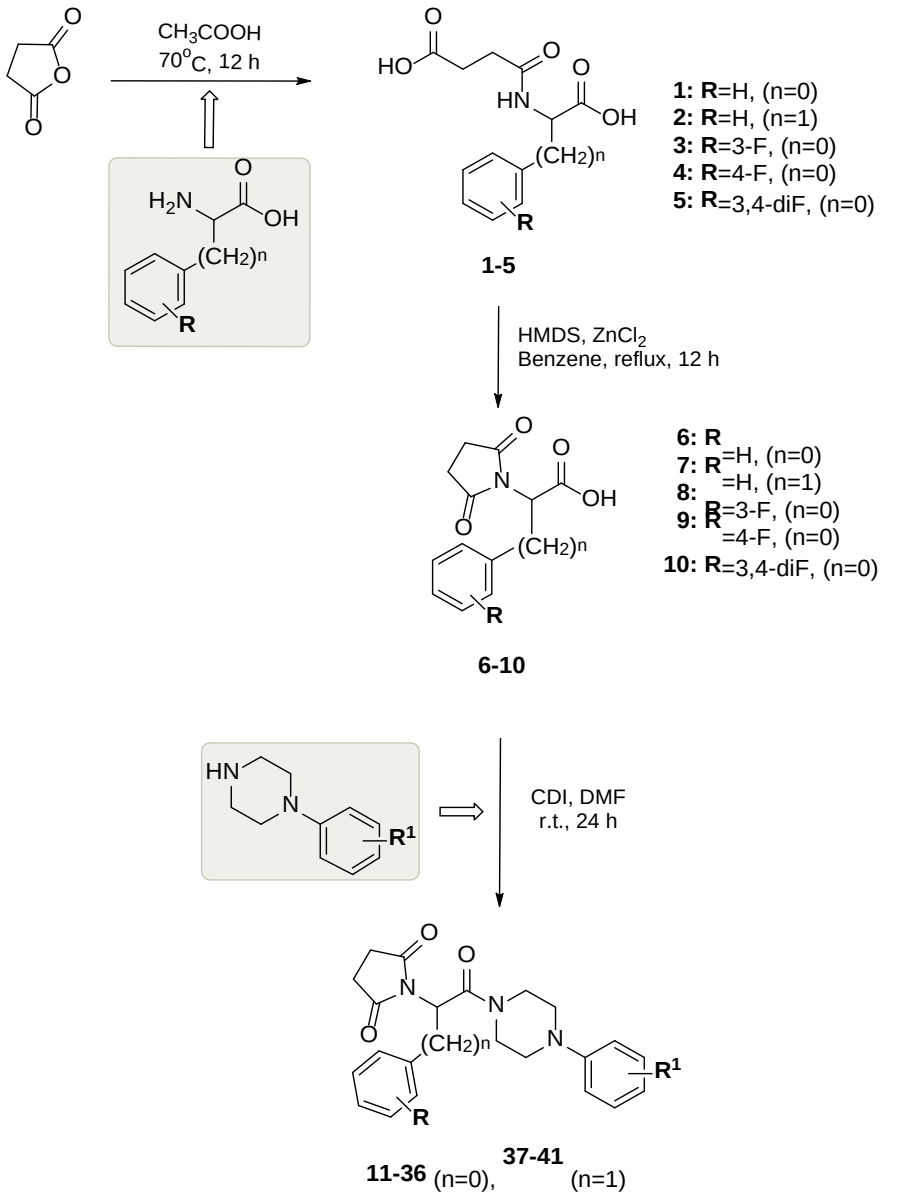


Figure 1. Design strategy and general structure of new hybrid molecules.

RESULTS AND DISCUSSION

Chemistry. The (monosubstituted)phenylpiperazinamides - compounds **11**–**41** (Series 1) were obtained by using a multi-step procedure according to Scheme 1. First, we synthesized succinamic acids (**1–5**) by reacting equimolar amounts of commercially available succinic anhydride with DL-phenylglycine (or its fluorinated analogs) or DL-phenylalanine. Next, the hexamethyldisilazane (HMDS)-promoted cyclization reaction of **1**–**5** was performed according to a previously reported method,[9] yielding intermediate monocarboxylic acids **6**–**10**. The final compounds **11**–**41** were obtained by coupling **6**–**10** with diverse (monosubstituted)phenylpiperazines (containing both electron-withdrawing or electron-donating atom/group) in the presence of carbonyldiimidazole (CDI) as the coupling reagent. This reaction was performed at room temperature in dry dimethylformamide (DMF), and its progress was monitored using the high-performance liquid chromatography (HPLC) (completion at ~24 h).



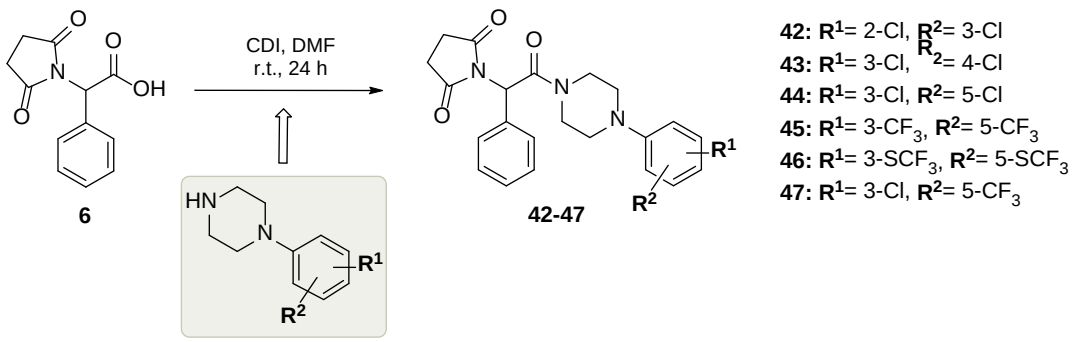
1: **R**=H, (n=0)
2: **R**=H, (n=1)
3: **R**=3-F, (n=0)
4: **R**=4-F, (n=0)
5: **R**=3,4-diF, (n=0)

6: **R**=H, (n=0)
7: **R**=H, (n=1)
8: **R**=3-F, (n=0)
9: **R**=4-F, (n=0)
10: **R**=3,4-diF, (n=0)

11: **R**= H, **R1**= H
12: **R**= H, **R1**= 2-Cl
13: **R**= H, **R1**= 3-Cl
14: **R**= H, **R1**= 4-Cl
15: **R**= H, **R1**= 2-F
16: **R**= H, **R1**= 3-F
17: **R**= H, **R1**= 4-F
18: **R**= H, **R1**= 2-CH_3
19: **R**= H, **R1**= 3-CH_3
20: **R**= H, **R1**= 4-CH_3
21: **R**= H, **R1**= 2-CF_3
22: **R**= H, **R1**= 3-CF_3
23: **R**= H, **R1**= 4-CF_3
24: **R**= H, **R1**= 3-OCH_3
25: **R**= H, **R1**= 3-OCF_3
26: **R**= H, **R1**= 3-SCH_3
27: **R**= H, **R1**= 3-SCF_3
28: **R**= H, **R1**= 3-CHF_2
29: **R**= H, **R1**= 3-NH_2
30: **R**= H, **R1**= 3-$CH(CH_3)_2$
31: **R**= H, **R1**= 3-$C(CH_3)_3$
32: **R**= H, **R1**= 3-C_5H_6
33: **R**= H, **R1**= 3-O-$CH_2C_5H_6$
34: **R**= 3-F, **R1**= 3-CF_3
35: **R**= 4-F, **R1**= 3-CF_3
36: **R**= 3,4-diF, **R1**= 3-CF_3
37: **R**= H, **R1**= H
38: **R**= H, **R1**= 3-Cl
39: **R**= H, **R1**= 3-CH_3
40: **R**= H, **R1**= 3-CF_3
41: **R**= H, **R1**= 3-OCF_3

Scheme 1. Synthesis of intermediates **1**–**10** and target compounds **11**–**41** (Series 1).

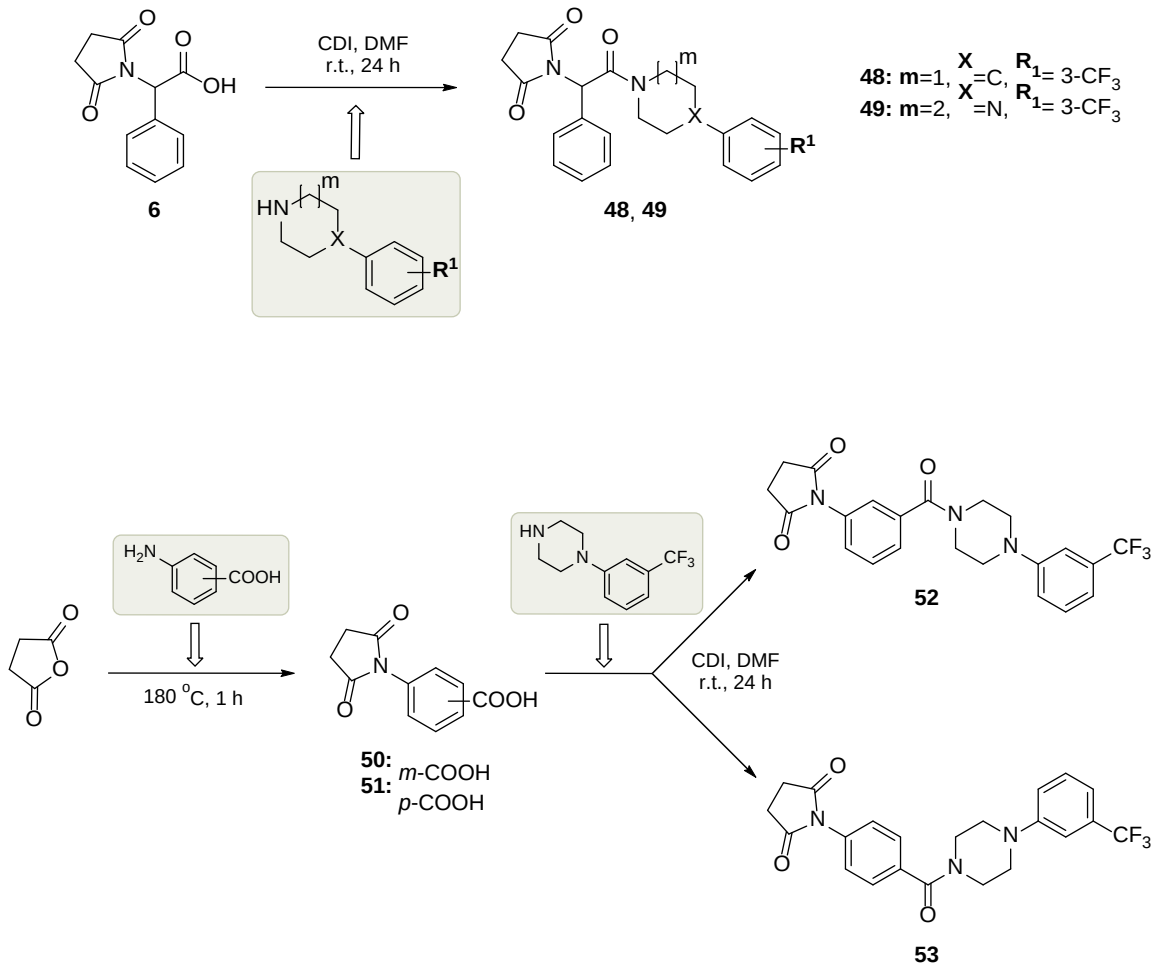
Based on the *in vivo* anticonvulsant activity data obtained for compounds representing Series 1, in the aim of finding the optimal substitution pattern of the phenylpiperazine moiety, the following structural modifications were focused on substances containing two electron withdrawing atoms/groups at the aromatic ring (Series 2). In this case we tested at first different position of chlorine atoms as follow 2,3-diCl (**42**), 3,4-diCl (**43**), and 3,5-diCl (**44**); notably for each compound we retained one chlorine at position-3 that appeared to be especially preferable for the anticonvulsant activity of the monochloro-derivative **13** (see Table S1 and Table 1). As a result, the most potent anticonvulsant activity was noticed for 3,5-diCl derivative **44**, therefore the substitution of other molecules was restricted only to position 3 and 5. The structures and synthetic pathway for compounds of Series 2 are depicted on Scheme 2.



Scheme 2. Synthesis of target compounds **42**–**47** (Series 2).

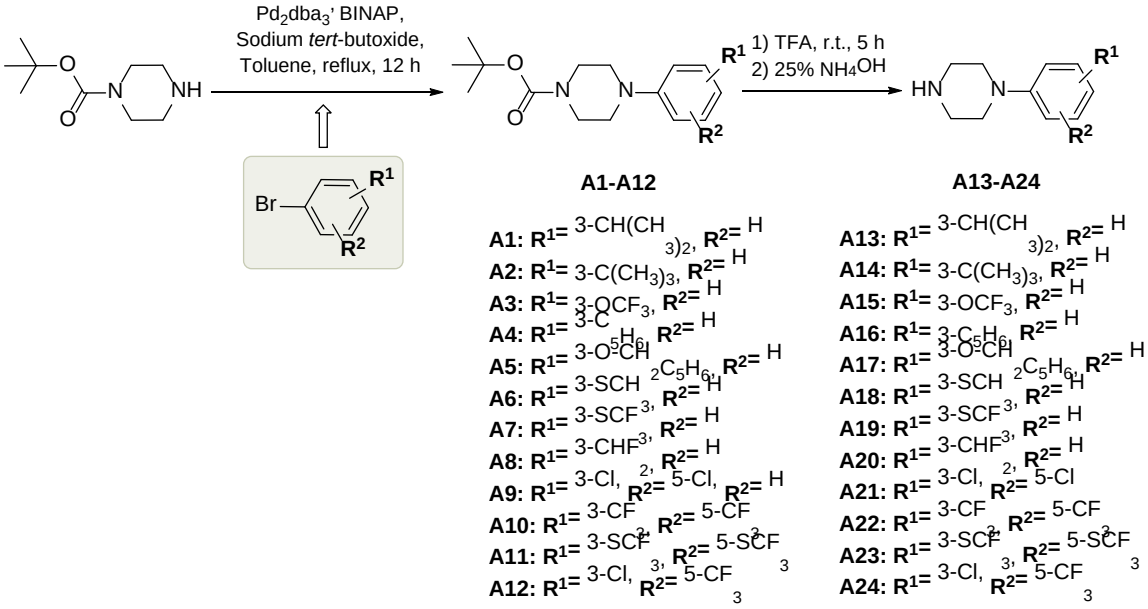
Finally, with the aim of examining the influence of the piperazine moiety on anticonvulsant activity for the most potent anticonvulsant from Series 1 that is, compound **22**, the respective piperidine (**48**) and diazepine (**49**) analogs were synthesized. Furthermore, for compound **22** we obtained also two derivatives **52** and **53** with the rigid benzene ring (Scheme 3). The latter compounds were synthesized according to the two-step procedure. Thus, the cyclocondensation reaction of succinic anhydride with 3-amino- or 4-aminobenzoic acid yielded corresponding intermediates **48** and **49**, which were then coupled with 1-[3-(trifluoro-

methyl)phenyl]piperazine in the presence of CDI to form the desired compounds **50** and **51**. The structures and synthetic routs for intermediates **50**, **51** and target compounds **48**, **49**, **52**, and **53** (Series 3) are depicted in Scheme 3.



Scheme 3. Synthesis of target compounds **48**, **49**, **52**, and **53** (Series 3).

In case of compounds **25**–**28**, **30**–**33**, and **44**–**47** the staring 4-phenylpiperazines (**A13**–**A24**) were previously synthesized using the respective aryl bromides and 1-Boc-piperazine in the Buchwald–Hartwig amination reaction in the nitrogen atmosphere.[17] Removal of the *tert*-butoxycarbonyl group (Boc) with trifluoroacetic acid (TFA) followed by the neutralization with 25% ammonium hydroxide produced the desired 4-phenylpiperazine derivatives **A13**–**A24** (Scheme 4).



Scheme 4. Synthesis of starting 4-phenylpiperazine derivatives **A13**–**A24**.

The target hybrid compounds were obtained in satisfying yield (>60%). The structures of initial 4-phenylpiperazine derivatives, carboxylic intermediates and desired molecules were confirmed by ^{1}H NMR, ^{13}C NMR (selected molecules), ^{19}F NMR (selected fluorinated final substances), and/or LC-MS spectra. The purity of target substances determined by UPLC method was ≥95%. The details are summarized in the Materials and Methods section and also Supporting Information.

Anticonvulsant activity. The screening approach applying animal models of epilepsy is an important and efficient pharmacological tool for the discovery of new anticonvulsants, as it enables the identification of both compounds with well-established mechanisms of action among known AEDs as well as compounds with novel and previously unidentified pharmacodynamics.[18] Therefore, the anticonvulsant properties of all final compounds was initially assessed using the MES test, which is a recognized model of tonic–clonic epilepsy in human.[19] This test allows identification of compounds able to prevent the spread of seizures when all neuronal circuits in the brain are paroxysmally active. It is worth noting that despite significant advances in epilepsy research during the past few decades, MES test is still

recognized as the most useful preclinical seizure model. It is widely employed during the early identification and screening of new AED candidates, as it may help to identify compounds with unique mechanisms of action.[20] In this study, all final compounds were initially tested after intraperitoneal (*i.p.*) administration at a fixed dose - 100 mg/kg in mice (in groups consisting of four animals) and the protection against MES seizures was observed at two pretreatment time points of 0.5 and 2 h.

According to the results of the MES test obtained for the (monosubstituted)-phenylpiperazinamides **11**–**41** (Series 1), at least 75% protection (3 mice protected from 4 tested) was demonstrated by compounds **11**, **13**, **19**, **20**, **22**, **23**, **25**, **27**, **28**, **32**, and **35**, which was found to be satisfactory (Table S1). No lethality was observed for these substances administrated at dose of 100 mg/kg. All aforementioned compounds were found to be active predominantly after a short pre-administration time (0.5 h), whereas compounds **19**, **22**, **25**, **27** and **32** were found to be active at both time points (0.5 and 2 h). Notably, 3-CF_3 (**22**), 3-OCF_3 (**25**), and 3-SCF_3 (**27**) derivatives provided maximal (100%) protection at 0.5 and 2 h. In the next step of the pharmacological characterization, eleven aforementioned MES-active compounds were studied in the mouse 6 Hz (32 mA) model of seizures, which is a well-established and commonly used preclinical model in the discovery of new AEDs effective in human focal epilepsy (Table S1).[18] According to the results, majority derivatives (**11**, **13**, **19**, **22**, **23**, **25**, **27**, **28**, **32**, and **35**) displayed satisfactory anticonvulsant activity protecting at least 75% of the mice especially at the time point of 0.5 h (only compound **13** showed potent protection at both pretreatment times). The maximal (100%) protection was observed for 3-Cl (**13**), 3-CH_3 (**19**), 3-CF_3 (**22**), 3-OCF_3 (**25**), 3-SCF_3 (**27**), and 3-phenyl (**32**) congeners. With the aim of finding broad-spectrum anticonvulsants during the preclinical tests, all effective compounds in the MES and 6 Hz (32 mA) models (**11**, **13**, **19**, **22**, **23**, **25**, **27**, **28**, **32**, and **35**) were further studied using the *sc*PTZ test. In the latter test seizures are induced chemically and

the latency and incidence of clonic seizures are the primary endpoints (Table S2). The *sc*PTZ model tends to identify drugs that may be clinically effective against generalized non-convulsive (myoclonic, absence) seizures.[21] In this test, compound **22** was most active and displayed 100% and 75% protection at 0.5 and 2 h, respectively. A satisfactory efficacy (75%) was also observed for compounds **11**, **13**, **19**, and **28** at 0.5 h. Surprisingly, the *in vivo* data for disubstituted piperazinamides **42**–**47** (Series 2) showed beneficial anticonvulsant properties only for 3,5-dichloroderivative (**44**), which protected mice in all tests applied; MES, 6 Hz (32 mA) as well as *sc*PTZ. During further structure-activity relationship analysis (SAR) of the aforementioned derivatives, we investigated the influence of the distance between the side aromatic ring and the acetamide linker on their anticonvulsant properties (modification B, Figure 1). Thus, structural modification of the selected potent phenylglycinamides (**11**, **13**, **19**, **22**, and **25**) resulted in the production of respective phenylalaninamides (**37**–**41**, see Scheme 1), which were subjected to the MES test. According to the results (Table S1), elongation of the distance between the aforementioned structural fragments (acetamide and side aromatic ring) caused a decrease in the anticonvulsant activity, which yielded compounds with efficacy equal to 50%. Regarding the influence of heterocyclic amine on the anticonvulsant activity (modification C, Figure 1), the presence of piperazine moiety proved to be especially beneficial, as we observed 75% protection in the MES and 6 Hz (32 mA) seizure models for piperidine analog **48** or lack of activity in case of diazepine derivative **49** (Table S3). Similar observations were noted for analogs with rigidified benzene ring **48** (*meta*-isomer) and **49** (*para*-isomer), which may be regarded as the stiffened analogs of the most potent compound **22** (Table S3, modification D, Figure 1).

In summary, based on the above screening data, (2,5-dioxopyrrolidin-1-yl)(phenyl)acetamides with unsubstituted phenylpiperazine (**11**) and respective 3-Cl (**13**), 3-CH_3, (**19**), 3-CF_3 (**22**), 3-CHF_2 (**28**), and **44** (3,5-diCl) analogs revealed broad-spectrum

anticonvulsant activity and potent protection in the MES, *sc*PTZ, and 6 Hz (32 mA) seizure models. The replacement of the methyl or trifluoromethyl groups onto *para*-position (**20**, **23**), the introduction of trifluoromethoxy (**25**), trifluorotiomethyl (**27**), and phenyl (**32**) moieties at *meta*-position, the introduction of fluorine atom at *para*-position of the side aromatic ring (**35**), or exchange of the piperazine ring to piperidine moiety (**48**) yielded compounds that are active predominantly in the MES and 6 Hz (32 mA) tests. All other structural modifications such as the other modes of substitution of both aromatic areas, insertion of the benzyl moiety at the acetamide linker, insertion of the diazepine in place of piperazine moiety, or the presence of benzene ring as the rigid fragment of molecules decreased the anticonvulsant activity or rendered the compounds inactive.

Continuing the preclinical characterization of the aforementioned hybrids, for compounds that showed at least of 75% protection in preliminary studies, their median effective doses (ED_{50}) in the given seizure model and median doses causing neurological impairment (TD_{50}) in the rotarod test were determined. These two pharmacological parameters were used to calculate the protective indexes (PIs), which are a measure of potential therapeutic window of the tested agent. In aim of assessing the potential for clinical utility of the compounds obtained herein, we performed the same experiments using AEDs with a narrow spectrum of efficacy in the preclinical studies (LEV – effective in 6 Hz, 32 mA test; ETX – effective in *sc*PTZ test); anticonvulsants with wider activity (LCS – effective in MES and 6 Hz (32/44 mA) tests); and with VPA, which is recognized as a broad-spectrum AED (active in MES, *sc*PTZ, and 6 Hz (32/44 mA) seizure models). Notably, VPA possesses multiple mechanisms of action and has efficacy against the widest range of epilepsy types and multiple epileptic syndromes. Table 1 summarizes the results obtained.

Table 1. The quantitative pharmacological parameters ED_{50}, TD_{50}, and PIs in mice *i.p.*

Compd	TPE (h)[a]	ED_{50} MES (mg/kg)[b]	ED_{50} 6 Hz (32 mA) (mg/kg)[c]	ED_{50} *sc*PTZ (mg/kg)[d]	TD_{50} (mg/kg)[e]	PI (TD_{50}/ED_{50})[f]
11	0.5	91.1 (84.5–98.5)	83.5 (65.9–105.7)	100.0 (87.3–114.5)	>300	>3.3 (MES) >3.6 (6 Hz) >3.0 (*sc*PTZ)
13	0.5	36.9 (32.2–42.4)	39.5 (28.3–55.2)	52.6 (37.7–73.5)	143.8 (113.8–181.9)	3.9 (MES) 3.6 (6 Hz) 2.7 (*sc*PTZ)
19	0.5	37.2 (33.2–41.8)	35.5 (25.6–49.4)	57.6 (34.0–97.9)	171.0 (157.2–185.9)	4.6 (MES) 4.8 (6 Hz) 3.0 (*sc*PTZ)
22	0.5	23.7 (18.4–31.2)	22.4 (17.4–28.8)	59.4 (37.5–94.1)	195.7 (132.7–288.6)	8.2 (MES) 8.7 (6 Hz) 3.3 (*sc*PTZ)
23	0.5	94.3 (85.0–104.8)	>100	–	>300	>3.2 (MES)
32	0.5	27.4 (23.0-32.5)	14.1 (14.2–48.5)	–	66.2 (52.6–83.2)	2.4 (MES) 4.7 (6 Hz)
27	0.5	36.2 (31.3–41.8)	15.8 (7.05–19.8)	–	150.1 (133.9–168.2)	4.1 (MES) 9.5 (6 Hz)
28	0.5	97.8 (82.0–116.6)	63.0 (59.7–66.5)	94.4 (85.0–104.8)	274.2 (257.8–291.7)	2.8 (MES) 4.4 (6 Hz) 2.9 (*sc*PTZ)
32	0.5	43.9 (40.8–47.3)	26.2 (8.4–23.5)	–	75.6 (65.1–87.8)	1.7 (MES) 2.9 (6 Hz)
35	0.5	56.4 (39.9–79.8)	48.3 (43.1–54.1)	–	>300	5.3 (MES) 6.2 (6 Hz)
44	0.5	68.5 (62.1–75.6)	17.7 (13.7–22.8)	>100	>300	>4.4 (MES) >17.0 (6 Hz)
48	0.5	81.8 (76.3–87.7)	41.1 (27.2–62.0)	–	254.3 (221.2–292.4)	3.1 (MES) 6.2 (6 Hz)
ETX[g]	0.25	n.a.	>200	140.4 (115.8–170.2)	318.0 (295.8–341.9)	2.3 (*sc*PTZ)
LCS[g]	0.5	9.2 (8.5–10.0)	5.3 (3.5–7.8)	n.a.	46.2 (44.5–48.0)	5.0 (MES) 8.8 (6 Hz)
LEV[g]	1.0	>500	15.7 (10.4–23.7)	n.a.	>500	>31.8 (6 Hz)
VPA[g]	0.5	252.7 (220.1–290.2)	130.6 (117.6–145.2)	239.4 (209.2–274.1)	430.7 (407.9–454.9)	1.7 (MES) 3.3 (6 Hz) 1.8 (*sc*PTZ)

Values in parentheses are 95% confidence intervals determined by probit analysis.[22]

[a] Time to peak effect.
[b] ED_{50} (MES-maximal electroshock seizure test).
[c] ED_{50} (6 Hz-seizure test, 32 mA).
[d] ED_{50} (*sc*PTZ- subcutaneous pentylenetetrazole seizure test).
[e] TD_{50} (NT-acute neurological deficit determined in the rotarod test).
[f] Protective index (TD_{50}/ED_{50}).
[g] Reference AEDs: Ethosuximide (ETX), Lacosamide (LCS), Levetiracetam (LEV), and Valproic acid (VPA) tested in the same conditions. TPEs for model AEDs taken from own experiments or literature.[23]
A dash indicates - not tested, n.a. - non-active.

The obtained quantitative data indicated five compounds **11**, **13**, **19**, **22**, and **28** as broad-spectrum anticonvulsants, which were effective in all the tests performed. These compounds revealed generally higher activity in electrically evoked seizure models, such as MES and 6 Hz (32 mA), compared to chemically induced convulsions, *sc*PTZ test. Among the aforementioned compounds, the most promising was compound **22**, which demonstrated distinctly more potent anticonvulsant activity in the MES, 6 Hz (32 mA), and *sc*PTZ models than that observed for the broad-spectrum AED, that is VPA, with ~10-fold (MES), ~6-fold (6 Hz, 32 mA), and ~4-fold (*sc*PTZ) higher potency. Moreover, **22** displayed substantially better therapeutic window (PI values) than those of VPA. Notably, VPA is still recognized as one of the most relevant first-line AEDs used for treatment of all types of seizures in patients. Compound **22** exhibited a lower potency than that of LCS (a dual-acting AED (MES/6 Hz (32 mA)) and LEV, which was found to be effective only in the 6 Hz (32 mA) model. Compound **22** was found to have a wider therapeutic window (PI) in the MES test and revealed comparable safety profile in the 6 Hz (32 mA) model than that of LCS. Unfortunately, compound **22** showed distinctly worse PI value than that of LEV, which is well-known to have an excellent tolerability profile, also clinically. Furthermore, compound **22** was found to be more potent in the *sc*PTZ test than both ETX and VPA, which are recognized as reference AEDs for this model of chemically-induced seizures. In the *sc*PTZ test, an anticonvulsant effect is considered when an animal is protected from the clonic seizures with loss of righting reflex. Figure 2 shows that compound **22**, compared to vehicle-treated group, prolonged the latency time to first seizure episode in a dose-dependent manner, from 485.4 ± 57.6 s (control) to 827.8 ± 218.9 s (70.54%), 1318 ±

253.1 s (171.53%), and 1504 ± 199.2 s (209.85%) for doses 30, 60, and 80 mg/kg, respectively. Similar effect was observed for ETX and VPA; however, these *sc*PTZ-active AEDs were effective in distinctly higher doses. Efficient dose-dependent prolongation of the latency time to first episode of clonic seizures was also observed for **11** and **19** (see Figure S1). Importantly, the most effective compounds (**11**, **13**, **19**, **22**), and reference AEDs effective in the *sc*PTZ model decreased the number and severity of seizures and decreased the lethality of mice in the aforementioned test (Table S4).

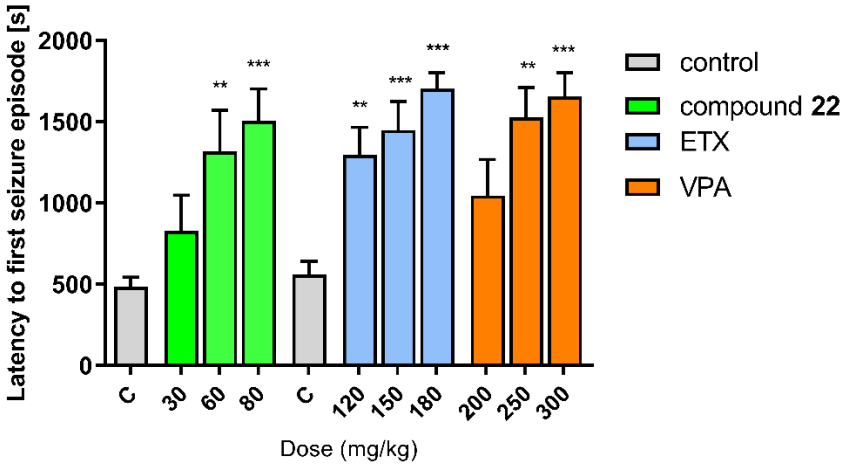


Figure 2. The latency time to first PTZ seizure episode for **22**, and reference AEDs: ETX and VPA. Each value represents the mean ± SEM obtained from 6–10 mice. Statistical analysis: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test. The compounds were administered *i.p.* 0.25 h (ETX) or 0.5 h before the test (**22**, VPA). Significant difference compared to the control group: **p < 0.01, ***p < 0.001. Data for ETX and VPA from ref.[13]

The 3-Cl (**13**) and 3-CH_3 (**19**) derivatives also showed potent and broad-spectrum anticonvulsant activity. Despite relatively strong neurological impairment, these derivatives displayed higher activity and also better PIs than that of VPA, but unfortunately showed weaker activity specifically in the MES and/or 6 Hz (32 mA) seizure models as well as less favorable PIs than that of LCS and LEV. Interesting results were obtained for 3-SCF_3 (**27**) and 3,5-diCl (**44**) derivatives that appeared to be especially effective in the 6 Hz (32 mA) test. In this seizure model, **27** and **44** were equally effective as LEV, and furthermore **44** did not affect motor

coordination of mice up to dose of 300 mg/kg that yielded a very favorable PI > 17 value. The other molecules tested revealed moderate efficacy (**13**, **28**), provided more narrow protection as demonstrated in the MES and 6 Hz seizures (**25**, **32**, **35**, and **48**), or revealed activity only in the MES test (**23**). Notably, the presence of 3-OCF_3 group (**25**) provided potent protection in the MES and 6 Hz (32 mA) seizure models, albeit with an increased neurological impairment. In summary, the introduction of the phenyl substituent in the side position on the acetamide linker, which was the most important structural modification proposed in this study (see Figure 1A), caused distinctly improved protection in the MES test for **22** compared to the respective methyl analog **KA-11**.[10] This modification also allowed to retain high activity in the 6 Hz (32 mA) and *sc*PTZ seizure models.

Antinociceptive activity. AEDs are of great importance in the pharmacotherapy of neuropathic pain and other neurological pain syndromes. Taking into consideration similarities in pathophysiology of epilepsy and pain (in particular neuropathic one), as well as the broad-spectrum and potent anticonvulsant activity of compound **22**, we hypothesized that it may also be a potent analgesic agent. To confirm this, we tested compound **22** for analgesic properties in the formalin test of tonic pain in mice. VPA was used as the reference AED, which showed broad-spectrum anticonvulsant activity, similar to compound **22**, in preclinical experiments (e.g., MES, *sc*PTZ, and 6 Hz test models). Pregabalin (PGB) was used as the second reference compound to compare the activity of **22**. In fact, PGB (together with gabapentin) is one of the two the most often used AEDs in the clinical management on neuropathic pain. Therefore, the antinociceptive activity of compound **22** may provide an essential clinical advantage over VPA, which is widely used in the pharmacotherapy of seizures, but its role in the treatment of pain is much less significant.

The formalin test is one of the most useful screening models for testing potentially clinically-relevant antinociceptive molecules because of its ease of administration and

standardization and validation with reference drugs.[24] Subcutaneous injection of formalin results in a focal injury that stimulates and then damages sensory endings. Two distinct phases of nociceptive response are associated with immediate activation of nociceptors and sensitization of spinal reflex circuits during phase I and phase II responses, respectively.[25] Moreover, it has been recently suggested that formalin injection results in pathological changes that resemble those observed in nerve injury.[26] Altered functions of sodium and calcium channels are probably involved in the mechanism of the both phases, thus making the formalin test a valuable method in the investigation of analgesic properties of anticonvulsant agents affecting sodium and/or calcium currents. Sodium channel blockers preferentially attenuate the nociceptive response in phase II of the test, whereas gabapentin or pregabalin (PGB), which bind to alpha$_2$-delta ($\alpha_2\delta$) site of voltage-gated calcium channels, are active in both phases.[27] As it is presented in Figure 3, the *i.p.* administration of compound **22** prior to the subcutaneous injection of formalin significantly attenuated the nociceptive response in mice in both phases of the test. Its ED_{50} value in phase I was found to be 28.5 mg/kg, whereas the ED_{50} value in phase II was found to be 12.4 mg/kg. Compound **22** was distinctly more effective in both phases compared to previously described **KA-11** (ED_{50} (phase I) = 71.2 mg/kg and ED_{50} (phase II) = 29.3 mg/kg) (for **KA-11** structure see Figure 1).[11] The results obtained in our laboratory for VPA revealed that this drug was ineffective in phase I, and the activity in phase II was moderate (ED_{50} = 132.9 mg/kg)[9], and much lower than that obtained for compound **22**. The administration of PGB resulted in a significant decrease of nociceptive response in the early phase only at the highest dose of 30 mg/kg, which is in contrast with the potent activity of compound **22**. Moreover, PGB was not as active as compound **22** in the late phase of the test (ED_{50} = 20.97 mg/kg). Taking into account the fact that PGB is clinically useful in the treatment of neuropathic pain, these results suggested highly relevant analgesic properties of compound **22**.

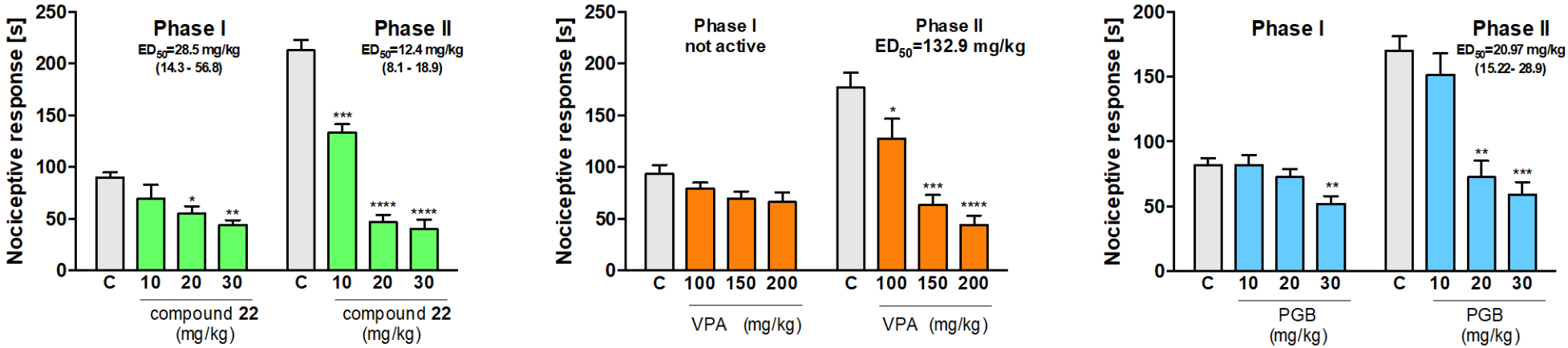


Figure 3. Figure 3. Antinociceptive activity of compound **22** and reference drugs; valproic acid (VPA), pregabalin (PGB) in the formalin test. Results are shown as time of licking in phase I (0–5 min after intraplantar injection of formalin) and in phase II (15–30 min after formalin injection). Each value represents the mean ± S.E.M. for 8-10 animals. Statistical analysis: one-way ANOVA followed by *post hoc* Dunnett's test. Statistical significance compared to vehicle-treated animals (Tween): *$p < 0.05$, **$p<0.01$, ***$p < 0.001$, ****$p < 0.0001$. C-control group.

Influence on spontaneous locomotor activity. We tested the influence of compound **22** on spontaneous locomotor activity to investigate its sedative properties. High sedative activity of anticonvulsants is considered to be an undesirable property, which may lead to an incorrect or ambiguous interpretation of the *in vivo* results. Moreover, it may prove to be an additional limitation in case of potential clinical application of new drug candidates. The data presented in the Figure S2 indicate that compound **22** has a moderate sedative activity (ED_{50} = 22.7 mg/kg), which is comparable to these dose producing analgesic effects. Therefore, the activity of compound **22** may be at least partially associated with sedative properties, however these effects do not dominate over its analgesic or anticonvulsant properties. Our observations are supported by the results obtained with PGB, which also decreased spontaneous locomotor activity of mice at the analgesic dose of 30 mg/kg. The obtained results show that the moderate sedative properties of this drug may be acceptable at the preclinical as well as clinical level.

***In vitro* radioligand binding studies and functional assays.** The traditional discovery and development process of potential therapeutic agents for the treatment of epilepsy often involves testing in animal seizure models prior to the determination of their possible mechanism of action.[28] This strategy enables to identify compounds acting on either well-known molecular targets for anticonvulsants or compounds with novel and unique mechanism of action. In addition, the currently used AEDs possess various mechanisms of action, and notably in many cases, their precise pharmacodynamic profiles were identified after their regulatory approval and introduction to the market (e.g. levetiracetam).

Compound **22** demonstrated broad-spectrum anticonvulsant and analgesic activity, thus, in order to identify the potential targets of compound **22**, we carried out binding studies with several voltage-gated or ligand-gated channels and GABA-transporter as the most common molecular targets for anticonvulsants. Moreover, considering the potential pro-arrhythmic activity, the interaction of **22** with potassium channel (hERG) was also investigated (Table 2).

Table 2. *In vitro* binding/functional assays for **22** and (concentration of 100 μM)

Binding studies	Source	% Inhibition of control specific binding[a]
Na^+ channel (site 2)	rat cerebral cortex	**82.5**
L-type Ca^{2+} (dihydropyridine site, antagonist radioligand)	rat cerebral cortex	**82.3**
L-type Ca^{2+} (diltiazem site, antagonist radioligand)	rat cerebral cortex	**69.6**
L-type Ca^{2+} (verapamil site, antagonist radioligand)	rat cerebral cortex	**58.2**
NMDA (antagonist radioligand)	rat cerebral cortex	26.2
Na^+ channel (site 1)	rat brain	21.0
N-type Ca^{2+} (antagonist radioligand)	rat cerebral cortex	5.2
GABA transporter (antagonist radioligand)	rat cerebral cortex	1.1
Potassium channel (hERG)	human recombinant (HEK-293 cells)	25.8
Functional studies		% Inhibition of control agonist response[a]
TRPV1 (VR1) *(h)* (antagonist effect)	human recombinant (CHO cells)	**71.7**
$Cav_{1.2}$ (L-type) *(h)* calcium ion channel cell based antagonist calcium flux assay	human recombinant (HEK-293 cells)	**104.0**

[a] **Results showing activity higher than 50% are considered to represent significant effects of the test compounds**; results showing an inhibition between 25% and 50% are indicative of weak effect; results showing an inhibition lower than 25% are not considered significant and mostly attributable to variability of the signal around the control level. Binding or functional studies were performed commercially in Cerep Laboratories (Poitiers, France). A dash indicates - not tested.

According to the obtained results, compound **22** revealed significant binding to voltage-gated sodium channels (site 2) and calcium channels (L-type, three sites) at a high concentration of 100 μM. Importantly, in case of the calcium channels, the highest affinity was observed for the dihydropyridine site, then the diltiazem site, and finally the verapamil site. Furthermore, the L-type calcium ion channel cell-based flux studies (see Table 2) revealed that **22** possesses an

antagonist activity. It should be stressed here that L-type voltage-gated calcium channels are widely distributed in the CNS, mostly post-synaptically and are responsible for regulating of neuronal firing. Notably, the most recent data indicate the involvement of central L-type calcium ion channels in pathological states associated with persistent pain (including neuropathic one).[29,30] The **22** molecule did not interact or revealed only weak interaction with NMDA receptors, voltage-gated sodium channels (site 1), N-type calcium channels, GABA transporter, and notably with potassium channel (hERG) at concentration of 100 μM; thus, we can conclude that it has a low risk of pro-arrhythmic activity. It should be stressed here that the aforementioned binding results, may only suggest voltage-gated sodium and calcium channels (L-type) as potential molecular targets for hybrid compounds reported herein. Consequently, there is a probability that both anticonvulsant and antinociceptive activity may result from additional, and currently undefined mode of action.

Compound **22** shares some structural similarities (e.g. aromatic ring position and amine fragment) with that of several TRPV1 antagonists (e.g., BCTC, see Figure 1). Therefore, in the next step, we determined the antagonist activity of compound **22** against TRPV1 receptor. Importantly, several recent neurobiological studies implicate central TRPV1 in the induction of epileptic seizures, and they have suggested that the inhibition of aforementioned channel is a new mechanism of action for anticonvulsants.[31,32] The results of functional assays (see Table 2 and Figure S3) confirmed the TRPV1 channel antagonist activity of compound **22** (IC_{50}=44 μM, K_B= 5.8 μM). Therefore, we hypothesize that TRPV1 antagonism may be involved, especially in the antinociceptive or/and in the anticonvulsant activity of compound **22**. Notably, it is suggested that TRPV1 desensitization, besides the influence on sodium and calcium conductance, is one of the crucial mechanisms of action responsible for anticonvulsant activity of cannabidiol (CBD),[33,34] which was proved to be effective against the drug-resistant epilepsy (especially in Dravet and Lennox-Gastaut syndromes in children).[35,36] It is worthy of note that

the comparison of the binding/functional profile for active as anticonvulsants vs. inactive compounds, **27**, **44** and **26** respectively, proved distinctly more p otent interaction with calcium channels (L-type), TRPV1 receptors as well as voltage-gated sodium channels (site 2) for **27** and **44**, which showed a potent protection in the MES and 6-Hz (32 mA) seizure tests (Table S5). In summary, the *in vitro* studies may indicate a multifunctional mechanism of action for the lead compound **22** that may explain its broad pharmacological activity *in vivo*.

***In vitro* ADME-Tox assays.** These studies are performed during the early stages of the drug discovery process, which enables us to evaluate the drug-like properties of the new compounds in parallel to the investigation of their efficacy. The selected ADME-Tox parameters of the most promising compound, **22**, were assessed *in vitro* using cytochrome (CYP) enzymes and human liver microsomes (HLMs). The results obtained summarized in Table 3.

Table 3. ADME-Tox parameters of **22** determined by *in vitro* methods

Human Cl_{int} (mL/min/kg)	5.8[a]
Metabolic pathways in human	dehydrogenation (**M1**)[b] hydroxylation (**M2**) hydroxylation with ketone reduction (**M3**)
CYP3A4 activity (% of control ± SD at 10 μM)	92.7 ± 8.2[c]
CYP2D6 activity (% of control ± SD at 10 μM)	120.4 ± 2.9[c]

[a] Data from four independent reactions, each terminated at different time point (5, 15, 30 or 45 min).
[b] Main metabolic pathway.
[c] Tested in triplicate.

The *in vitro* metabolic stability of compound **22** was determined by using HLMs. The Cl_{int} values were calculated by measuring the disappearance of compound **22** with time, according to the previously described protocols and formulas.[37–39] The results have shown an excellent, low Cl_{int} values (5.8 ml/min/kg) after incubating compound **22** with HLMs. Moreover, the metabolic pathways and the most probable structure of metabolites were

determined by using ultra performance liquid chromatography-mass spectrometer (UPLC-MS), supported by the predicted *in silico* (MetaSite 5.1.1 program), which indicates the most probable sites of metabolism of compound **22**. As a result, three metabolites **M1**–**M3** were obtained after the process of dehydrogenation (**M1**), hydroxylation (**M2**), and hydroxylation with ketone reduction (**M3**) and were identified using *in vitro* models (Table 3, Figures S4–S8).

We performed luminescence-based assays for the most important isoforms of cytochrome P450, namely, CYP3A4 and CYP2D6, which are responsible for the metabolism of ~40%−50% of all marketed drugs. As a result, the effect of compound **22** on the activity of CYPs may be a potential source of clinically important DDIs. Both CYP3A4 and CYP2D6 assays were performed according to the manufacturer's protocol (see Supplementary material). According to the results, compound **22** showed no effect on CYP3A4, whereas CYP2D6 showed slight induction effect at concentrations ≥10 μM (Table 3) (Figures S9 and S10). In conclusion, none or slight effect of compound **22** on the most relevant CYP subtypes, as well as its influence on Pgp (P-glycoprotein) efflux transporter activity determined only at the highest used concentration 100 μM, indicates low risk of potential DDI.

CONCLUSION

In the present study we have synthetized and characterized a new series of anticonvulsants with hybrid structure based on the pyrrolidine-2,5-dione core fragment. The preclinical data in mice revealed that these hybrid compounds exhibited potent protection and showed broad-spectrum activity in different animal models of seizures, such as the MES, *sc*PTZ, and 6 Hz (32 mA). The most promising anticonvulsant properties were found for compound **22**, which revealed its potential as a broad-spectrum antiepileptic agent. Notably, compound **22** showed better protection and more favorable safety profile than that of VPA, which is recognized as the most clinically valuable broad-spectrum AED. The more detailed

pharmacological evaluation indicated a potent antinociceptive activity of compound **22** in the formalin-induced tonic pain model in mice. The results from *in vitro* studies may suggest that the mechanism of action of compound **22** is probably multifunctional. Functional assays showed antagonist activity of the tested compound toward the TRPV1 receptor and L-type calcium channels. Compound **22** revealed high metabolic stability on HLMs. This molecule showed no or very slight influence on CYP3A4, CYP2D6, but only at high concentration. Overall, the obtained results indicate that compound **22** may be a candidate for further development with potential therapeutic utility in epilepsy and neuropathic pain conditions.

MATERIALS AND METHODS

Chemistry. All reagents and solvents came from commercial suppliers. Melting points were measured in melting point apparatus (Büchi Labortechnik, Flawil, Switzerland) in open capillaries. The purity/homogeneity of the intermediates and target compounds was assessed by TLC and UPLC methods. TLC was performed on silica gel 60 F_{254} pre-coated aluminum sheets (Macherey-Nagel, Düren, Germany). The following developing systems were used: S_1–DCM:MeOH (9:0.2; *v/v*), S_2–DCM:MeOH (9:0.3; *v/v*), S_3–DCM:MeOH (9:0.5; *v/v*), S_4–DCM:MeOH (9:1; *v/v*). The spots were observed in UV light (λ = 254 nm). The mass spectra were obtained on Waters ACQUITY™ TQD system (Waters, Milford, CT, USA) equipped with the MS-TQ detector and UV-Vis-DAD eλ detector. The ACQUITY UPLC BEH C18, 1.7 μm (2.1 × 100 mm) column was used with the VanGuard Acquity UPLC BEH C18, 1.7 μm (2.1 × 5 mm) (Waters, Milford, CT, USA). For UPLC/MS analyses the 1 mg/mL solutions of each substance were prepared MeCN/water mixture (1:1; *v/v*). The following conditions were applied: eluent A (water + 0.1% HCOOH), eluent B (MeCN + 0.1% HCOOH), flow rate of 0.3 mL/min, gradient of 5–100% B over 10 min, injection volume - 10 μl. The UPLC retention times (t_R) are given in minutes. Preparative column chromatography was carried out using silica

gel 60 (particle size 0.063–0.200; 70–230 Mesh ATM) (Macherey-Nagel, Düren, Germany). Elemental analyses (C, H, and N) for target compounds were performed using the elemental Vario EI III Elemental analyzer (Hanau, Germany). The results obtained were within ±0.4% of the calculated values. The NMR spectra were recorded in a Varian Mercury spectrometer (Varian Inc., Palo Alto, CA, USA), in $CDCl_3$ or DMSO-D_6 operating at 300 MHz (^{1}H NMR), 75 MHz (^{13}C NMR), and 282 MHz (^{19}F NMR) or in a JEOL-500 spectrometer (JEOL USA, Inc. MA, USA), in $CDCl_3$ operating at 500 MHz (^{1}H NMR) and 126 MHz (^{13}C NMR). Chemical shifts are given as δ values (ppm). TMS δ = 0 (^{1}H) was used as internal standard. The following abbreviations were applied in the spectra description: s (singlet), br. s (broad singlet), d (doublet), dd (double doublet), ddd (double double doublet), t (triplet), dt (doublet of triplets), td (triplet of doublets), m (multiplet).

Synthetic procedure for succinamic acids 1-5. Succinic anhydride (3.0 g, 30 mmol, 1 eq) was dissolved in 15 ml of glacial acetic acid and afterwards equimolar amount of DL-phenylglycine (4.53 g) DL-phenylalanine (4.95 g), DL-(3-fluorophenyl)glycine (5.10 g), DL-(4-fluorophenyl)glycine (5.10 g) or DL-(3,4-difluorophenyl) glycine (5.61 g) was added. The reaction mixture was heated to 70°C and next stirred for 12 h. After this time acetic acid was evaporated to dryness. The intermediates **1**–**5** were obtained in solid form after wash up with diethyl ether.

4-((Carboxy(phenyl)methyl)amino)-4-oxobutanoic acid (**1**): White solid. Yield: 87% (6.55 g); mp. 199.4–200.6ºC; TLC: R_f = 0.25 (S_4); UPLC (purity 100%): t_R = 2.77 min. LC-MS (ESI): *m/z* calcd for $C_{12}H_{13}NO_5$ $(M+H)^+$ 252.08, found 252.1.

Synthetic procedure for monocarboxylic acids 6-10. To a suspension of the appropriate succinamic acid **1**–**5** (20 mmol, 1 eq) in dry benzene (100 mL) $ZnCl_2$ (2.73 g, 20 mmol, 1 eq) was added, and the reaction mixture was heated to 80°C. Afterwards, HMDS (4.84 g, 6.25 mL, 30 mmol, 1.5 eq) in dry benzene (15 mL) was instilled over 30 min, and the reaction

was continued in reflux for additional 12 h. Next, organic solvent was evaporated under reduced pressure. After concentration, the residual solid was dissolved in DCM and extracted with 0.1 N HCl (3 × 50 mL), water (3 × 50 mL), saturated brine (3 × 50 mL). The organic solvent was dried over anhydrous Na_2SO_4 and afterwards evaporated to dryness. Intermediates **6**–**10** were obtained as solids after wash up with diethyl ether.

2-(2,5-Dioxopyrrolidin-1-yl)-2-phenylacetic acid (**6**): White solid. Yield: 90% (4.20 g); mp. 198.2–199.5ºC; TLC: R_f = 0.45 (S_4); UPLC (purity 100%): t_R = 3.41 min. LC-MS (ESI): *m/z* calcd for $C_{12}H_{11}NO_4$ $(M+H)^+$ 234.08, found 234.1. ^{1}H NMR (300 MHz, DMSO-D_6) δ 2.73 (s, 4H, imide), 5.76 (s, 1H, C<u>H</u>-COOH), 7.26–7.35 (m, 3H, ArH) 7.36–7.45 (m, 2H, ArH), 13.22 (br. s, 1H COO<u>H</u>).

Synthetic procedure for monocarboxylic acids 50 and 51. Succinic anhydride (2.0 g, 20 mmol) was suspended in 5 mL of water and 3-aminobenzoic acid or 4-aminobenzoicacid (2.74 g, 20 mmol) was gradually added. The mixture was heated at 180°C for 1 h (after distilling of water) in a term–regulated sand bath (ST 72 Roth, Karlsruhe, Germany). The oily crude products were purified by crystallization from MeOH.

3-(2,5-Dioxopyrrolidin-1-yl)benzoic acid (**50**): White solid. Yield: 78% (3.42 g); mp. 236.2–237.1ºC; TLC: R_f = 0.71 (S_4); UPLC (purity 92%): t_R = 2.73 min. LC-MS (ESI): *m/z* calcd for $C_{11}H_9NO_4$ $(M+H)^+$ 220.05, found 220.2. ^{1}H NMR (300 MHz, DMSO-D_6) δ 2.79–2.94 (m, 4H, imide), 7.46–7.66 (m, 2H, ArH), 7.89–8.17 (m, 2H, ArH), 11.75 (s, 1H, COO<u>H</u>).

Synthetic procedure for target compounds 11–47, 52, **and 53.** Carbonyldiimidazole (1.17 g, 7.2 mmol, 1.2 eq) was dissolved in 5 mL of dry DMF. Afterwards, this solution was added to solution of given intermediate **6–10**, **50** or **51** (6 mmol, 1 eq) in 10 mL of anhydrous DMF (while stirring). After 0.5 h the appropriate secondary amine (6 mmol, 1 eq) in 5 mL of anhydrous DMF was instilled. The reaction was continued for approximately 24 h at an ambient temperature and next organic solvent was evaporated to dryness. The column chromatography

was applied for purification of crude products, using the following developing systems: S_1 (**13**, **16**, **22**, **31**), S_2 (**11**, **12**, **14**–**21**, **23**–**25**, **28**, **30**, **33**, **42**–**44**, **52**, **53**), S_3 (**26**, **27**, **29**, **32**, **34**–**41**, **45**–**49**). The desired compounds were obtained as solids after evaporation of organic solvents.

1-(2-Oxo-1-phenyl-2-(4-phenylpiperazin-1-yl)ethyl)pyrrolidine-2,5-dione (**11**). White solid. Yield: 84% (1.90 g); mp. 156.7–157.4ºC; TLC: R_f = 0.35 (S_2); UPLC (purity 100%): t_R = 5.93 min. LC-MS (ESI): *m/z* calcd for $C_{22}H_{23}N_3O_3$ $(M+H)^+$ 378.17, found 378.2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.58–2.81 (m, 5H; 4H, imide, 1H, piperazine), 2.95–3.15 (m, 2H, piperazine), 3.17–3.42 (m, 3H, piperazine), 3.63–3.76 (m, 1H, piperazine), 3.92–4.05 (m, 1H, piperazine), 6.12 (s, 1H, C<u>H</u>CO), 6.80–6.91 (m, 3H, ArH), 7.19–7.28 (m, 2H, ArH), 7.29–7.47 (m, 5H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.1, 42.4, 45.8, 48.9, 49.2, 56.8, 116.5, 116.6, 120.6, 128.6, 128.6, 128.9, 129.1, 129.2, 129.8, 129.9, 133.0, 150.7, 165.0, 176.3. Anal. calcd for $C_{22}H_{23}N_3O_3$ (377.44): C: 70.01, H: 6.14, N: 11.13; Found C: 70.10, H: 6.18, N: 11.10.

Synthetic procedure for starring 4-phenylpiperazine derivatives A13–A24. Appropriate aryl bromide (10 mmol, 1 eq), Pd_2dba_3 (0.37 g, 0.4 mmol, 0.04 eq), BINAP (0.37 g, 0.59 mmol, 0.06 eq), sodium *tert*-butoxide (1.35 g, 14 mmol, 1.4 eq) and Boc-piperazine (3.74 g, 20 mmol, 2 eq) were suspended under inert gas (nitrogen) atmosphere in 50 mL of dry toluene. The reaction mixture was refluxed for 12 h, subsequently cooled and filtered through Celite 545 Merck (Darmstadt, Germany). The organic layer was washed with water and next with brine, dried over anhydrous Na_2SO_4, an finally concentrated in vacuo. The Boc-protectd amines **A1**–**A12** were purified by column chromatography using the following developing systems: S_2 (**A1**, **A3**–**A4**, **A6**–**A12**) or S_3 (**A2**, **A5**). The aforementioned intermediates were obtained as light oils. In the next step, **A1**–**A12** were dissolved in 5 mL of DCM, treated with TFA (3 eq) and stirred at room temperature for 5 h. Afterwards, the organic solvents were evaporated in vacuo. The resulting oil residue was dissolved in water (20 mL) and then 25% ammonium hydroxide was carefully added to pH = 8. The aqueous layer was extracted with

DCM (3 × 20 mL), dried over Na_2SO_4, and concentrated in vacuo to give the starting 4-phneylpiperazine derivatives **A13**–**A24** as light oils, which were used without further purification.

***Tert*-butyl 4-(3-isopropylphenyl)piperazine-1-carboxylate** (**A1**): Yellow oil, yield 87% (2.65 g); TLC: R_f = 0.45 (S_2); UPLC (purity 83%): t_R = 8.90 min. LC-MS (ESI): *m/z* calcd for $C_{18}H_{28}N_2O_2$ $(M+H)^+$ 305.22, found 305.2.

1-(3-Isopropylphenyl)piperazine (**A13**): Yellow oil, yield 99% (1.75 g); TLC: R_f = 0.28 (S_3); UPLC (purity 82%): t_R = 4.07 min. LC-MS (ESI): *m/z* calcd for $C_{13}H_{20}N_2$ $(M+H)^+$ 205.16, found 205.2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.16–1.31 (m, 7H; 6H, $CH(C\underline{H}_3)_2$, 1H, piperazine), 2.57–3.42 (m, 9H; 8H, piperazine, 1H, $C\underline{H}$-$(CH_3)_2$), 6.65–6.90 (m, 3H, ArH), 7.06–7.33 (m, 1H, ArH).

Anticonvulsant activity and acute neurotoxicity. Experiments were performed on adult male CD-1 mice weighing 22-26 g purchased from the Animal House at the Faculty of Pharmacy, Jagiellonian University Medical College, Cracow, Poland. The animals were kept at room temperature of 20 ± 2˚C under standard conditions. Initial qualitative efficacy screening was conducted in groups of four mice. To obtain the ED_{50} (*median effective dose*), at least three groups of six mice were injected with various doses of tested compounds. The anticonvulsant activity studies were approved by the Local Ethical Committee in Cracow, Poland (No 111/2016 and 73/2015) and conducted in compliance with the European Union Directive of 22 September 2010 (2010/63/EU). The substances tested were suspended in a 1% solution of Tween 80 and administered by the intraperitoneal (*i.p.*) route in a volume of 10 mL/kg body weight.

ETX and VPA were purchased in Sigma-Aldrich (St. Louis, MO, USA) and LCS and LEV from UCB Pharma (Braine l'Alleud, Belgium). The pretreatment times for the aforementioned reference AEDs were based upon information from the National Institute of

Neurological Disorders and Stroke (NINDS) Epilepsy Therapy Screening Program (ETSP).[40] Other experimental conditions including mice strain and devices applied were established in our laboratory and described in details before.[10]

The *in vivo* procedures are reported elsewhere, as follow: maximal electroshock seizure test (MES),[10] subcutaneous pentylenetetrazole seizure test (*sc*PTZ),[10] the 6 Hz (32 mA) seizure model,[41] the rotarod test for acute neurological toxicity.[42]

Antinociceptive activity. The experimental groups consisted of 6–12 adult male Albino Swiss mice (CD-1, 18–25 g). Each animal was tested only once. Immediately after the assay, the animals were sacrificed by cervical dislocation. Behavioral measurements were observed by trained observers. The *in vivo* antinociceptive assays were in accordance to Polish regulations and European Union Directive of 22 September 2010 (2010/63/EU). All procedures were carried out according to the rules of the International Council on Laboratory Animal Science (ICLAS) and were approved by the Local Ethical Committee in Cracow, Poland (No 104/2015). The tested and reference substances were suspended in 1% aqueous solution of Tween 80 and were injected *i.p.* 30 min prior to the test. Control group animals (negative control) were administered with an appropriate amount of vehicle (Tween 80, 1% aqueous solution, *i.p.*) 30 min prior to the test.

The reference drugs PGB and VPA were obtained from Sigma-Aldrich (St. Louis, MO, USA). The other substances were purchased from: 5% glucose solution (Polfa Kutno, Poland), formalin (Formalinum; P.O.Ch., Poland).

The experimental *in vivo* procedures are reported in the following literature; formalin test.[43] Each substance was tested in three doses: compound **22** and PGB (10, 20, and 30 mg/kg), VPA (100, 150, and 200 mg/kg). Before formalin application different groups of animals were injected *i.p.* with vehicle (10 mL/kg, negative control).

Influence on spontaneous locomotor activity. This test was carried out according to the procedure described elsewhere.[44] The animals were injected (*i.p.*) with **22** at doses 10, 20, 30, and 40 mg/kg and placed in the activity cages individually (30 min before experiment). The number of light-beam crossings was counted in each group during the next 30 min in 10-min intervals.

Data analysis. *Anticonvulsant activity and neurotoxicity studies.* The probit analysis[20] was used to calculate the ED_{50} and TD_{50} values with 95% confidence limits. The PI values (protective index) for the tested substance and standard AEDs were calculated by dividing the TD_{50} value, as determined in the rotarod test, by the respective ED_{50} value, as determined in the MES, *sc*PTZ, or 6 Hz (32 mA or 44 mA) tests. In aim of assessment of influence of substance on the latency time to first PTZ seizure episode the one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test was applied. The criterion for significance was set at $p < 0.05$.

Antinociceptive activity studies. Data are presented as means ± standard error of the mean (SEM). The GraphPad Prism Software (v.5) was used to analyze the vast majority of data. Statistically significant differences between groups were calculated using one-way analysis of variance (ANOVA) and the *post hoc* Dunnett's multiple comparison test or two-way analysis of variance (ANOVA) and the *post hoc* Tukey's comparison when appropriate. The criterion for significance was set at $p < 0.05$. The log-probit method was applied to statistically determine the ED_{50} values with 95% confidence limits.

***In vitro* pharmacology and ADMETox studies.** *Radioligand binding/functional assays.* Binding/functional studies were carried out commercially in Cerep Laboratories (Poitiers, France) using testing procedures reported previously. The general information is listed in Supporting Information.

Metabolic stability. These assays were performed on human liver microsomes (HLMs), purchased form Sigma-Aldrich (St. Louis, MO, USA), according to literature.[38]

Influence on recombinant human CYP3A4 and CYP2D6 P450 cytochromes. The luminescent CYP3A4 P450-Glo™ and CYP2D6 P450-Glo™ assays and protocols were provided by Promega (Madison, WI, USA).[45] The detailed procedures are reported in the literature (CYP3A4[10] and CYP2D6[38]).

The *in silico* study. The metabolic biotransformations pathways of compound **22** was predicted *in silico* by MetaSite 5.1.1 provided by Molecular Discovery Ltd (Hertfordshire, UK). The metabolic pathways and probable metabolites' structures were studied by liver computational model.[46]

Safety

No hazards or risks are associated with the reported work.

Abbreviations

ADME-Tox, absorption, distribution, metabolism, excretion, toxicity; AEDs, antiepileptic drugs; CDI, carbonyldiimidazole; DCM, dichloromethane; DMF, dimethylformamide; ETX, Ethosuximide; HLMs, human liver microsomes; HMDS, hexamethyldisilazane; 6 Hz, six-Hertz seizure test; LCS, Lacosamide; LEV, Levetiracetam; MeCN, acetonitrile; MES, maximal electroshock seizure test; MeOH, methanol; Pgp, P-glycoprotein; PGB, Pregabalin; PI, protective index (TD_{50}/ED_{50}); *sc*PTZ, subcutaneous pentylenetetrazole seizure test; SV2A, synaptic vesicle glycoprotein 2A; TFA, trifluoroacetic acid; TPE, time of peak effect; TRPV1, transient receptor potential cation channel vanilloid type 1; VPA, Valproic acid.

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Author Contributions:

M.A.: Synthesis and purification of the intermediates and final compounds, physicochemical and spectral characterization of compounds, preparation of the manuscript and Supplementary material. A.R.: *In vivo* studies – anticonvulsant and neurotoxic activity. S.M.: *In vivo* studies – antinociceptive and neurotoxic activity. G.L. and A.L.: *In vitro* studies: metabolic stability on human liver microsomes (HLMs), influence on recombinant human CYP3A4 and 2D6 cytochromes, *in silco* studies, description of the results in the manuscript, preparation of the Supplementary material. R.M.K.: Interpretation and critical review of the data. K.K.: Design of compounds, data analysis, structure–activity relationship discussion, preparation of the manuscript and Supplementary material.

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Conflict of Interest:

The authors declare no conflict of interest.

Notes

The authors declare no competing financial interest. The results are subject of the international WIPO patent application no PCT/PL2020/050001 (submitted on 5 February 2020).

Supporting Information

Physicochemical and spectral data for the starting materials (amines), intermediates, and target compounds, UPLC traces and MS spectra for target compounds, supplemental *in vivo* and *in vitro* pharmacology, ADME-Tox assays methodology, *in silico* metabolic biotransformations pathways, metabolism data (ion fragment analysis and structures of probable metabolites).

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