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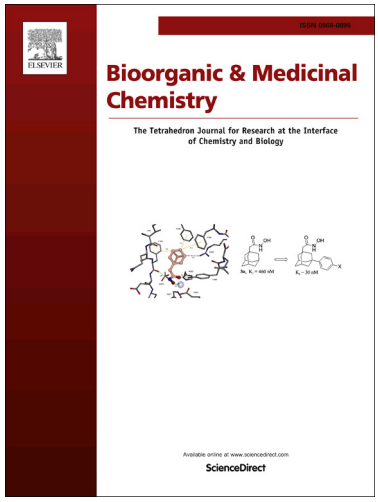

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Design, synthesis and biological evaluation of new hybrid anticonvulsants derived from *N*-benzyl-2-(2,5-dioxopyrrolidin-1-yl)propanamide and 2-(2,5-dioxopyrrolidin-1-yl)butanamide derivatives

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Abstract

The purpose of this study was to synthetize the library of 33 new *N*-benzyl-2-(2,5-dioxopyrrolidin-1-yl)propanamides, 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamides, and 2-(2,5-dioxopyrrolidin-1-yl)butanamides as potential new hybrid anticonvulsant agents. These hybrid molecules join the chemical fragments of well-known antiepileptic drugs (AEDs) such as ethosuximide, levetiracetam, and lacosamide. The coupling reaction of the 2-(2,5-dioxopyrrolidin-1-yl)propanoic acid, 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanoic acid, or 2-(2,5-dioxopyrrolidin-1-yl)butanoic acid with the appropriately substituted benzylamines in the presence of the coupling reagent, *N*,*N*-carbonyldiimidazole (CDI) generated the final compounds **4**–**36**. Spectral data acquired via ^{1}H NMR, ^{13}C NMR, and LC-MS confirmed the chemical structures of the newly prepared compounds. The initial anticonvulsant screening was performed in mice intraperitoneally (*i.p.*), using the maximal electroshock seizure (MES) and subcutaneous pentylenetetrazole (*sc*PTZ) seizure tests. The rotarod test determined the acute neurological toxicity (NT). The results of preliminary pharmacological screening revealed that 25 compounds showed protection in half or more of the animals tested in the MES and/or *sc*PTZ seizure models at the fixed dose of 100 mg/kg. The broad spectra of activity across the preclinical seizure models displayed compounds **4**, **7**, **8**, **13**, **15**, **16**–**18**, **24**, and **26**. The quantitative pharmacological studies in mice demonstrated the highest protection for compounds **4** (ED_{50} MES = 67.65 mg/kg, ED_{50} *sc*PTZ = 42.83 mg/kg); **8** (ED_{50} MES = 54.90 mg/kg, ED_{50} *sc*PTZ = 50.29 mg/kg); and **20** (ED_{50} *sc*PTZ = 47.39 mg/kg). These compounds were distinctly more potent and provided better safety profiles in the rotarod test compared to valproic acid or ethosuximide, which were used as model AEDs. Compound **8** underwent only a slight metabolic change by the human liver microsomes (HLMs), and also did not affect the activity of human cytochrome P450 isoform, CYP3A4, in the *in vitro* assays.

Keywords: Pyrrolidine-2,5-dione; Hybrid compounds; Anticonvulsant activity; *In vivo* studies; *In vitro* studies; Metabolic stability

1. Introduction

Epilepsy affects approximately 50 million people worldwide, making it the second most common neurological disorder after stroke [1,2]. For many years, epilepsy was considered a disorder of the young, as the first symptoms occur usually before the age of 10. However, current data show that the prevalence increases with the age, being 0.7% in range of 55–64 years and 1.2% in population over the age of 85 [3,4]. This is a very serious fact owing to the demographic changes related to the aging societies in industrialized countries. Despite the significant advances that have been made in epilepsy research, convulsions in 30% of epileptics are still inadequately controlled by standard drug therapy [5,6]. Furthermore, compliance is often limited by adverse side effects most notably related to the central nervous system (CNS) exposure, such as diminished attention, executive function, intelligence, language skills, memory, and processing speed [7]. Data collected from eight of the biggest markets show that therapy-resistant epilepsy affects about 1.8 million people worldwide [8]. During the last twenty years, several new drugs such as levetiracetam, felbamate, lamotrigine, gabapentin, and topiramate have been implemented for the treatment of epilepsy. Although these drugs have been shown to be effective in many patients with epileptic syndromes, their efficacy does not appear to be superior to that of the established antiepileptic drugs (AEDs). Therefore, the ideal AED should prevent different types of seizures without producing side effects that adversely affect patients' quality of life. Considering the aforementioned facts, the continued search for safer and more effective AEDs is urgently necessary.

The incomplete information on the pathogenesis of human epilepsy and the complex mechanism of action of majority AEDs make it difficult to use the rational drug design technique of drug development that is based on the three-dimensional structure of the biological target. Conceptually, there are two methods of obtaining new AEDs, ligand-based approach and screening approach [9,10]. The ligand-based approach relies on the use of

existing biological data for old and new drugs or other anticonvulsant-active compounds. This approach is applied mainly for structural modifications of the currently available AEDs, with the aim of obtaining more efficacious drugs that will suppress different types of seizures and/or drugs with minimal or no adverse effects compared to maternal AEDs. It was successfully used in the discovery of several third-generation AEDs (e.g., eslicarbazepine, fluorofelbamate, pregabalin) as well as compounds currently available in Phase 3 of clinical trials (e.g., brivaracetam or seletracetam). The screening approach involves a comprehensive screening process of either diverse or focused compound libraries and utilizes rodent models of human epilepsy [11,12].

Our previous studies performed in the laboratory identified pyrrolidine-2,5-diones differently substituted at position-1 and -3 as targets for new AEDs [13–17]. Many of these compounds were found to be effective in the maximal electroshock (MES) and subcutaneous pentylenetetrazole (*sc*PTZ) screens that are still recognized as the "gold standard" in the early stages of testing new drug candidates. Studies on the structure–activity relationship (SAR) demonstrated the potent and wide spectrum of anticonvulsant activity (MES, *sc*PTZ, 6 Hz tests), exclusively for the 2-(2,5-dioxopyrrolidin-1-yl)acetamides containing phenylpiperazines with highly electronegative chlorine, fluorine, or trifluoromethyl substituents at the amide function [18].

In the present study, the library of new *N*-benzyl-2-(2,5-dioxopyrrolidin-1-yl)propionamides, 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propionamides, and 2-(2,5-dioxopyrrolidin-1-yl)butanamides was synthesized. These compounds were designed as hybrids combining the chemical fragments of well-established, old generation AED–ethosuximide and the newest AEDs, such as levetiracetam and lacosamide (Fig. 1).

Fig. 1.

It should be stressed that each of mentioned drugs possesses different indications as well as various mechanisms of pharmacodynamic activity. Ethosuximide is a prototypical anti-absence medication [19], the mechanism of action of which has been attributed to a reduction of T-type Ca^{2+} current in thalamocortical relay neurons [20–22]. Levetiracetam involves a presynaptic mechanism of action distinct from that of the other AEDs. Levetiracetam acts by modulating the exocytotic function of SV2A protein of synaptic vesicles. Thus, on the one hand, it possibly enhances the release of inhibitory neurotransmitters (GABA- and Gly-gated currents) on the other, it reduces the exocytosis of excitatory amino acids. Studies performed to date demonstrate the efficacy of levetiracetam in epilepsies with pharmaco-resistance to other AEDs. This was approved for add-on therapy in partial seizures with and without secondary generalization. Seizure control was also shown in idiopathic generalized epilepsies [23,24]. Lacosamide is the first drug to come from a class of compounds known as functionalized amino acids [25–28]. The detailed mechanism of action is at present unknown; however, the most plausible is the influence on slow inactivation of sodium channels [29]. Lacosamide has been recently approved for the adjunctive treatment of partial-onset seizures with or without secondary generalization and diabetic neuropathic pain in humans [30,31].

Considering the aforementioned facts, our aim in the present study was to obtain anticonvulsants with a broad spectrum of activity in “classic” animal models of epilepsy, MES and *sc*PTZ seizure tests. Considering the drug safety evaluation, which is important in the preclinical identification of new drug candidates, for the most promising molecule, its antiproliferative potential and influence on function of recombinant human cytochrome P450 isoform, CYP3A4, were studied *in vitro*. Furthermore, the metabolic stability was determined using the *in silico* and *in vitro* methods.

2. Results and discussion

2.1. Chemistry

The final compounds **4**–**36** were synthesized using a two-step reaction according to Scheme 1. In the first step, the condensation reaction of commercially purchased succinic anhydride or 3-methylsuccinic acid, with DL-α-alanine or DL-2-aminobutyric acid, yielded corresponding intermediates **1**–**3**. In the next step, these intermediates were converted to final compounds **4**–**13**, **15**–**24**, **26**–**35** in the coupling reaction with unsubstituted benzylamine or benzylamines with electronegative substituents such as chlorine, and fluorine atoms or trifluoromethyl group. This substitution mode was based on the previous research that showed that the presence of mentioned electronegative substituents enhanced anticonvulsant activity in several series of succinimide derivatives. Furthermore, with the aim of investigating the role of aromatic region localized at the amide fragment, three *N*-cyclohexylmethyl-analogs (**14**, **25**, **36**) were obtained. The reaction was carried out in dry tetrahydrofuran as solvent at room temperature in the presence of carbonyldiimidazole (CDI), which is a commonly used reagent for the synthesis of amides from carboxylic acids and amines through the acyl imidazole intermediate [32,33]. The progress of the reaction was monitored using HPLC (completion at ~24 h). Final compounds **4**–**36** were obtained with yield ranging between 55% and 83%. All the compounds were prepared as racemic mixtures. The intermediates **1**–**3** and final compounds **4**–**36** were fully characterized by C/H/N elemental analysis, and ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, LC–MS spectra. The detailed physicochemical and analytical data are listed in the experimental section (see also supplementary materials).

Scheme 1

2.2. Anticonvulsant activity

The preclinical development of new chemical agents for the treatment of epilepsy is based on the use of predictable animal seizure models, which correspond to different types of human epilepsies. Such models can be categorized into two main categories: models of acute seizures (non-epileptic animals induced to have a seizure by an electrical or chemical stimulus) and models of chronic epilepsy (animals induced to have enhanced seizure susceptibility or spontaneous seizures). For practical reasons, screening is often carried out using acute seizure models, although various types of kindling models, a class of chronic model, are commonly included in the battery of tests to which early stage compounds are subjected. Despite the diversity of models that could potentially be used to screen for anticonvulsant activity, the MES model and the *sc*PTZ model remain the "gold standards" in the early stages of testing. The MES test is the mechanism-independent animal seizure model which enables identification of compounds preventing seizure spread. This test uses an electrical stimulus to produce generalized tonic–clonic seizures, and thus is thought to be an experimental model of tonic–clonic epilepsy and of partial convulsions with or without secondary generalization in humans. The *sc*PTZ test employs chemically induced myoclonic seizures and is proposed to identify the agents raising the seizure threshold. This test is related to human generalized absence seizures [34–36]. Considering the aforementioned fact, all final substances **4–36** were screened in the MES and *sc*PTZ tests. Furthermore, in addition to the primary anticonvulsant screening, the acute neurological toxicity (NT) was determined in mice by the rotorod test.

Compounds **4–36** were administered in mice intraperitoneally (*i.p.*) at the fixed dose of 100 mg/kg, and the anticonvulsant protection was observed at two pretreatment times–30 and 120 min. This method allowed the determination of the number of animals (in a group consisting of four mice) protected against electrically (MES) or chemically (*sc*PTZ) induced

seizures, as well as the onset and the length of anticonvulsant effect. The results of the primary screening are summarized in Table 1.

Table 1.

The preliminary pharmacological data revealed that 25 compounds (**4**–**9**, **12**, **13**, **15**–**24**, **26**–**28**, **30**, **31**, and **35**) showed protection in half or more of the animals tested in the MES or/and *sc*PTZ tests. As is shown in Table 1, 6 molecules displayed prolonged (both time points: **16**, **18**, **19**) or delayed activity (time point of 120 min: **13**, **22**, **35**), except of MES-active **35**, exclusively in the PTZ seizures. Furthermore, the activity in both time intervals was visible only for the substances representing the 2-(2,5-dioxopyrrolidin-1-yl)butanamides (**16**, **18**, **19**). Other active compounds revealed protection at earlier time point–30 min. These results indicate the rapid onset of anticonvulsant action, however short in duration. It should be stressed that no acute neurological impairment was observed in the rotarod test at the fixed dose of 100 mg/kg. The primary pharmacological screening identified 10 substances (**4**, **7**, **8**, **13**, **15**–**18**, **24**, **26**) to be active in both seizure models. The other substances showed protection exclusively in either the MES (**5**, **6**, **9**, **12**, **27**, **28**, **30**, **31**, **35**) or *sc*PTZ (**19**–**23**) seizure test.

As shown in Table 1, the more beneficial anticonvulsant properties were observed for 2-(2,5-dioxopyrrolidin-1-yl)butanamides (**15**–**25**). Except inactive cyclohexane derivative **25**, all other compounds showed protection in at least one seizure model. In this series, the unsubstituted compound **15** and chloro- derivatives **16**–**18** showed activity in both tests, whereas fluoro and trifluoromethyl analogs were active predominantly in the pentylenetetrazole seizures. Replacement of the 2-(2,5-dioxopyrrolidin-1-yl)butanamide core fragment (**15**–**25**) with 2-(2,5-dioxopyrrolidin-1-yl)propanamide (**4**–**14**) decreased the

protection in PTZ test; however, it increased the activity in the MES seizures. Among the aforementioned compounds, the most beneficial properties were showed by *N*-benzyl-2-(2,5-dioxopyrrolidin-1-yl)propanamide (**4**) and its *para*-chlorine (**7**), *ortho*-fluorine (**8**), and *para*-trifluoromethyl (**13**) analogs, which were effective in both the tests. The rest of the active compounds (**5**, **6**, **9**, **12**) revealed protection only in the MES seizures. In this series, three compounds (**10**, **11**, **14**) were completely devoid of activity. The introduction of methyl group in position-3 of pyrrlolidine-2,5-dione ring–series of 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamides (**26**–**36**)– slightly decreased the anticonvulsant activity compared to the 3-unsubstituted analogs. Among these compounds, only **26** showed protection in both tests, whereas **27**, **28**, **30**, **31**, and **35** were active only in electrically induced seizures. It should be stressed that similar to 2-(dioxopyrrolidin-1-yl)propanamide analogs, the preferential for anticonvulsant activity was the presence of unsubstituted benzylamine or its chloro or fluoro derivatives (especially *ortho* and *meta* substitution).

Based on the preliminary results, in the next step of the pharmacological studies, the median effective doses (ED_{50}, in the MES and *sc*PTZ tests) and the median neurotoxic doses (TD_{50}, in the rotarod test) were determined for all active compounds. The data were used to calculate the protective indexes (PI), which are the measure of the benefit-to-risk ratio of the therapeutic agent. In parallel, the same tests were performed for model AEDs active in the MES (carbamazepine, lacozamide) or PTZ seizure (ethosuximide), as well as effective in both MES and PTZ seizure tests (valproic acid). The studies were carried out in mice after *i.p.* injection at time of peak activity taken from the screening data. The quantitative pharmacological results are summarized in Table 2.

Table 2.

The analysis of quantitative pharmacological data showed the widest protection, and the best ED_{50} values for 2-(2,5-dioxopyrrolidin-1-yl)propanamides containing the benzylamine (**4**) or 2-fluorobenzylamine (**8**) as an amine function. These molecules revealed distinctly higher activity in comparison with MES/*sc*PTZ-active AED (valproic acid) as follows: 3.73-fold (**4**) and 4.60-fold (**8**) in the MES test, and 5.59-fold (**4**) and 4.76-fold (**8**) in the pentylenetetrazole seizures. It should be stressed that both substances (**4** and **8**) showed relatively low acute NT in the rotarod test that yielded remarkably more favorable PI than the mentioned reference drug. The introduction of chlorine atom (**7**) or trifluoromethyl group (**13**) in *para* position caused similar activity in the *sc*PTZ test, however decreased the protection in the MES seizures (ED_{50} > 90 mg/kg) in comparison with the unsubstituted or 2-fluoro derivatives **4** and **8**. Moreover, at the same time, the presence of substituents in *para* position increased the NT that led to worsening of PIs. The rest of 2-(2,5-dioxopyrrolidin-1-yl)propanamides with the chlorine (**5**, **6**) or fluorine (**9**) atoms and trifluoromethyl group (**12**) at the benzyl moiety showed protection exclusively in the MES test. These substances showed lower activity than model AEDs active in the MES seizures–carbamazepine and lacosamide. It should be emphasized that compound **9** did not impair the motor coordination of mice up to a dose of 500 mg/kg, thus revealing a distinctly better PI than both model AEDs. Compounds **5** and **6** provided more favorable safety profiles than carbamazepine, and only slightly worse compared to lacosamide. Encouraging results were obtained for 2-(2,5-dioxopyrrolidin-1-yl)butanamides (**15**–**21**, **24**). Except on *N*-benzyl derivative **15**, other compounds were appreciably more effective in the *sc*PTZ model than in the MES test or showed activity only in the chemically induced seizures. In this series, all the aforementioned substances showed higher activity in the *sc*PTZ test in comparison to ethosuximide, which is known to be a model antiepileptic for PTZ seizures. Among these, the most potent anti-PTZ protection was observed for 3-fluoro derivative (**20**), which showed ED_{50} of 47.39 mg/kg (2.96-fold better

than ethosuximide). In parallel, this molecule revealed 3.05-fold superior PI compared to the mentioned reference drug. The introduction of fluorine atom in *ortho* (**19**) or *para* (**21**) position, its exchange to chlorine (**16**–**18**) or trifluoromethyl group (**23**, **24**), and removal of any substituents from benzyl moiety (**15**) decreased the activity in PTZ seizures. On the other hand, all the aforementioned compounds were more effective than ethosuximide from which **16**, **18**, **19**, and **21** also revealed distinctly better PI values. It should be stressed that the benzyl derivative **15** and *para*-trifluoromethyl analog **24** showed additionally potent activity in the MES seizures. The weaker anti-MES protection was also observed for chlorine derivatives (**16**–**18**). Furthermore, all substances active in both MES and PTZ seizures were more effective and at the same time showed better or comparable safety profiles compared to valproic acid. The screening data for 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamides (Table 1) showed activity mainly in the MES test. The ED_{50} values revealed that the most potent were *ortho*-chloro (**27**) and *ortho*-fluoro (**30**) derivatives. The introduction of chlorine or fluorine atoms into *meta* position (**28**, **31**), as well as their removal (**26**), decreased anti-MES protection. Furthermore, in this series of compounds, only unsubstituted *N*-benzyl derivative **26** showed protection also in the PTZ seizures, and was more effective and safer than valproic acid. It is noticeable that all 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamides revealed higher NT and in consequence lower PI values compared to respective (2,5-dioxopyrrolidin-1-yl) analogs.

Pentylenetetrazol is known to be a CNS stimulant with epileptogenic properties. It has been used to study seizure phenomenon and to identify pharmaceuticals that may control seizure susceptibility. As a noncompetitive GABA antagonist, PTZ is specifically used in seizure assays as a method of assessing the excitability of the CNS and GABA activity. After administration of an *s.c.* bolus of PTZ, mice are observed for seizure profile ranking and measurement of latencies to twitches and seizures (clonic/tonic). Seizure profiles are assessed for 30 min post-PTZ administration, for signs such as immobility, abnormal limb splay,

ataxia, straub tail, clonic/tonic seizures, muscle fasciculation, loss of righting reflex, and status epilepticus. In the present study, 14 compounds (**4**, **7**, **8**, **13**, **15**, **16**, **17**, **18**, **19**, **20**, **21**, **23**, **24**, and **26**) revealed prominent anticonvulsant activity in the *sc*PTZ seizures with the ED_{50} values ranging from 42.83 to 90.26 mg/kg (Table 2). As shown in Fig. 2 A–G, these substances, when compared to vehicle-treated group, prolonged significantly in a dose-dependent manner the latency time to first seizure episode starting at the dose of 30 mg/kg (**4**, **7**, **18**), 60 mg/kg (**8**, **19**, **20**), 80 mg/kg (**15**–**17**, **23**, **24**, **26**), and 100 mg/kg (**13**, **21**). It should be stresses that mentioned substances provided distinctly better protection in comparison with standard AEDs active in the *sc*PTZ test – ethosuximide (120 mg) and valproic acid (250 mg) (Fig. 2 H).

Fig. 2 A–G.

Based on the aforementioned pharmacological data, several SAR conclusions can be deduced. In general, except a few compounds (**4**, **7**, **8**, **13**, and **26**), the presence of 2-(2,5-dioxopyrrolidin-1-yl)propanamide or 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide core was preferential for MES protection. The replacement of propanamide with butanamide moiety extended notably the protection to PTZ seizures. Regarding the substitution mode of the benzylamine moiety, the most favorable were unsubstituted derivatives and compounds with chlorine or fluorine atoms (especially in *ortho* and *meta* position). An exception was observed for chloro-substituted-2-(2,5-dioxopyrrolidin-1-yl)butanamides (**16**–**18**), for which the highest protection in both tests showed *para*-chloro derivative **18**. The introduction of trifluoromethyl group yielded active molecules (**12**, **13**, **23**, **24**); however, on the other hand, the presence of the mentioned structural element increased remarkably the neurotoxic properties in the rotarod test (**13**, **23**, **24**). These molecules offered lower PIs in comparison to model AEDs.

2.3. *In vitro* and *in silico* assays

In the modern approach to the drug development process, drug-like properties of the new compounds should be evaluated in parallel with the study on their efficacy at early discovery phases [37,38]. In the present study, compound **8**, as the most promising in the pharmacological studies, was selected for preliminary *in vitro* assays of selected ADME–Tox parameters, such as antiproliferative activity and metabolic stability. Additionally, the bioluminescence assay was used for the prediction of the influence of compound **8** on the activity of recombinant cytochrome P450 3A4, considering the potential drug–drug interactions (DDIs).

2.3.1. Antiproliferative and cytotoxic assays

The *in vitro* cytotoxicity and antiproliferative assays are a reliable alternative to *in vivo* methods, leading to a reduction of animal use (3 R's approach) and also allowed the study of more number of compounds [39]. Moreover, the identification of cytotoxic agents against the cancer cell lines is a useful tool in anticancer drug discovery and development [40]. During this study, the formazan dye–based EZ4U assay was used for the preliminary safety test of compound **8**. As shown in Fig. 3 (left), no antiproliferative effect of compound **8** against HEK-293 cell line, even in the highest concentrations, was determined, whereas doxorubicin, a reference antiproliferative drug, showed IC_{50} value of 0.46 μM. Thus, the results obtained qualify compound **8** as safe in relation to the antiproliferative effect. Additionally, no cytotoxicity was observed against cancer IMR-32 neuroblastoma cell line (Fig. 3, right).

Fig. 3.

2.3.2. Metabolic stability

The metabolic stability of compound **8** was examined first *in silico* by using MetaSite software [41]. The plot of MetaSite predictions for sites of metabolism of compound **8** by liver computational model is shown in Fig. 4. According to *in silico* data, the highest probability of metabolism will occur at the position-4 of benzyl moiety. Moreover, compound **8** will be likely metabolized also at the alkylamide linker and at pyrrolidine-2,5-dione ring.

Fig. 4.

The metabolic stability of compound **8** was also evaluated *in vitro* using human liver microsomes (HLMs). A full scan chromatogram of the reaction mixture after 2 h of incubation of **8** with HLMs showed the presence of only one metabolite (M1). As shown at the UPLC spectrum (Fig. 5 A), compound **8** seems to be a very stable molecule because only 1.19% of the substrate was metabolized. The LC–MS analysis provided also the molecular mass of obtained metabolite M1 $[M+H]^+$ = 297.13 *m/z*. The analysis of the obtained fragmentation patterns of compound **8** and M1 under the fragment UPLC conditions and the most plausible *in silico* metabolites (not shown) allowed to determine the structure of metabolite M1. However, the *in vitro* study did not confirm the *in silico* data, where the position-4 of benzyl moiety was shown as the most probable site of metabolism. Thus, HLMs metabolic pathways of compound **8** included the hydroxylation of the alkylamide linker with the reduction of one carbonyl group of pyrrolidine-2,5-dione ring (Fig. 5 B).

Fig. 5 A.

Fig. 5 B.

2.3.3. Influence on recombinant human CYP3A4 P450 cytochrome

The cytochromes P450 (CYPs) inhibition, as a source of potential DDI, has become an important concern for the Food and Drug Administration (FDA) and pharmaceutical companies. DDI occurs if one drug affects the activity of CYPs and the other, coadministrated drug (victim), is excluded from metabolism, thus increasing the toxic concentration [38]. The FDA's draft guidance document for DDI recommends evaluating the CYPs' inhibition potential of new biologically active compounds at an earlier stage in drug development to avoid developing molecules with the potential to yield adverse drug interactions [42]. During this study, the luminescence assay was used to evaluate the potential inhibition or induction of cytochrome CYP3A4 by compound **8**. This assay is based on the conversion of the beetle D-luciferin derivative into D-luciferin by recombinant human isoenzyme CYP3A4. After addition of the firefly luciferase, the measured amount of light produced was proportional to obtained D-luciferin concentration in the presence of compound **8** [43]. The comparison with the luminescence obtained in the control reaction allowed evaluating the effect of compound **8** on CYP3A4 activity. Previous experiments showed that CYP3A4 inhibitor ketoconazole inhibited completely the CYP3A4 activity at 10 μM with calculated IC_{50} = 0.14 μM [44]. As shown in Fig. 6, compound **8** did not change the CYP3A4 activity, even at a very high concentration of 25 μM (Fig. 6).

Fig. 6.

3. Conclusion

In the present study, the focused library of 33 new *N*-benzyl-2-(2,5-dioxopyrrolidin-1-yl)propanamides, 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamides, and 2-(2,5-

dioxopyrrolidin-1-yl)butanamides was synthesized and tested for anticonvulsant activity in the MES and subcutaneous pentylenetetrazole (*sc*PTZ) seizure tests. The quantitative pharmacological data in mice showed that majority of molecules displayed remarkable activities in the mentioned "classic" animal models of epilepsy. Among these substances, 10 revealed broad spectra of protection in both MES and *sc*PTZ seizures. Moreover, several compounds revealed substantial safety profiles in the rotarod test for NT and emerged as anticonvulsants with very favorable PIs. The *in vitro* assays for the most promising 2-(2,5-dioxopyrrolidin-1-yl)-*N*-(2-fluorobenzyl)propanamide proved the lack of influence on function of human CYP3A4 isoform of cytochrome P450. This compound underwent only a slight metabolic change by the HLMs. The SAR studies showed that anticonvulsant protection depended on the structure of alkylamide moiety and also on the substitution mode of aromatic ring. Due to the high anticonvulsant activity in the MES and *sc*PTZ tests, as well as the favorable safety profiles of compounds described here, further biological characterization in subsequent animal epilepsy models such as psychomotor 6 Hz seizures is urgently necessary.

4. Experimental

4.1. Chemistry

4.1.1. General remarks

All chemicals and solvents were purchased from Sigma–Aldrich (St. Louis, USA) and were used without further purification. Melting points (mp.) 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 by TLC and gradient HPLC chromatography. The thin–layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} pre–coated aluminium sheets (Merck; Darmstadt, Germany), using

developing system consisted of: S_1 – dichloromethane : methanol (9 : 1; *v/v*), S_2 – dichloromethane : methanol (9 : 0.3; *v/v*). Spots were detected by their absorption under UV light (λ = 254 nm). HPLC analyses were run on the HPLC Waters 2695 Separation Module (Waters, Milford, USA) equipped with photodiode array detector Waters 2998 (Waters, Milford, USA). The Chromolith RP–18 SpeedROD column (4.6 × 50 mm) was used. Conditions applied were as follow: eluent A (water/0.1% TFA), eluent B (acetonitrile/0.1% TFA); flow rate of 5 ml/min, gradient of 0–100% B over 3 min were used, injection volume was 10 μL. Standard solutions (1 mg/ml) of each compound were prepared in analytical grade acetonitrile and analyzed at wave lengths 214 and 254 nm. Retention times (t_R) are given in minutes. Elemental analysis for C, H, and N were carried out by a micro method using the elemental Vario 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 $CDCl_3$ operating at 300 MHz (^{1}H NMR), 75 MHz (^{13}C NMR), and 282 MHz (^{19}F NMR). Chemical shifts are reported in δ values (ppm) relative to TMS δ = 0 (^{1}H), as internal standard. The *J* values are expressed in Hertz (Hz). Signal multiplicities are represented by the following abbreviations: s (singlet), br.s. (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet). The mass spectra (LC–MS) were obtained on Waters ACQUITY™ TQD system with the TQ Detector (Waters, Milford, USA). The ACQUITY UPLC BEH C18, 1.7 μm, 2.1 × 50 mm column was used (Waters, Milford, USA). Preparative column chromatography was performed using silica gel 60 (particle size 0,063–0,200; 70–230 Mesh ATM) purchased from Merck (Darmstadt, Germany).

4.1.2. General procedure for the preparation of the 2-(2,5-dioxopyrrolidin-1-yl)propanoic acid (1), 2-(2,5-dioxopyrrolidin-1-yl)butanoic acid (2) and 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanoic acid (3)

Succinic anhydride (5.0 g, 0.05 mol, intermediates **1** and **2**) or 2-methylsuccinic anhydride (5.7 g, 0.05 mol, intermediate **3**) were suspended in 20 ml of water and DL-α-alanine (4.4 g, 0.05 mol, intermediates **1** and **3**) or DL-2-aminobutyric acid (5.2 g, 0.05 mol, intermediate **2**) was gradually added. The mixture was heated in term–regulated sand bath (ST 72 Roth, Karlsruhe, Germany), with simultaneous distillation of water. After the water was completely removed, the temperature of the reaction was maintained at 180°C for 1 h. The crude products were purified by column chromatography (dichloromethane : methanol, 9 : 1, *v/v*) to afford intermediates **1**–**3** as yellow oils which solidified after standing in room temperature.

4.1.2.1. 2-(2,5-Dioxopyrrolidin-1-yl)propanoic acid (1)

Yellow oil. Yield 75%; TLC: R_f = 0.61 (S_1); HPLC: t_R = 0.278 min; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (d, 3H, *J*=6.9 Hz, –CH_3), 2.81 (s, 4H, imide), 4.55 (q, 1H, *J*=7.3 Hz, ≡CH), 9.20 (br. s., 1H, –COOH). ESI–MS: 172.1 ($C_7H_9NO_4$ $[M+H]^+$). Anal calcd for $C_7H_9NO_4$ (171.15): C, 49.12; H, 5.30; N, 8.18. Found: C, 49.20; H, 5.18; N, 8.25.

4.1.2.2. 2-(2,5-Dioxopyrrolidin-1-yl)butanoic acid (2)

Yellow oil. Yield 72%; TLC: R_f = 0.71 (S_1); HPLC: t_R = 0.524 min; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (t, 3H, *J*=7.4 Hz, $\underline{CH_3}$–CH_2–), 2.08–2.30 (m, 2H, CH_3–$\underline{CH_2}$–), 2.77 (s, 4H, imide), 4.86 (dd, 1H, *J*=10.4, 5.1 Hz, ≡CH), 9.24 (br. s., 1H, –COOH). ESI–MS: 186.0 ($C_8H_{11}NO_4$ $[M+H]^+$). Anal calcd for $C_8H_{11}NO_4$ (185.15): C, 51.89; H, 5.99; N, 7.56. Found: C, 51.95; H, 5.90; N, 7.66.

4.1.2.3. 2-(3-Methyl-2,5-dioxopyrrolidin-1-yl)propanoic acid (3)

Yellow oil, yield 75%; TLC: R_f = 0.75 (S_1); HPLC: t_R = 0.445 min; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (d, 3H, *J*=7.2 Hz, –CH_3), 1.55 (d, 3H, *J*=7.4 Hz, –CH_3,), 2.32–2.38 (m, 1H, imide), 2.80–2.90 (m, 2H, imide), 4.50 (q, 1H, *J*=7.1 Hz, ≡CH), 9.27 (br.s., 1H, –COOH). ESI–MS: 185.2 ($C_8H_{11}NO_4$ $[M+H]^+$). Anal calcd for $C_8H_{11}NO_4$ (185.15): C, 51.89; H, 5.99; N, 7.56. Found: C, 51.99; H, 6.10 ; N, 7.69.

4.1.3. General method for the preparation of 2-(2,5-dioxopyrrolidin-1-yl)propionamides (4–14), 2-(2,5-dioxopyrrolidin-1-yl)butanamides (15–25) and 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propionamides (26–36)

Carbonyldiimidazole (0.97 g, 0.006 mol) in 5 mL of dry THF was added under stirring to a solution of intermediate **1** (1.02g, 0.006 mol), **2** (1.11, 0.006 mol) or **3** (1.11g, 0.006 mol) dissolved in 10 mL of anhydrous THF. After the end of gaseous (carbon dioxide) evolution (ca. 0.5 h), the respective benzylamine (0.006 mol) or cyclohexylmethylamine dissolved in 5 mL of anhydrous THF was added dropwise in 5 min. The mixture was stirred overnight at room temperature and evaporated to dryness. The crude product was purified by column chromatography (dichloromethane : methanol, 9 : 0.3, *v/v*). After concentration of organic solvents under reduced pressure the final amides were obtained as solid substances.

4.1.3.1. *N*-Benzyl-2-(2,5-dioxopyrrolidin-1-yl)propanamide (4)

White solid. Yield: 61%; mp. 106–107ºC; TLC: R_f = 0.52 (S_2); HPLC: t_R = 1.060 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.41 (d, 3H, *J*=7.2 Hz, –CH_3), 2.61 (s, 4H, imide), 4.26 (d, 2H, *J*=6.2 Hz, –NH-$\underline{CH_2}$–), 4.57 (q, 1H, *J*=7.2 Hz, ≡CH), 7.13–7.36 (m, 5H, ArH), 8.32–8.36 (m, 1H, –NH–); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3, 28.1, 43.6, 49.5, 127.4, 127.5, 128.6, 137.8,

168.6, 176.9. ESI–MS: 261.3 ($C_{14}H_{16}N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{16}N_2O_3$ (260.29): C: 64.60, H: 6.20, N:10.76; Found C: 64.70, H: 6.32, N: 10.62.

4.1.3.2. *N*-(2-Chlorobenzyl)-2-(2,5-dioxopyrrolidin-1-yl)propanamide (5)

White solid. Yield: 81%; mp. 144–145ºC; TLC: R_f = 0.56 (S_2); HPLC: t_R = 1.070 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.61 (d, 3H, *J*=1.0 Hz, –CH_3), 2.73 (s, 4H, imide), 4.53 (t, 2H, *J*=5.8 Hz, –NH-$\underline{CH_2}$–), 4.80 (q, 1H, *J*=7.1 Hz, ≡CH), 6.42 (br. s., 1H, –NH–), 7.16–7.31 (m, 2H, ArH), 7.33–7.42 (m, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 28.2, 43.5, 49.7, 125.6, 128.1, 128.6, 131.2, 134.3, 139.7, 168.3, 176.5. ESI–MS: 295.1 ($C_{14}H_{15}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{15}ClN_2O_3$ (294.73): C: 57.05, H: 5.13, N:9.50; Found C: 57.15, H: 5.22, N: 9.62.

4.1.3.3. *N*-(3-Chlorobenzyl)-2-(2,5-dioxopyrrolidin-1-yl)propanamide (6)

White solid. Yield: 73%; mp. 97–98ºC; TLC: R_f = 0.53 (S_2); HPLC: t_R = 1.072 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.57 (dd, 3H, *J*=7.2, 1.0 Hz, –CH_3), 2.69 (d, 4H, *J*=1.5 Hz, imide), 4.36 (d, 2H, *J*=5.8 Hz, –NH-$\underline{CH_2}$–), 4.77 (q, 1H, *J*=7.2 Hz, ≡CH), 6.52 (br. s., 1H, –NH–), 7.13 (d, 1H, *J*=5.9 Hz, ArH), 7.18–7.28 (m, 3H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 28.1, 43.1, 49.7, 125.5, 127.5, 127.6, 130.0, 134.4, 139.9, 168.7, 176.7. ESI–MS: 295.2 ($C_{14}H_{15}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{15}ClN_2O_3$ (294.73): C: 57.05, H: 5.13, N:9.50; Found C: 57.18, H: 5.20, N: 9.52.

4.1.3.4. *N*-(4-Chlorobenzyl)-2-(2,5-dioxopyrrolidin-1-yl)propanamide (7)

White solid. Yield: 78%; mp. 124–125ºC; TLC: R_f = 0.53 (S_2); HPLC: t_R = 1.068 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.59 (d, 3H, *J*=7.3 Hz, –CH_3), 2.73 (s, 4H, imide), 4.40 (d, 2H, *J*=5.8 Hz, –NH-$\underline{CH_2}$–), 4.80 (q, 1H, *J*=7.3 Hz, ≡CH), 6.33 (s, 1H, –NH–), 7.17–7.23 (m, 2H,

ArH); 7.27–7.33 (m, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 28.1, 43.1, 49.7, 128.8, 129.0, 133.3, 136.3, 168.5, 176.7. ESI–MS: 295.1 ($C_{14}H_{15}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{15}ClN_2O_3$ (294.73): C: 57.05, H: 5.13, N:9.50; Found C: 57.19, H: 5.18, N: 9.63.

4.1.3.5. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-(2-fluorobenzyl)propanamide (8)

White solid. Yield: 71%; mp. 109–110ºC; TLC: R_f = 0.64 (S_2); HPLC: t_R = 0.849 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.55 (d, 3H, *J*=7.1 Hz, –CH_3), 2.67 (s, 4H, imide), 4.43 (d, 2H, *J*=5.9 Hz, –NH-$\underline{CH_2}$–), 4.74 (q, 1H, *J*=7.4 Hz, ≡CH), 6.56 (br. s., 1H, –NH–), 6.94–7.12 (m, 2H, ArH), 7.17–7.37 (m, 2H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –119.18 (s, 1F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 28.2, 43.2, 49.7, 114.3, 114.6, 123.4, 130.1, 130.4, 140.3, 161.2, 164.8, 168.7, 176.3. ESI–MS: 279.1 ($C_{14}H_{15}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{15}FN_2O_3$ (278.28): C: 60.42, H: 5.43, N: 10.07; Found C: 60.51, H: 5.25, N: 9.92.

4.1.3.6. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-(3-fluorobenzyl)propanamide (9)

White solid. Yield: 75%; mp. 96–97ºC; TLC: R_f = 0.59 (S_2); HPLC: t_R = 0.880 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.61 (d, 3H, *J*=7.3 Hz, –CH_3), 2.74 (s, 4H, imide), 4.44 (d, 2H, *J*=5.8 Hz, –NH-$\underline{CH_2}$–), 4.82 (q, 1H, *J*=7.3 Hz, ≡CH), 6.92 (br. s., 1H, –NH–), 6.94–7.12 (m, 2H, ArH), 7.17–7.37 (m, 2H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –119.20 (br. s., 1F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 28.1, 43.2, 49.7, 114.2, 114.5, 123.0, 130.1, 130.2, 140.3, 161.3, 164.5, 168.6, 176.7. ESI–MS: 279.1 ($C_{14}H_{15}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{15}FN_2O_3$ (278.28): C: 60.42, H: 5.43, N: 10.07; Found C: 60.53, H: 5.38, N: 10.08.

4.1.3.7. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-(4-fluorobenzyl)propanamide (10)

White solid. Yield: 62%; mp. 126–127ºC; TLC: R_f = 0.58 (S_2); HPLC: t_R = 0.889 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.58 (d, 3H, *J*=7.3 Hz, –CH_3), 2.70 (s, 4H, imide), 4.34–4.42 (m,

2H, –NH-$\underline{CH_2}$–), 4.81 (q, 1H, *J*=7.1 Hz, ≡CH), 6.27 (br. s., 1H, –NH–), 6.90–7.05 (m, 2H, ArH), 7.15–7.29 (m, 2H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –115.22 (br. s., 1F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2, 28.1, 42.9, 50.3, 115.5, 129.2, 133.5, 163.7, 168.7, 177.0. ESI–MS: 279.2 ($C_{14}H_{15}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{15}FN_2O_3$ (278.28): C: 60.42, H: 5.43, N: 10.07; Found C: 60.55, H: 5.45, N: 10.18.

4.1.3.8. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-[2-(trifluoromethyl)benzyl]propanamide (11)

White solid. Yield: 68%; mp. 124–125ºC; TLC: R_f = 0.62 (S_2); HPLC: t_R = 1.159 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.55 (d, 3H, *J*=7.1 Hz, –CH_3), 2.69 (s, 4H, imide), 4.57 (br. s., 2H, –NH-$\underline{CH_2}$–), 4.74 (q, 1H, *J*=7.4 Hz, ≡CH), 6.51 (br. s., 1H, –NH–), 7.29–7.41 (m, 1H, ArH), 7.51 (d, 2H, *J*=4.2 Hz, ArH), 7.61 (d, 1H, *J*=7.6 Hz, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –59.69 (s, 3F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2, 28.1, 40.3, 40.4, 49.6, 122.5, 125.9, 126.0, 127.6, 130.2, 132.3, 136.0, 168.7, 176.7. ESI–MS: 329.2 ($C_{15}H_{15}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{15}F_3N_2O_3$ (328.29): C: 58.25, H: 4.89, N: 9.06; Found C: 58.33, H: 4.96, N: 9.08.

4.1.3.9. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-[3-(trifluoromethyl)benzyl]propanamide (12)

White solid. Yield: 66%; mp. 137–138ºC; TLC: R_f = 0.67 (S_2); HPLC: t_R = 1.203 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.56 (d, 3H, *J*=7.1 Hz, –CH_3), 2.67 (s, 4H, imide), 4.41 (d, 2H, *J*=5.9 Hz, –NH-$\underline{CH_2}$–), 4.76 (q, 1H, *J*=7.1 Hz, ≡CH), 6.68 (br. s., 1H, –NH–),7.39–7.56 (m, 4H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –62.63 (s, 3F). ESI–MS: 329.2 ($C_{15}H_{15}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{15}F_3N_2O_3$ (328.29): C: 58.25, H: 4.89, N: 9.06; Found C: 58.33, H: 4.96, N: 9.08.

4.1.3.10. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-[4-(trifluoromethyl)benzyl]propanamide (13)

White solid. Yield: 70%; mp. 110–111ºC; TLC: R_f = 0.66 (S_2); HPLC: t_R = 1.225 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.56 (d, 3H, *J*=7.4 Hz, –CH_3), 2.67 (s, 4H, imide), 4.41 (d, 2H, *J*=6.1 Hz, –NH-$\underline{CH_2}$–), 4.76 (q, 1H, *J*=7.1 Hz, ≡CH), 6.66 (t, 1H, *J*=5.5 Hz, –NH–), 7.34 (d, 2H, *J*=7.9 Hz, ArH), 7.55 (d, 2H, *J*=8.2 Hz, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –62.55 (s, 3F). ESI–MS: 329.2 ($C_{15}H_{15}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{15}F_3N_2O_3$ (328.29): C: 58.25, H: 4.89, N: 9.06; Found C: 58.30, H: 4.98, N: 9.02.

4.1.3.11. *N*-(Cyclohexylmethyl)-2-(2,5-dioxopyrrolidin-1-yl)propanamide (14)

White solid. Yield: 75%; mp. 150–151ºC; TLC: R_f = 0.60 (S_2); HPLC: t_R = 1.201 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.77–0.97 (m, 2H, cyclohexane), 1.06–1.29 (m, 2H, cyclohexane), 1.32–1.49 (m, 2H, cyclohexane), 1.57 (d, 3H, *J*=7.4 Hz, –CH_3), 1.60–1.76 (5H, m, cyclohexane), 2.71 (s, 4H, imide), 2.98–3.14 (m, 2H, –NH-$\underline{CH_2}$–), 4.73 (q, 1H, *J*=7.4 Hz, ≡CH), 6.18 (br. s., 1H, –NH–). ESI–MS: 267.3 ($C_{14}H_{22}N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{14}H_{22}N_2O_3$ (266.34): C: 63.13, H: 8.33, N: 10.52; Found C: 63.15, H: 8.25, N: 10.55.

4.1.3.12. *N*-Benzyl-2-(2,5-dioxopyrrolidin-1-yl)butanamide (15)

White solid. Yield: 71%; mp. 110–111ºC; TLC: R_f = 0.60 (S_2); HPLC: t_R = 0.961 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*=7.3 Hz, –CH_3), 1.98–2.29 (m, 2H, CH_3-$\underline{CH_2}$–), 2.75 (s, 4H, imide), 4.49 (t, 2H, *J*=1.0 Hz, –NH-$\underline{CH_2}$–), 4.62 (q, 1H, *J*=7.1 Hz, ≡CH), 6.54 (br. s., 1H, –NH–), 6.99–7.15 (m, 2H, ArH), 7.21–7.36 (m, 3H, ArH). ESI–MS: 275.2 ($C_{15}H_{18}N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{18}N_2O_3$ (274.31): C: 65.68, H: 6.61, N:10.21; Found C: 65.60, H: 6.62, N: 10.32.

4.1.3.13. *N*-(2-Chlorobenzyl)-2-(2,5-dioxopyrrolidin-1-yl)butanamide (16)

White solid. Yield: 81%; mp. 155–156ºC; TLC: R_f = 0.70 (S_2); HPLC: t_R = 1.153 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*=7.4 Hz, –CH_3), 2.00–2.30 (m, 2H, CH_3-$\underline{CH_2}$–), 2.76 (s, 4H, imide), 4.52 (dd, 2H, *J*=10.1, 5.9 Hz, –NH-$\underline{CH_2}$–), 4.62 (dd, 1H, *J*=10.3, 6.1 Hz, ≡CH), 7.17–7.29 (m, 3H, ArH), 7.31–7.41 (m, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2, 28.1, 42.9, 49.5, 50.3, 115.5, 129.2, 163.7, 168.7, 177.0. ESI–MS: 309.2 ($C_{15}H_{17}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}ClN_2O_3$ (308.76): C: 58.35, H: 5.55, N:9.07; Found C: 58.40, H: 5.65, N: 9.01.

4.1.3.14. *N*-(3-Chlorobenzyl)-2-(2,5-dioxopyrrolidin-1-yl)butanamide (17)

White solid. Yield: 65%; mp. 95–96ºC; TLC: R_f = 0.60 (S_2); HPLC: t_R = 1.184 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.90 (t, 3H, *J*=7.4 Hz, –CH_3), 2.01–2.29 (m, 2H, CH_3-$\underline{CH_2}$–), 2.77 (s, 4H, imide), 4.42 (t, 2H, *J*=5.7 Hz, –NH-$\underline{CH_2}$–), 4.64 (q, 1H, *J*=10.4 Hz, ≡CH), 6.55 (br. s., 1H, –NH–), 6.90–7.07(m, 3H, ArH), 7.20–7.26 (m, 1H, ArH). ESI–MS: 309.2 ($C_{15}H_{17}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}ClN_2O_3$ (308.76): C: 58.35, H: 5.55, N:9.07; Found C: 58.40, H: 5.65, N: 9.01.

4.1.3.15. *N*-(4-Chlorobenzyl)-2-(2,5-dioxopyrrolidin-1-yl)butanamide (18)

White solid. Yield: 83%; mp. 72–73ºC; TLC: R_f = 0.61 (S_2); HPLC: t_R = 1.214 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.89 (t, 3H, *J*=7.3 Hz, –CH_3), 2.00–2.29 (m, 2H, CH_3-$\underline{CH_2}$–), 2.76 (s, 4H, imide), 4.41 (t, 2H, *J*=5.1 Hz, –NH-$\underline{CH_2}$–), 4.63 (q, 1H, *J*=10.4 Hz, ≡CH), 6.51 (br. s., 1H, –NH–), 7.13–7.37 (m, 4H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.8, 21.4, 28.0, 43.0, 56.8, 128.8, 128.9, 133.3, 136.3, 168.3, 177.1. ESI–MS: 309.2 ($C_{15}H_{17}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}ClN_2O_3$ (308.76): C: 58.35, H: 5.55, N:9.07; Found C: 58.42, H: 5.64, N: 9.10.

4.1.3.16. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-(2-fluorobenzyl)butanamide (19)

White solid. Yield: 75%; mp. 109–110ºC; TLC: R_f = 0.62 (S_2); HPLC: t_R = 1.010 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.89 (t, 3H, *J*=7.3 Hz, –CH_3), 1.97–2.34 (m, 2H, CH_3-$\underline{CH_2}$–), 2.76 (s, 4H, imide), 4.45 (t, 2H, *J*=6.0 Hz, –NH-$\underline{CH_2}$–), 4.64 (q, 1H, *J*=10.4 Hz, ≡CH), 6.44 (br. s., 1H, –NH–), 7.20–7.41 (m, 4H, ArH). ESI–MS: 292.2 ($C_{15}H_{17}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}FN_2O_3$ (292.31): C: 61.63, H: 5.86, N:9.58; Found C: 61.50, H: 5.95, N: 9.48.

4.1.3.17. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-(3-fluorobenzyl)butanamide (20)

White solid. Yield: 79%; mp. 91–92ºC; TLC: R_f = 0.63 (S_2); HPLC: t_R = 1.043 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.90 (t, 3H, *J*=7.4 Hz, –CH_3), 1.98–2.31 (m, 2H, CH_3-$\underline{CH_2}$–), 2.77 (s, 4H, imide), 4.44 (t, 2H, *J*=5.3 Hz, –NH-$\underline{CH_2}$–), 4.65 (q, 1H, *J*=10.4 Hz, ≡CH), 6.55 (br. s., 1H, –NH–), 6.90–7.07 (m, 3H, ArH), 7.20–7.28 (m, 1H, ArH). ESI–MS: 292.1 ($C_{15}H_{17}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}FN_2O_3$ (292.31): C: 61.63, H: 5.86, N:9.58; Found C: 61.68, H: 5.97, N: 9.60.

4.1.3.18. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-(4-fluorobenzyl)butanamide (21)

White solid. Yield: 77%; mp. 117–118ºC; TLC: R_f = 0.61 (S_2); HPLC: t_R = 1.045 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.89 (t, 3H, *J*=1.0 Hz, –CH_3), 2.00–2.29 (m, 2H, CH_3-$\underline{CH_2}$–), 2.75 (s, 4H, imide), 4.40 (t, 2H, *J*=5.1 Hz, –NH-$\underline{CH_2}$–), 4.62 (q, 1H, *J*=10.4 Hz, ≡CH), 6.50 (br. s., 1H, –NH–), 6.96–7.05 (m, 2H, ArH), 7.18–7.29 (m, 2H, ArH). ESI–MS: 292.2 ($C_{15}H_{17}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}FN_2O_3$ (292.31): C: 61.63, H: 5.86, N:9.58; Found C: 61.70, H: 5.92, N: 9.64.

4.1.3.19. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-[2-(trifluoromethyl)benzyl]butanamide (22)

White solid. Yield: 65%; mp. 161–162ºC; TLC: R_f = 0.63 (S_2); HPLC: t_R = 1.283 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.85 (t, 3H, *J*=1.0 Hz, –CH_3), 1.89–2.32 (m, 2H, CH_3-$\underline{CH_2}$–), 2.72 (s, 4H, imide), 4.45–4.74 (m, 3H, –NH-$\underline{CH_2}$–, ≡CH), 6.68 (br. s., 1H, –NH–), 7.49 (d, 2H, *J*=1.0 Hz, ArH), 7.62 (d, 2H, *J*=7.6 Hz, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –59.66 (s, 3F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.8, 21.4, 28.1, 43.2, 56.8, 125.5, 125.6, 125.6, 125.9, 127.7, 141.7, 168.6, 177.1. ESI–MS: 343.2 ($C_{16}H_{17}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{16}H_{17}F_3N_2O_3$ (342.31): C: 56.14, H: 5.01, N:8.18; Found C: 56.21, H: 5.19, N: 8.17.

4.1.3.20. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-[3-(trifluoromethyl)benzyl]butanamide (23)

White solid. Yield: 67%; mp. 165–166ºC; TLC: R_f = 0.61 (S_2); HPLC: t_R = 1.314 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.85 (t, 3H, *J*=7.4 Hz, –CH_3), 1.97–2.24 (m, 2H, CH_3-$\underline{CH_2}$–), 2.71 (s, 4H, imide), 4.34–4.51 (m, 2H, –NH-$\underline{CH_2}$–), 4.60 (q, 1H, *J*=10.3 Hz, ≡CH), 6.84 (br. s., 1H, –NH–), 7.34–7.58 (m, 4H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –62.64 (s, 3F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.8, 21.4, 28.0, 43.1, 56.8, 125.5, 125.6, 125.6, 125.7, 127.7, 141.9, 168.5, 177.1. ESI–MS: 343.2 ($C_{16}H_{17}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{16}H_{17}F_3N_2O_3$ (342.31): C: 56.14, H: 5.01, N:8.18; Found C: 56.21, H: 5.19, N: 8.17.

4.1.3.21. 2-(2,5-Dioxopyrrolidin-1-yl)-*N*-[4-(trifluoromethyl)benzyl]butanamide (24)

White solid. Yield: 75%; mp. 59–60ºC; TLC: R_f = 0.59 (S_2); HPLC: t_R = 1.331 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.90 (t, 3H, *J*=7.3 Hz, –CH_3), 1.97–2.31 (m, 2H, CH_3-$\underline{CH_2}$–), 2.77 (s, 4H, imide), 4.45–4.55 (m, 2H, –NH-$\underline{CH_2}$–), 4.66 (q, 1H, *J*=10.3 Hz, ≡CH), 6.63 (br. s., 1H, –NH–), 7.38 (d, 2H, *J*=7.9 Hz, ArH), 7.59 (d, 2H, *J*=7.9 Hz, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –61.63 (s, 3F). ESI–MS: 343.2 ($C_{16}H_{17}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{16}H_{17}F_3N_2O_3$ (342.31): C: 56.14, H: 5.01, N:8.18; Found C: 56.24, H: 5.10, N: 8.20.

4.1.3.22. *N*-(Cyclohexylmethyl)-2-(2,5-dioxopyrrolidin-1-yl)butanamide (25)

White solid. Yield: 79%; mp. 107–108ºC; TLC: R_f = 0.70 (S_2); HPLC: t_R = 1.028 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.86 (t, 3H, *J*=7.2 Hz, –CH_3), 1.04–1.26 (m, 4H, cyclohexane), 1.31–1.51 (m, 1H, cyclohexane), 1.56–1.74 (m, 5H, cyclohexane), 1.95–2.35 (m, 2H, CH_3-$\underline{CH_2}$-), 2.73 (s, 4H, imide), 2.93–3.14 (m, 2H, –NH-$\underline{CH_2}$–), 4.55 (q, 1H, *J*=10.5 Hz, ≡CH), 6.38 (br. s., 1H, –NH–). ESI–MS: 281.3 ($C_{15}H_{24}N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{24}N_2O_3$ (280.36): C: 64.26, H: 8.63, N: 9.99; Found C: 64.11, H: 8.80, N: 9.90.

4.1.3.23. *N*-Benzyl-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (26)

White solid. Yield: 81%; mp. 98–99ºC; TLC: R_f = 0.68 (S_2); HPLC: t_R = 0.952 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.30 (d, 3H, *J*=6.1 Hz, –CH_3), 1.57 (d, 3H, *J*=7.1 Hz, –CH_3), 2.20–2.30 (m, 1H, imide), 2.71–2.97 (m, 2H, imide), 4.40 (d, 2H, *J*=5.3 Hz, –NH-$\underline{CH_2}$–), 4.75 (q, 1H, *J*=7.1 Hz, ≡CH), 6.43 (br. s., 1H, –NH–), 7.17–7.38 (m, 5H, ArH). ESI–MS: 275.2 ($C_{15}H_{18}N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{18}N_2O_3$ (274.31): C: 65.68, H: 6.61, N: 10.21; Found C: 65.60, H: 6.70, N: 10.23.

4.1.3.24. *N*-(2-Chlorobenzyl)-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (27)

White solid. Yield: 60%; mp. 94–95ºC; TLC: R_f = 0.61 (S_2); HPLC: t_R = 1.122 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.30 (d, 3H, *J*=4.8 Hz, –CH_3), 1.57 (d, 3H, *J*=7.4 Hz, –CH_3), 2.30 (d, 1H, *J*=7.4 Hz, imide), 2.76–2.96 (m, 2H, imide), 4.40–4.48 (m, 2H, –NH-$\underline{CH_2}$–), 4.74 (q, 1H, *J*=7.4 Hz, ≡CH), 6.57 (br. s., 1H, –NH–), 7.17–7.39 (m, 4H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 16.7, 34.6, 36.3, 41.8, 49.7, 127.1, 129.0, 129.4, 130.0, 135.1, 135.1, 168.5, 175.9, 180.1. ESI–MS: 309.1 ($C_{15}H_{17}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}ClN_2O_3$ (308.76): C: 58.35, H: 5.55, N: 9.07; Found C: 58.27, H: 5.65, N: 9.11.

4.1.3.25. *N*-(3-Chlorobenzyl)-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (28)

White solid. Yield: 56%; mp. 79–80ºC; TLC: R_f = 0.67 (S_2); HPLC: t_R = 1.179 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.31 (d, 3H, *J*=7.1 Hz, –CH_3), 1.57 (d, 3H, *J*=7.1 Hz, –CH_3), 2.25–2.30 (m, 1H, imide), 2.80–2.89 (m, 2H, imide), 4.29–4.36 (m, 2H, –NH-$\underline{CH_2}$–), 4.75 (q, 1H, *J*=7.2 Hz, ≡CH), 6.55 (br. s., 1H, –NH–), 7.07–7.16 (m, 1H, ArH), 7.20–7.29 (m, 3H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 16.6, 34.6, 36.4, 41.8, 49.6, 127.0, 129.1, 129.5, 130.1, 135.1, 135.3, 168.6, 175.7, 180.0. ESI–MS: 309.2 ($C_{15}H_{17}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}ClN_2O_3$ (308.76): C: 58.35, H: 5.55, N: 9.07; Found C: 58.33, H: 5.67, N: 9.18.

4.1.3.26. *N*-(4-Chlorobenzyl)-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (29)

White solid. Yield: 60%; mp. 79–80ºC; TLC: R_f = 0.67 (S_2); HPLC: t_R = 1.190 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.30 (d, 3H, *J*=6.9 Hz, –CH_3), 1.57 (d, 3H, *J*=6.9 Hz, –CH_3), 2.30 (d, 1H, *J*=7.9 Hz, imide), 2.75–2.96 (m, 2H, imide), 36 (br. s., 2H, –NH-$\underline{CH_2}$–), 4.73 (q, 1H, *J*=1.0 Hz, ≡CH), 6.51 (br. s., 1H, –NH–), 7.16 (d, 2H, *J*=1.0 Hz, ArH), 7.26 (d, 2H, *J*=1.0 Hz, ArH). ESI–MS: 309.2 ($C_{15}H_{17}ClN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}ClN_2O_3$ (308.76): C: 58.35, H: 5.55, N: 9.07; Found C: 58.38, H: 5.69, N: 9.10.

4.1.3.27. *N*-(2-Fluorobenzyl)-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (30)

Yellow solid. Yield: 59%; mp. 109–110ºC; TLC: R_f = 0.62 (S_2); HPLC: t_R = 1.000 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.30 (d, 3H, *J*=7.1 Hz, –CH_3), 1.56 (d, 3H, *J*=7.4 Hz, –CH_3), 2.10–2.23 (m, 1H, imide), 2.79–2.86 (m, 2H, imide), 4.44 (br. s., 2H, –NH-$\underline{CH_2}$–), 4.73 (q, 1H, *J*=7.4 Hz, ≡CH), 6.54 (br. s., 1H, –NH–), 6.95–7.12 (m, 2H, ArH), 7.18–7.34 (m, 2H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –119.17 (s, 1F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 16.6, 34.6, 36.3, 37.8, 49.7, 115.4, 124.3, 124.6, 129.3, 130.1, 159.2, 162.5, 168.6, 175.9, 180.1.

ESI–MS: 293.3 ($C_{15}H_{17}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}FN_2O_3$ (292.30): C: 61.63, H: 5.86, N: 9.58; Found C: 61.55, H: 5.99, N: 9.60.

4.1.3.28. *N*-(3-Fluorobenzyl)-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (31)

White solid. Yield: 62%; mp. 110–111ºC; TLC: R_f = 0.60 (S_2); HPLC: t_R = 1.017 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.32 (d, 3H, *J*=7.1 Hz, $-CH_3$), 1.58 (d, 3H, *J*=7.4 Hz, $-CH_3$), 2.28–2.35 (m, 1H, imide), 2.85–2.90 (m, 2H, imide), 4.35–4.42 (m, 2H, –NH-$\underline{CH_2}$–), 4.77 (q, 1H, *J*=7.2 Hz, ≡CH), 6.50 (br. s., 1H, –NH–), 6.95 (d, 1H, *J*=9.2 Hz, ArH), 7.01 (d, 2H, *J*=7.6 Hz, ArH), 7.27 (q, 1H, *J*=1.0 Hz, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –112.70 (s, 1F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3, 16.7, 34.4, 36.3, 37.8, 49.2, 115.5, 124.2, 124.7, 129.3, 130.2, 159.1, 162.5, 168.6, 175.8, 180.1. ESI–MS: 293.3 ($C_{15}H_{17}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}FN_2O_3$ (292.30): C: 61.63, H: 5.86, N: 9.58; Found C: 61.60, H: 5.95, N: 9.50.

4.1.3.29. *N*-(4-Fluorobenzyl)-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (32)

White solid. Yield: 64%; mp. 114–115ºC; TLC: R_f = 0.58 (S_2); HPLC: t_R = 0.996 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.18 (d, 3H, *J*=6.1 Hz, $-CH_3$), 1.57 (d, 3H, *J*=7.4 Hz, $-CH_3$), 2.25–2.32 (m, 1H, imide), 2.80–2.93 (m, 2H, imide), 4.35–4.38 (m, 2H, –NH-$\underline{CH_2}$–), 4.74 (q, 1H, *J*=7.1 Hz, ≡CH), 6.45 (br. s., 1H, –NH–), 6.98 (t, 2H, *J*=1.0 Hz, ArH), 7.20 (t, 2H, *J*=1.0 Hz, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –115.02 (br. s., 1F); ESI–MS: 293.3 ($C_{15}H_{17}FN_2O_3$ $[M+H]^+$). Anal. calcd for $C_{15}H_{17}FN_2O_3$ (292.30): C: 61.63, H: 5.86, N: 9.58; Found C: 61.60, H: 5.95, N: 9.50.

4.1.3.30. 2-(3-Methyl-2,5-dioxopyrrolidin-1-yl)-*N*-[2-(trifluoromethyl)benzyl]propanamide (33)

White solid. Yield: 55%; mp. 102–103ºC; TLC: R_f = 0.71 (S_2); HPLC: t_R = 1.362 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.28–1.36 (m, 3H, –CH_3), 1.60 (d, 3H, *J*=7.4 Hz, –CH_3), 2.05–2.16 (m, 1H, imide), 2.79–3.03 (m, 2H, imide), 4.63 (d, 2H, *J*=5.3 Hz, –NH-$\underline{CH_2}$–), 4.78 (q, 1H, *J*=7.1 Hz, ≡CH), 6.32 (br. s., 1H, –NH–), 7.23–7.41 (m, 1H, ArH), 7.50–7.57 (m, 2H, ArH), 7.64 (d, 1H, *J*=6.9 Hz, ArH). ESI–MS: 343.2 ($C_{16}H_{17}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{16}H_{17}F_3N_2O_3$ (342.31): C: 56.14, H: 5.01, N: 8.18; Found C: 56.21, H: 5.18, N: 8.10.

4.1.3.31. 2-(3-Methyl-2,5-dioxopyrrolidin-1-yl)-*N*-[3-(trifluoromethyl)benzyl]propanamide (34)

White solid. Yield: 71%; mp. 115–116ºC; TLC: R_f = 0.68 (S_2); HPLC: t_R = 1.312 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.32 (d, 3H, *J*=3.0 Hz, –CH_3), 1.60 (d, 3H, *J*=4.8 Hz, –CH_3), 2.25–2.31 (m, 1H, imide), 2.76–3.03 (m, 2H, imide), 4.48 (br. s., 2H, –NH-$\underline{CH_2}$–), 4.79 (br. s., 1H, ≡CH), 6.53 (br. s., 1H, –NH–), 7.45–7.60 (m, 4H, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –62.62 (br. s., 3F). ESI–MS: 343.2 ($C_{16}H_{17}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{16}H_{17}F_3N_2O_3$ (342.31): C: 56.14, H: 5.01, N: 8.18; Found C: 56.16, H: 5.15, N: 8.12.

4.1.3.32. 2-(3-Methyl-2,5-dioxopyrrolidin-1-yl)-*N*-[4-(trifluoromethyl)benzyl]propanamide (35)

White solid. Yield: 81%; mp. 132–133ºC; TLC: R_f = 0.69 (S_2); HPLC: t_R = 1.331 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 1.33 (d, 3H, *J*=6.9 Hz, –CH_3), 1.60 (d, 3H, *J*=7.1 Hz, –CH_3), 2.33 (d, 1H, *J*=8.2 Hz, imide), 2.81–2.99 (m, 2H, imide), 4.48 (br. s., 2H, –NH-$\underline{CH_2}$–), 4.79 (q, 1H, *J*=7.1 Hz, ≡CH), 6.48 (br. s., 1H, –NH–), 7.37 (d, 2H, *J*=7.4 Hz, ArH), 7.58 (d, 2H, *J*=7.5 Hz, ArH); ^{19}F NMR (282 MHz, $CDCl_3$) δ –62.55 (s, 3F); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4, 16.5, 33.9, 43.1, 49.6, 125.5, 127.5, 142.0, 168.9, 176.0, 180.1. ESI–MS: 343.2

($C_{16}H_{17}F_3N_2O_3$ $[M+H]^+$). Anal. calcd for $C_{16}H_{17}F_3N_2O_3$ (342.31): C: 56.14, H: 5.01, N: 8.18; Found C: 56.10, H: 5.17, N: 8.15.

4.1.3.33. *N*-(Cyclohexylmethyl)-2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide (36)

White solid. Yield: 59%; mp. 112–113ºC; TLC: R_f = 0.50 (S_2); HPLC: t_R = 1.191 min; ^{1}H NMR (300MHz, $CDCl_3$) δ 0.81–0.97 (m, 2H, cyclohexane), 1.11–1.25 (m, 2H, cyclohexane), 1.34 (d, 3H, *J*=7.1 Hz, –CH_3), 1.39–1.53 (m, 2H, cyclohexane), 1.59 (d, 3H, *J*=7.1 Hz, –CH_3), 1.62–1.78 (m, 5H, cyclohexane), 2.29–2.37 (m, 1H, imide), 2.88–2.99 (m, 2H, imide), 3.02–3.15 (m, 2H, –NH-$\underline{CH_2}$–), 4.73 (q, 1H, *J*=7.2 Hz, ≡CH), 6.08 (br. s., 1H, –NH–); ESI–MS: 281.3 ($C_{15}H_{24}N_2O_3$ $[M+H]^+$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3, 14.4, 34.6, 36.3, 43.1, 49.6, 122.2, 125.5, 127.5, 129.4, 142.0, 168.9, 169.0, 176.02, 180.1. Anal. calcd for $C_{15}H_{24}N_2O_3$ (280.36): C: 64.26, H: 8.63, N: 9.99; Found C: 64.20, H: 8.69, N: 10.10.

4.2. *In vivo* pharmacology

4.2.1. General

The initial anticonvulsant evaluations were performed at the Department of Pharmacodynamics, Faculty of Pharmacy, Jagiellonian University Medical College (Cracow, Poland). All the experiments were performed according to the procedures of the Anticonvulsant Screening Program pursued originally in the National Institute of Neurological Disorders and Stroke, National Institute of Health, Rockville, USA [45]. Preliminary studies involved three tests: maximal electroshock (MES), subcutaneous pentylenetetrazole (*sc*PTZ) and rotorod test for acute neurological toxicity (NT). 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 4 animals and each mouse was used only once. The experiments were performed between 8 a.m. and 3 p.m. Procedures involving animals and their care were conducted in accordance with current European Community and Polish legislation on animal experimentation. The experimental protocols and procedures described in this manuscript were approved by the Local Ethics Committee at the Jagiellonian University Medical College and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The compounds were suspended in 0.5% methylcellulose/water mixture (Loba Chemie, Germany). All substances were administered intraperitoneally into mice in volumes 0.1 ml per 10 g body weight. The animals were administered with a constant dose of 100 mg/kg of each compound with anticonvulsant activity and neurotoxicity assessment at 0.5 and 2 hours after injection. Control animals were given appropriate amounts of vehicle (methylcellulose).

4.2.2. Maximal electroshock seizure test

The MES test was performed according to procedure originally described by Toman et al. [46]. Briefly, the mice received an electrical stimulus of sufficient intensity (25 mA, 500 V, 50 Hz) delivered by the electroshock generator (Hugo Sachs rodent shocker, Germany) 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 procedure caused immediate hindlimb tonic extension. Mice not displaying hind-limb tonic extension were considered to be protected from seizure.

4.2.3. Subcutaneous pentylenetetrazole seizure test (scPTZ)

Pentylenetetrazole (PTZ) were purchased from Sigma–Aldrich (St. Louis, USA). PTZ was dissolved in saline solution. *Sc*PTZ-induced seizures were performed by subcutaneously injection of PTZ (100 mg/kg). This produced clonic convulsions lasting for at least five seconds, with accompanying loss of the righting reflex. PTZ was administered 0.5 and 2 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 4–16 min of observation. The absence of clonic convulsions in the observed time period of 0.5 and 2 h was interpreted as the compound's ability to protect against PTZ-induced seizure [47]. Moreover, the latency time to first clonus episodes was noted and compared between vehicle-treated and drug-treated groups.

4.2.4. Minimal motor impairment test (NT)

Minimal motor impairment was established in mice by standard rotarod procedure [48]. Mice were trained to balance on an accelerating rotarod that rotated at ten 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 2 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 one minute.

4.2.4. 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 6 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 [49]. The protective indexes for the investigated compounds were calculated by dividing a TD_{50} value, as determined in the rotarod test, by the respective ED_{50} value, as determined in the MES or *sc*PTZ tests. The protective index is considered as an index of the margin of safety and tolerability between anticonvulsant doses and doses of the compounds exerting acute adverse effects e.g. sedation, motor coordination impairment, ataxia or other neurotoxic manifestations [50].

4.3. *In vitro* pharmacology

4.3.1. Antiproliferative assay

Human embryonic kidney HEK-293 cell line (ATCC CRL-1573) was kindly donated by Prof. Dr. Christa Müller (Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn). Neuroblastoma IMR-32 cell line was provided by Department of Oncogenomics, Academisch Medisch Centrum, Amsterdam, Holland [51,52]. The cell lines were seeded in 96-well plates at a concentration of 1.5×10^4 cells/well (HEK-293) and 2×10^4 cells/well (IMR-32) and cultured in 200 µl of Dulbecco's Modified Eagle's Medium - DMEM (Gibco) with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, 100 U/ml penicillin at 37°C in an atmosphere containing 5% CO_2 for 24 h to reach 60% confluence. The stock solution of compound **8** in DMSO (25 mM) was diluted into fresh growth medium and added to the wells at final concentrations 0.01 µM – 250 µM. DMSO concentration did not exceed 1%. After 48 h of incubation, the EZ4U (EZ4U Non-radioactive cell proliferation and cytotoxicity assay**,** Biomedica) labeling mixture (20 µl) was added to each well and the cells were incubated

under the same conditions for 5 h. The absorbance of the samples was measured using a microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA) at 492 nm. The activity of the reference compound doxorubicin (DX) was estimated as described previously [53].

4.3.2. Metabolic stability

4.3.2.1. Reaction with recombinant human liver microsomes (HLM)

The biotransformation was carried out using 1 mg/ml of commercial, pooled, human (adult male & female) liver microsomes (HLMs) provided by Sigma-Aldrich (St. Louis, USA) in 200 µl of reaction buffer containing 0.1 M Tris-HCl (pH 7.4), NADPH Regeneration System (Promega, Madison, WI, USA) and compound **8** with final volume 50 µM. The reaction mixture was preincubated at 37°C for 5 min and then, the reaction was initiated by adding 50 µl of Regeneration System. The reaction was conducted for 120 min and was terminated after addition of 200 µl of cold methanol. The mixture was centrifuged at 13 000 rpm for 15 min and next the UPLC-MS/MS analyses of the supernatant was performed. Mass spectra was recorded on UPLC-MS/MS system consisted of a Waters Acquity UPLC (Waters, Milford, USA), coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole).

4.3.2.2. CYP3A4 P450-Glo™ Assay

The luminescent CYP3A4 P450-Glo™ Assay and protocol was provided by Promega (Madison, WI, USA). The enzymatic reactions were performed in white polystyrene, flat-bottom Nunc™ MicroWell™ 96-Well Microplates (Thermo Scientific, Waltham, MA, USA) with the final concentrations of compound **8** 0.025 µM – 25 µM. The luminescence signal was measured with a microplate reader in luminescence mode (EnSpire, PerkinElmer, Waltham,

MA, USA). The IC_{50} value of the CYP3A4 inhibitor ketoconazole was calculated as reported previously [54].

4.3. *In silico* studies

The computational procedure MetaSite 4.1.1 provided by Molecular Discovery Ltd was used for prediction of compound **8** metabolic biotransformations. MetaSite identifies the most likely sites of metabolism in examined compound and predicts the structures of the metabolites. The highest metabolism probability sites were analyzed during this study using computational liver model [41].

Acknowledgments

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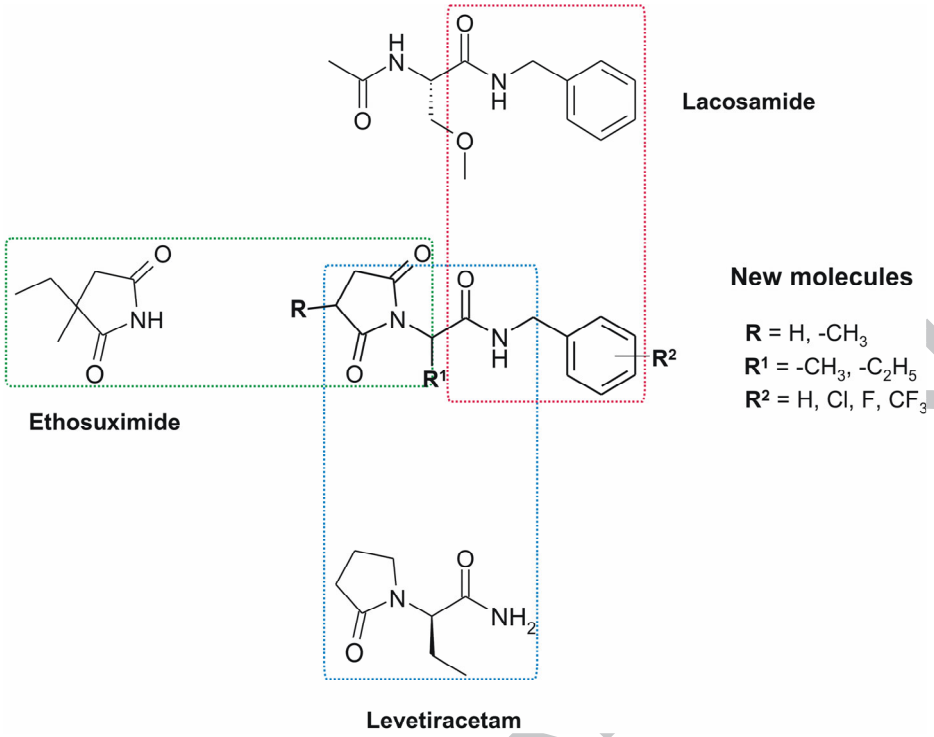


Figure 1. The current design and the general structure of new compounds.

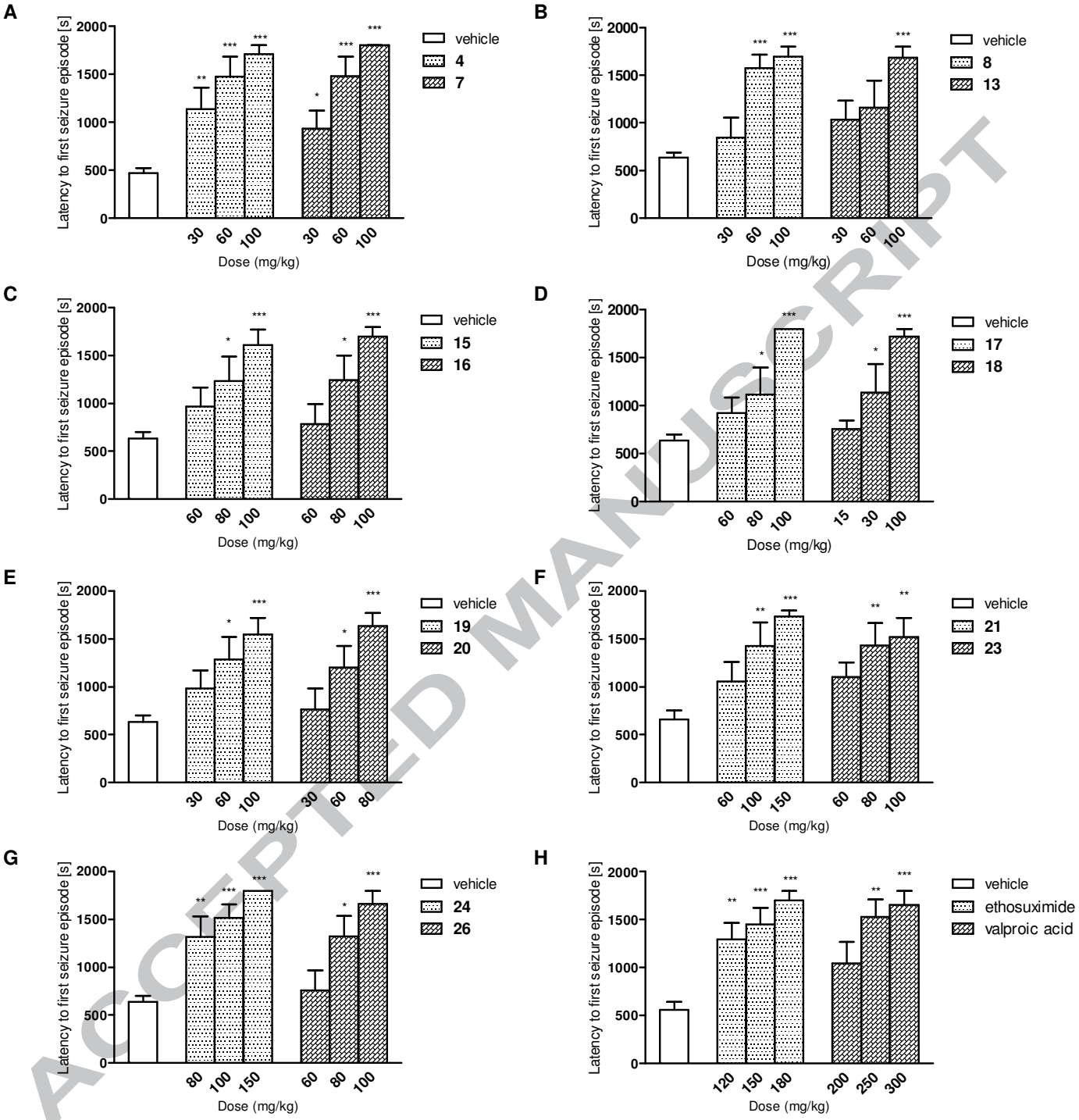


Figure 2 A-H. Anticonvulsant activity of compounds **4**, **7**, **8**, **13**, **15–21**, **23**, **24**, **26**, and reference AEDs: ethosuximide and valproic acid in the *sc*PTZ test. Each value represents the

mean ± SEM obtained from 6–8 mice. Statistical analysis: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test: $F[3,26] = 19.84$, $p < 0.0001$ (**4**); $F[3,26] = 27.99$, $p < 0.0001$ (**7**); $F[3,24] = 18.08$, $p < 0.0001$ (**8**); $F[3,24] = 7.76$, $p < 0.001$ (**13**); $F[3,25] = 7.56$, $p < 0.001$ (**15**); $F[3,25] = 10.45$, $p < 0.0001$ (**16**); $F[3,24] = 14.98$, $p < 0.0001$ (**17**); $F[3,25] = 11.77$, $p < 0.0001$ (**18**); $F[3,25] = 7.72$, $p < 0.001$ (**19**); $F[3,25] = 14.13$, $p < 0.0001$ (**20**); $F[3,20] = 7.71$, $p < 0.01$ (**21**); $F[3,21] = 6.42$, $p < 0.01$ (**23**); $F[3,21] = 13.51$, $p < 0.0001$ (**24**); $F[3,22] = 9.10$, $p < 0.001$ (**26**); $F[3,20] = 12.71$, $p < 0.0001$ (ethosuximide); $F[3,20] = 8.94$, $p < 0.001$ (valproic acid). The compounds were administered *i.p.* 0.25 h (ethosuximide), 0.5 h (valproic acid, **4**, **7**, **8**, **15**–**21**, **23**, **24**, **26**) or 2 h (**13**) before the test. Significant difference compared to the vehicle-treated group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

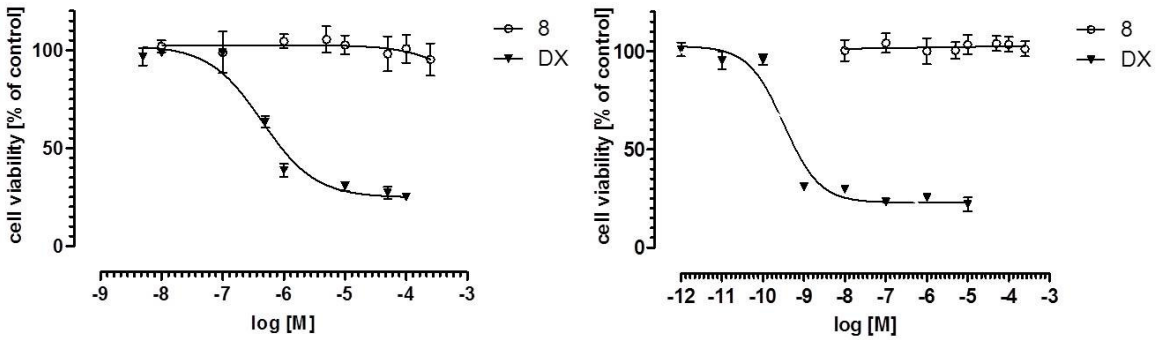


Figure 3. Activity of the reference doxorubicin (DX) and compound **8** against HEK-293 (left) and neuroblastoma IMR-32 (right) cell lines.

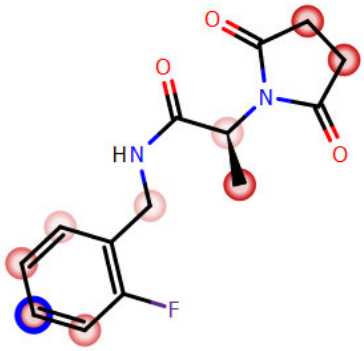


Figure 4. The plot of MetaSite predictions for sites of metabolism of compound **8**. The darker red color of the marked functional group indicates its higher probability to be involved in the metabolism pathway. The blue circle marked the site of **8** which will be involved in metabolism with the highest probability.

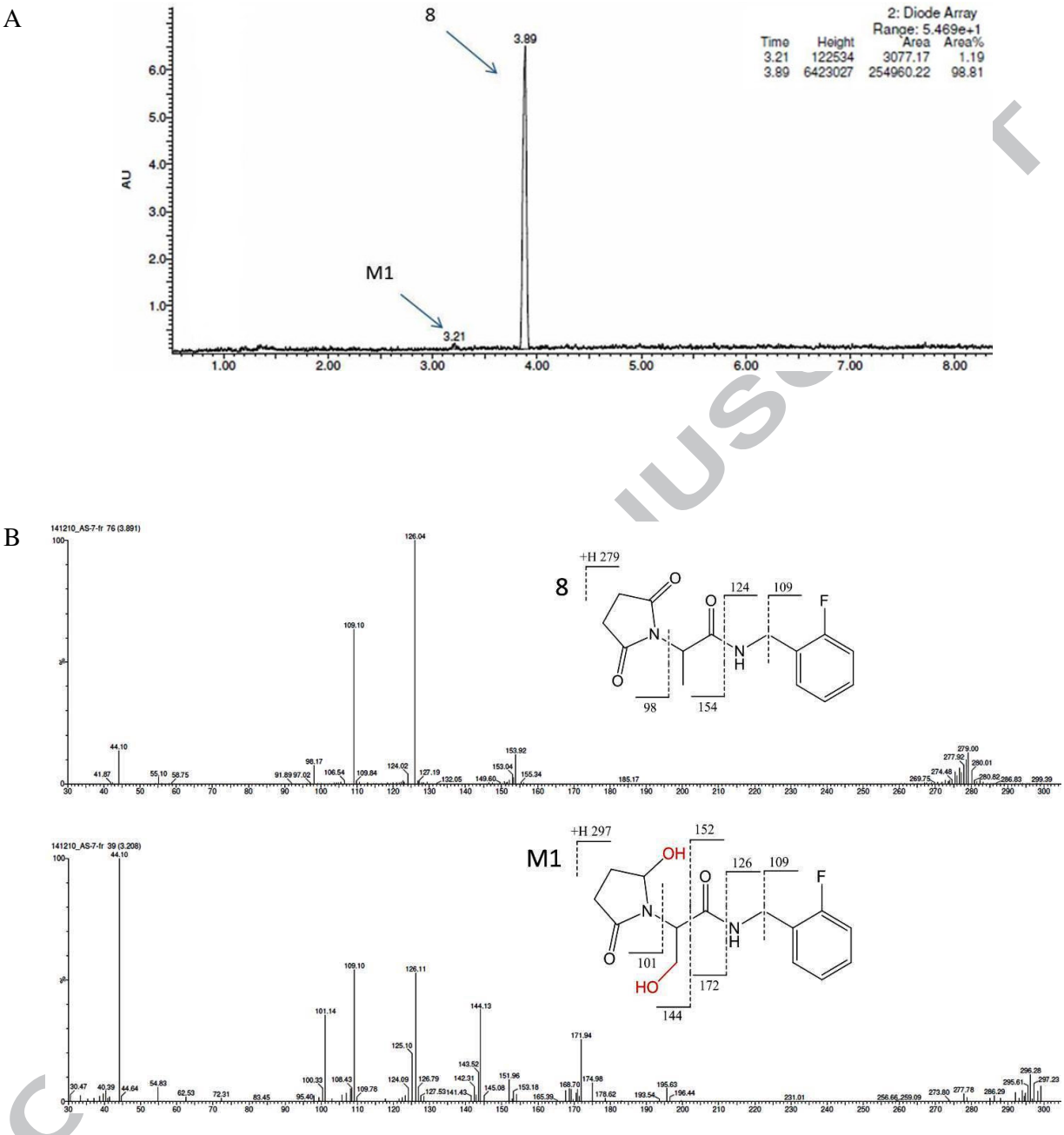


Figure 5. **A** - The UPLC spectrum after 2 h reaction of **8** with HLMs. **B** - MS/MS spectrums and ion fragments analysis of compound **8** and M1 in the total ion chromatogram.

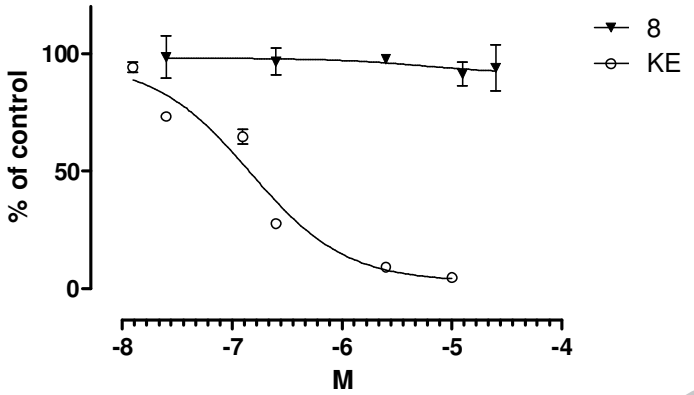
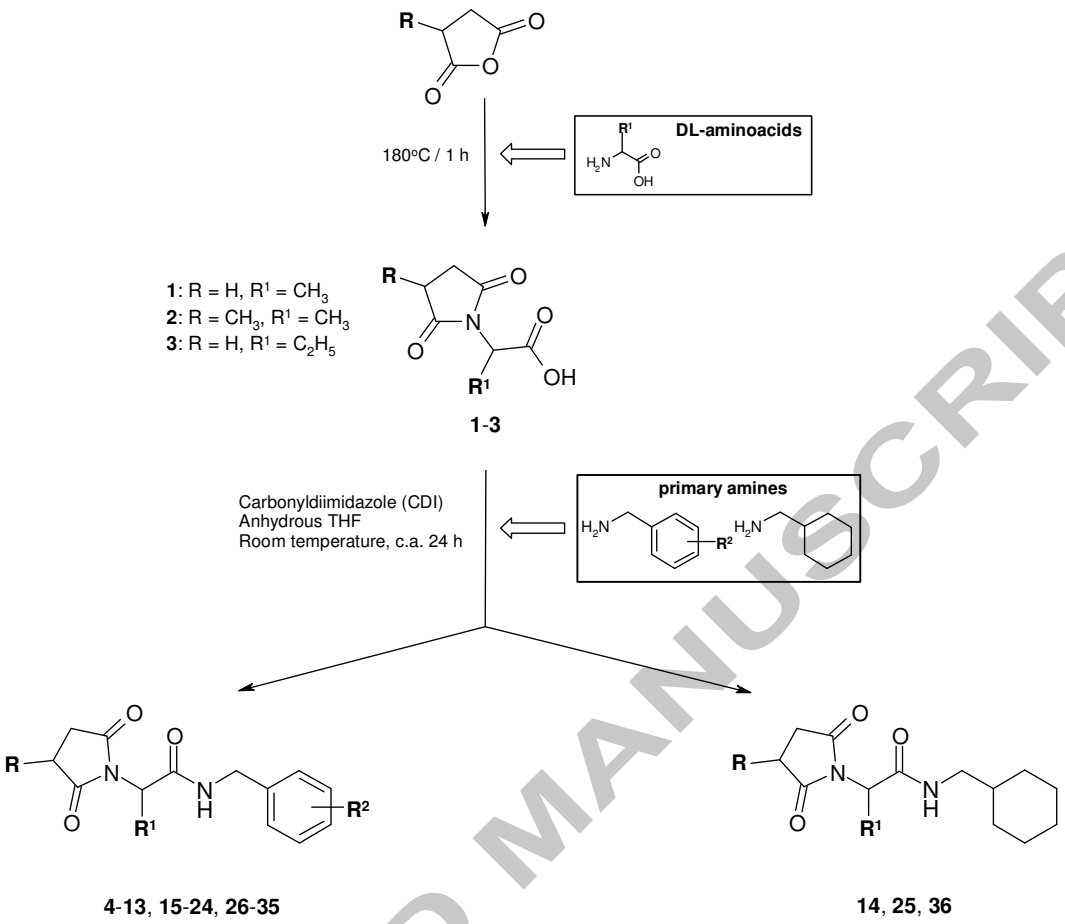


Figure 6. The effect of ketoconazole (KE) (CYP3A4 inhibitor) and compound **8** on CYP3A4 P450 cytochrome activity.



R = H (**4**-**14**)

4: $R^1 = CH_3$, R^2 = H
5: $R^1 = CH_3$, R^2 = 2-Cl
6: $R^1 = CH_3$, R^2 = 3-Cl
7: $R^1 = CH_3$, R^2 = 4-Cl
8: $R^1 = CH_3$, R^2 = 2-F
9: $R^1 = CH_3$, R^2 = 3-F
10: $R^1 = CH_3$, R^2 = 4-F
11: $R^1 = CH_3$, R^2 = 2-CF_3
12: $R^1 = CH_3$, R^2 = 3-CF_3
13: $R^1 = CH_3$, R^2 = 4-CF_3

R = H (**15**-**25**)

15: $R^1 = C_2H_5$, R^2 = H
16: $R^1 = C_2H_5$, R^2 = 2-Cl
17: $R^1 = C_2H_5$, R^2 = 3-Cl
18: $R^1 = C_2H_5$, R^2 = 4-Cl
19: $R^1 = C_2H_5$, R^2 = 2-F
20: $R^1 = C_2H_5$, R^2 = 3-F
21: $R^1 = C_2H_5$, R^2 = 4-F
22: $R^1 = C_2H_5$, R^2 = 2-CF_3
23: $R^1 = C_2H_5$, R^2 = 3-CF_3
24: $R^1 = C_2H_5$, R^2 = 4-CF_3

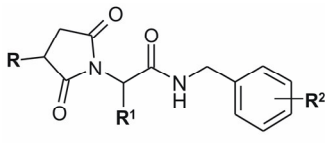
R = CH_3 (**26**-**36**)

26: $R^1 = C_2H_5$, R^2 = H
27: $R^1 = C_2H_5$, R^2 = 2-Cl
28: $R^1 = C_2H_5$, R^2 = 3-Cl
29: $R^1 = C_2H_5$, R^2 = 4-Cl
30: $R^1 = C_2H_5$, R^2 = 2-F
31: $R^1 = C_2H_5$, R^2 = 3-F
32: $R^1 = C_2H_5$, R^2 = 4-F
33: $R^1 = C_2H_5$, R^2 = 2-CF_3
34: $R^1 = C_2H_5$, R^2 = 3-CF_3
35: $R^1 = C_2H_5$, R^2 = 4-CF_3

Scheme 1. Synthesis of intermediates **1–3** and target compounds **4–36**.

Table 1

Anticonvulsant activity (MES, *sc*PTZ) and acute neurotoxicity (rotarod test) following *i.p.* administration in mice (dose of 100 mg/kg)



No	R	R^1	R^2	Pretreatment times					
				MES[a]		PTZ[b]		NT[c]	
				0.5 h	2 h	0.5 h	2 h	0.5 h	2 h
4	H	CH_3	H	100	–	100	–	–	–
5	H	CH_3	2-Cl	100	–	–	–	–	–
6	H	CH_3	3-Cl	100	–	–	–	–	–
7	H	CH_3	4-Cl	100	–	100	–	–	–
8	H	CH_3	2-F	100	–	100	–	–	–
9	H	CH_3	3-F	100	–	–	–	–	–
10	H	CH_3	4-F	–	–	–	–	–	–
11	H	CH_3	2-CF_3	–	–	–	–	–	–
12	H	CH_3	3-CF_3	100	–	–	–	–	–
13	H	CH_3	4-CF_3	100	–	–	100	–	–
14	H	CH_3	–	–	–	–	–	–	–
15	H	C_2H_5	H	100	–	100	–	–	–
16	H	C_2H_5	2-Cl	100	–	100	100	–	–
17	H	C_2H_5	3-Cl	100	–	100	–	–	–
18	H	C_2H_5	4-Cl	100	–	100	100	–	–
19	H	C_2H_5	2-F	–	–	100	100	–	–
20	H	C_2H_5	3-F	–	–	100	–	–	–
21	H	C_2H_5	4-F	–	–	100	–	–	–
22	H	C_2H_5	2-CF_3	–	–	–	100	–	–
23	H	C_2H_5	3-CF_3	–	–	100	–	–	–
24	H	C_2H_5	4-CF_3	100	–	100	–	–	–
25	H	C_2H_5	–	–	–	–	–	–	–
26	CH_3	CH_3	H	100	–	100	–	–	–
27	CH_3	CH_3	2-Cl	100	–	–	–	–	–
28	CH_3	CH_3	3-Cl	100	–	–	–	–	–

29	CH_3	CH_3	4-Cl	–	–	–	–	–	–
30	CH_3	CH_3	2-F	100	–	–	–	–	–
31	CH_3	CH_3	3-F	100	–	–	–	–	–
32	CH_3	CH_3	4-F	–	–	–	–	–	–
33	CH_3	CH_3	2-CF_3	–	–	–	–	–	–
34	CH_3	CH_3	3-CF_3	–	–	–	–	–	–
35	CH_3	CH_3	4-CF_3	–	100	–	–	–	–
36	CH_3	CH_3	–	–	–	–	–	–	–

Data indicate the minimum dose whereby anticonvulsant activity or motor impairment (neurotoxicity) were demonstrated in half or more animals in a group consisting of four mice. The animals were examined at two pretreatment times–0.5 h, and 2 h. A dash indicates the absence of anticonvulsant activity or neurotoxicity at dose of 100 mg/kg.

[a] MES–maximal electroshock seizures test.

[b] PTZ–subcutaneous pentylenetetrazole seizures test.

[c] NT– neurotoxicity screening–rotorod test.

Table 2

The quantitative pharmacological parameters ED_{50}, TD_{50}, and PI values following *i.p.* administration in mice

No	TPE (h)[a]	ED_{50} MES (mg/kg)[b]	ED_{50} PTZ (mg/kg)[c]	TD_{50} (mg/kg)[d]	PI (TD_{50}/ED_{50})[e]
4	0.5	67.65 (56.36–81.20)	42.83 (24.47–74.99)	347.63 (307.54–392.88)	5.14 (MES) 8.12 (PTZ)
5	0.5	74.49 (61.80–89.79)	–	340.30 (309.04–374.72)	4.57 (MES)
6	0.5	81.95 (70.45–95.32)	–	346.82 (308.82–389.49)	4.23 (MES)
7	0.5	91.84 (67.56–124.86)	43.58 (35.74–53.15)	228.08 (191.73–271.32)	2.48 (MES) 5.23 (PTZ)
8	0.5	54.90 (48.34–62.35)	50.29 (34.79–72.68)	300.89 (256.76–352.61)	5.48 MES) 5.98 (PZT)
9	0.5	77.43 (61.94–96.79)	–	> 500	> 6.46 (MES)
12	0.5	92.37 (82.80–103.05)	–	331.67 (301.58–364.77)	3.59 (MES)
13	0.5 (MES) 2.0 (PTZ)	80.05 (68.88–93.03)	54.50 (37.42–79.37)	142.86 (132.74–153.77)	1.78 (MES) 2.62 (PTZ)
15	0.5	73.26 (57.41–93.48)	90.26 (66.48–122.56)	312.46 (304.68–320.48)	4.26 (MES) 3.46 (PTZ)
16	0.5	113.02 (97.74–130.68)	76.39 (66.48–122.56)	417.96 (370.95–470.93)	3.70 (MES) 5.47 (PTZ)

17	0.5	96.55 (87.60–106.41)	78.85 (74.67–83.26)	180.03 (161.17–201.11)	1.12 (MES) 2.28 (PTZ)
18	0.5	95.35 (86.54–105.05)	50.77 (36.57–70.49)	174.22 (155.71–194.94)	1.83 (MES) 3.43 (PTZ)
19	0.5	–	65.71 (37.69–114.57)	346.24 (335.88–356.92)	5.27 (PTZ)
20	0.5	–	47.39 (38.69–58.04)	326.78 (311.35–342.97)	6.89 (PTZ)
21	0.5	–	83.43 (54.64–127.40)	268.38 (251.54–286.35)	3.22 (PTZ)
22*	0.5	> 150	–	–	–
23	0.5	–	77.37 (66.37–90.21)	163.02 (146.62–181.24)	2.12 (PTZ)
24	0.5	69.40 (57.38–83.94)	80.37 (69.11–93.47)	112.30 (104.88–120.24)	1.62 (MES) 1.40 (PTZ)
26	0.5	106.27 (85.42–132.20)	78.30 (65.82–93.13)	355.22 (321.93–391.97)	3.34 (MES) 4.54 (PTZ)
27	0.5	85.85 (56.70–129.97)	–	275.12 (235.26–321.73)	3.20 (MES)
28	0.5	90.26 (66.48–122.54)	–	246.61 (214.94–282.97)	2.73 (MES)
30	0.5	80.45 (69.20–93.49)	–	239.37 (209.05–274.09)	2.97 (MES)
31	0.5	129.64 (114.29–147.05)	–	297.20 (265.19–333.07)	2.29 (MES)

35*	2.0	> 150	–	–	–
Carbamazepine[f]	0.5[g]	14.20 (12.78–15.91)	> 100	49.30 (41.61–58.40)	3.47 (MES)
Ethosuximide[f]	0.25[g]	> 500	140.40 (115.81–170.21)	318.01 (295.80–341.89)	2.26 (PTZ)
Lacosamide[f]	0.5[g]	9.24 (8.53–10.0)	> 500	46.20 (44.48–48.00)	5.00 (MES)
Valproic acid[f]	0.5[g]	252.74 (220.10–290.22)	239.45 (209.18–274.10)	430.77 (407.92–454.90)	1.70 (MES) 1.80 (PTZ)

Values in parentheses are 95% confidence intervals determined by probit analysis [49].
[a] Time to peak effect. TPEs taken from literature [34].
[b] ED_{50} (MES-maximal electroshock seizure test)
[c] ED_{50} (*sc*PTZ-pentylenetetrazole seizure test)
[d] TD_{50} (NT-acute neurological toxicity determined in the rotarod test).
[e] Protective index (TD_{50}/ED_{50}).
[f] Reference AEDs tested in the same conditions.
* It was not possible to determine the ED_{50} values up to dose of 150 mg/kg.
A dash indicates-not tested.

List of captions:

Figure 1. The current design and the general structure of new compounds.

Figure 2 A-H. Anticonvulsant activity of compounds **4**, **7**, **8**, **13**, **15–21**, **23**, **24**, **26**, and reference AEDs: ethosuximide and valproic acid in the *sc*PTZ test. Each value represents the mean ± SEM obtained from 6–8 mice. Statistical analysis: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test: $F[3,26] = 19.84$, $p < 0.0001$ (**4**); $F[3,26] = 27.99$, $p < 0.0001$ (**7**); $F[3,24] = 18.08$, $p < 0.0001$ (**8**); $F[3,24] = 7.76$, $p < 0.001$ (**13**); $F[3,25] = 7.56$, $p < 0.001$ (**15**); $F[3,25] = 10.45$, $p < 0.0001$ (**16**); $F[3,24] = 14.98$, $p < 0.0001$ (**17**); $F[3,25] = 11.77$, $p < 0.0001$ (**18**); $F[3,25] = 7.72$, $p < 0.001$ (**19**); $F[3,25] = 14.13$, $p < 0.0001$ (**20**); $F[3,20] = 7.71$, $p < 0.01$ (**21**); $F[3,21] = 6.42$, $p < 0.01$ (**23**); $F[3,21] = 13.51$, $p < 0.0001$ (**24**); $F[3,22] = 9.10$, $p < 0.001$ (**26**); $F[3,20] = 12.71$, $p < 0.0001$ (ethosuximide); $F[3,20] = 8.94$, $p < 0.001$ (valproic acid). The compounds were administered *i.p.* 0.25 h (ethosuximide), 0.5 h (valproic acid, **4**, **7**, **8**, **15–21**, **23**, **24**, **26**) or 2 h (**13**) before the test. Significant difference compared to the vehicle-treated group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 3. Activity of the reference doxorubicin (DX) and compound **8** against HEK-293 (left) and neuroblastoma IMR-32 (right) cell lines.

Figure 4. The plot of MetaSite predictions for sites of metabolism of compound **8**. The darker red color of the marked functional group indicates its higher probability to be involved in the metabolism pathway. The blue circle marked the site of **8** which will be involved in metabolism with the highest probability.

Figure 5. **A** - The UPLC spectrum after 2 h reaction of **8** with HLMs. **B** - MS/MS spectrums and ion fragments analysis of compound **8** and M1 in the total ion chromatogram.

Figure 6. The effect of ketoconazole (KE) (CYP3A4 inhibitor) and compound **8** on CYP3A4 P450 cytochrome activity.

Scheme 1. Synthesis of intermediates **1–3** and target compounds **4–36**.

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Table 2. The quantitative pharmacological parameters ED_{50}, TD_{50}, and PI values following *i.p.* administration in mice.

Graphical abstract