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Design, synthesis, and anticonvulsant activity of new *N*-Mannich bases derived from spirosuccinimides and spirohydantoins

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ABSTRACT

The synthesis and anticonvulsant properties of new *N*-Mannich bases of [7,8-*f*]benzo-2-aza-spiro[4.5]decane-1,3-diones (**5a**–**h**) and [7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-diones (**7a**–**h**) were described. Initial anticonvulsant screening was performed using intraperitoneal (ip) maximal electroshock (MES) and subcutaneous pentylenetetrazole (*sc*PTZ) seizures tests. The neurotoxicity was determined applying the rotarod test. The majority of compounds were effective in the MES or/and *sc*PTZ screen. The quantitative studies showed that several molecules were more potent than phenytoin, used as reference drug. Selected derivatives were screened in the 6-Hz test and also assessed for potential activity against nerve agents using the Pilocarpine Induced Status Prevention model. To explain the possible mechanism of anticonvulsant action, for chosen active derivatives, their influence on voltage-dependent Na^+ channel were tested in vitro.

1. Introduction

The past decades have demonstrated many attempts to identify the structural features of compounds crucial for anticonvulsant activity. It's well documented that one of the important core fragments of anticonvulsants is defined by nitrogen heteroatomic system, usually a cyclic imide, at least of one carbonyl group and phenyl or alkyl groups attached to the heterocyclic system.[1–3] This common template is present in the structures of two old, however well-established anticonvulsants (AEDs) such as ethosuximide and phenytoin as well as among the newest drugs, for example, levetiracetam, brivaracetam, or seletracetam[4–6] (Fig. 1).

The incomplete information about the cellular mechanism of human epilepsy as well as the complex mechanism of action of most AEDs makes it difficult to use of rational methodologies of discovery. Therefore an alternative rational design of new AEDs is based on mentioned pharmacophoric patterns and enables to overcome the above difficulties. The currently used AEDs can be classified into four categories on the basis of the main molecular mechanisms of action, as follow: (i) modulation of voltage-dependent Na^+ and/or Ca^{2+} channels, (ii) enhancement of GABA-mediated inhibition or other effect on the GABA system, (iii) inhibition of synaptic excitation mediated by ionotropic glutamate receptors, and (iv) modulation of synaptic release.[7] In general, the discovery of new anticonvulsants is still particularly focused on targeting voltage-dependent Na^+ channels since they play a fundamental role in establishing and regulation the excitability of neurons within the central nervous system (CNS). Furthermore the changes in the expression pattern and behavior of different Na^+ channels isoforms are observed in epilepsy and could play a role in seizure generation and spread.[8,9]

Previous research in our group has identified the pyrrolidine-2,5-dione system differently substituted at position-1 and -3 as targets for new antiepileptic drugs. Many of these compounds were effective in the maximal electroshock (MES) or/and subcutaneous pentylenetetrazole (*sc*PTZ) screens that are known as the most popular seizure models in the early stages of the search of new anticonvulsants.[10–12]

Recent studies from our laboratory have demonstrated the potent anticonvulsant activity among the *N*-(4-arylpiperazin-1-yl)-alkyl-azaspiranes.[13,14] In this group of derivatives the most active was compound **1** with the ED_{50} value of 23 mg/kg and its trifluoromethyl analogue **2** that showed the ED_{50} of 205 mg/kg in the MES test (Fig. 1). Taking into consideration the above, as part of our efforts to design new anticonvulsant agents, in the present study we have synthesized a new series of compounds with the [7,8-*f*]benzo-2-aza-spiro[4.5]decane-1,3-dione (**5a–h**) system as a core fragment. Based on results obtained previously,[13,14] at the nitrogen atom of succinimide we have introduced, by methylene spacer amines favorable for anticonvulsant activity: 4-phenyl-piperazines

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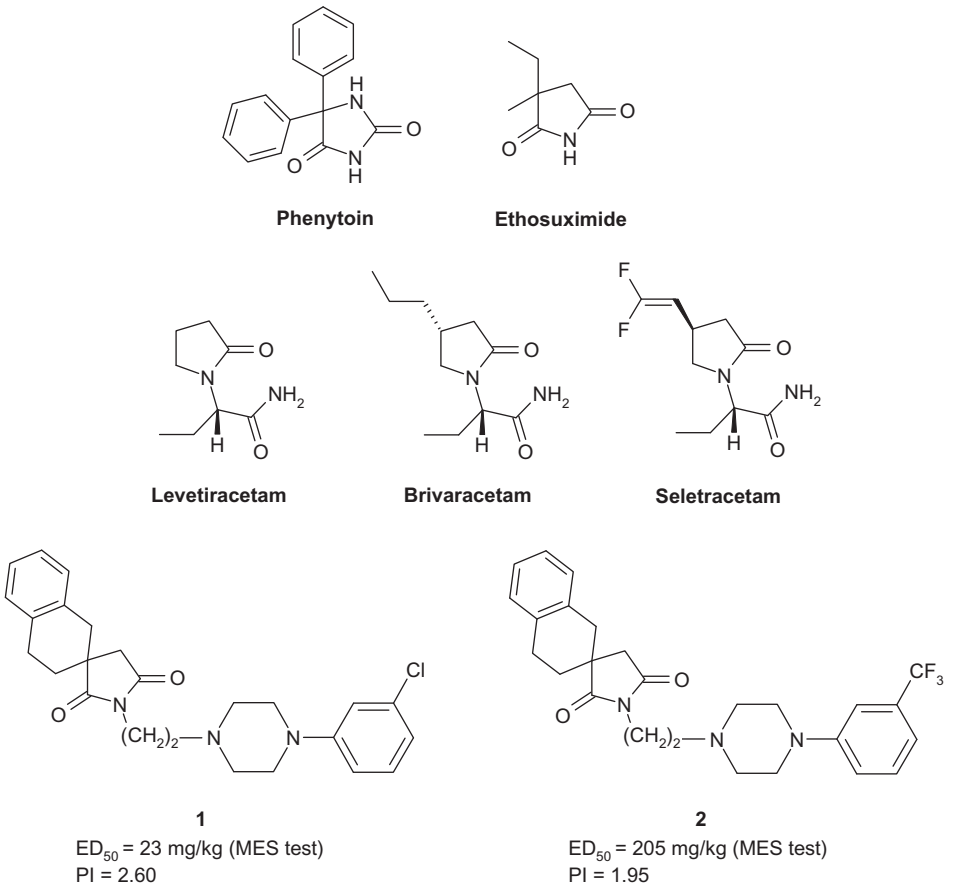


Figure 1. Lead structures for target compounds **5a**–**h** and **7a**–**h**.

(unsubstituted or with electron-withdrawing Cl, F atoms or CF_3 group), benzyl-piperazine, 2-hydroxyethyl-piperazine or morpholine. On the other hand, taking into account well documented anticonvulsant properties many of hydantoins[15–17] and spirohydantoins,[18–20] as a second step of our investigation, we have synthesized a series of analogues **5a–h** in which the succinimide core fragment has been changed into the hydantoin ring (**7a–h**). Such modification enabled the evaluation of the role of mentioned heterocyclic rings for the anticonvulsant activity.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **5a**–**h** and **7a**–**h** was accomplished as shown in Scheme 1. The starting materials 2-carboxy-1,2,3,4-tetrahydronaphtalene-2-carboxylic acid (**3**) and [7,8-*f*]benzo-2-azaspiro[4.5]decane-1,3-dione (**4**) were prepared using methods reported by Faust et al.[21] The β-tetralonehydantoin ([7,8-*f*]benzo-1,3-diaza-spiro-decane-2,4-dione) (**6**) was prepared from the 3,4-dihydronaphtalen-2-on (β-tetralone) by the Bücherer–Berg reaction with modifications described by Goodson et al.[22] In the next step, we prepared two series of new *N*-Mannich bases derived from spirosuccinimide (**5a**–**h**) or spirohydantoin (**7a**–**h**). For aminoalkylation of the acidic proton, in the presence of formaldehyde, we used various 4-substituted piperazines or morpholine. The reaction was carried out in ethanol at a room temperature for ca. 12 h. The final **5a**–**h** and **7a**–**h** compounds were obtained in yields ranging from 62% to 88%. Their purity was assessed by TLC chromatography. The structures of compounds synthesized were confirmed by both spectral and elemental analysis. The detailed physical and analytical data are listed in the experimental section.

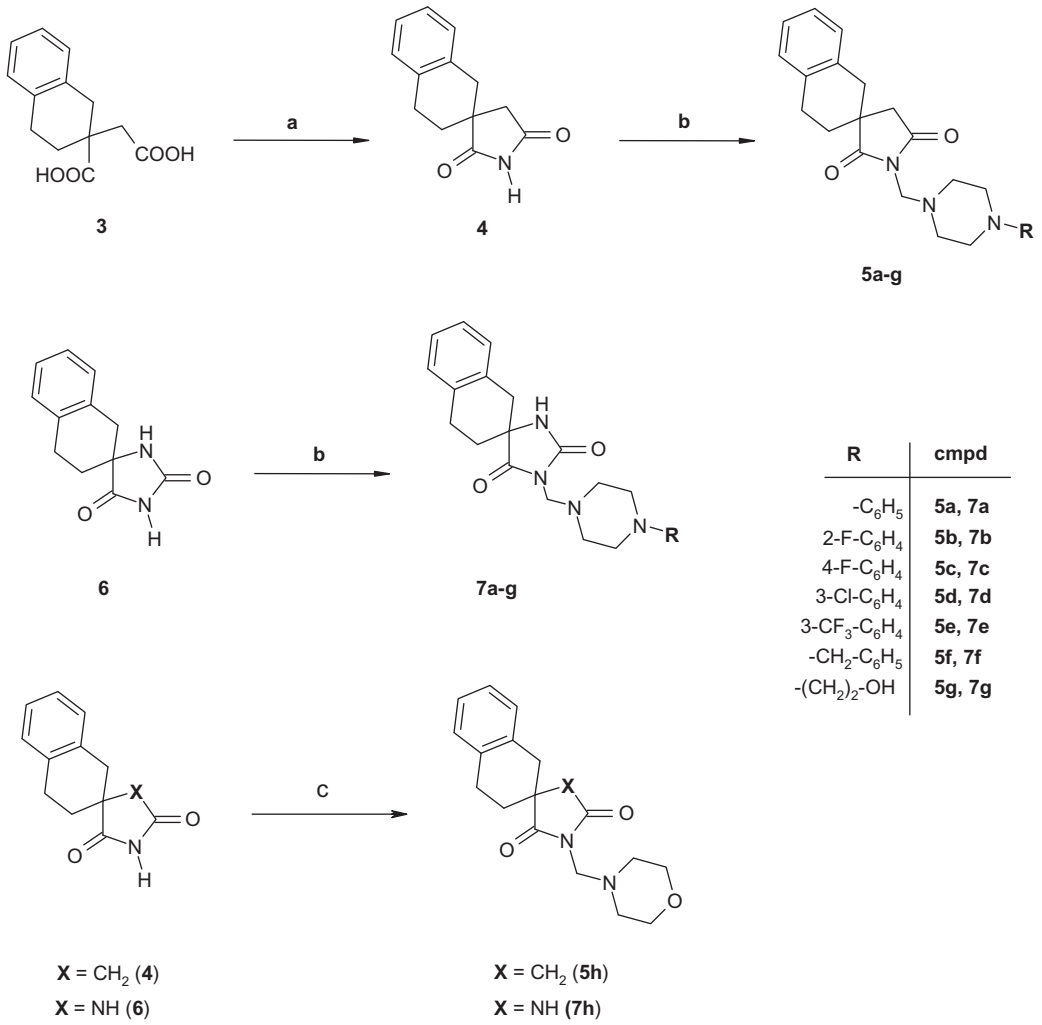
As usually in our practices,[23–26] the molecular structure of selected compounds, **5a** and **5h**, has been crystallographically confirmed. In both molecules the mutual positions of [7,8-*f*]benzo-2-aza-spiro[4.5]decane-1,3-dione moiety with respect to 4-arylpiperazine (**5a**) or morpholine (**5h**) are almost the same (Fig. 2).

2.2. Anticonvulsant activity

The pre-clinical discovery and development of new chemical agents for the treatment of epilepsy are based mainly on the use of predictable animal models. At the present time there are three in vivo screens used routinely that include the maximal electroshock seizure (MES), the subcutaneous pentylenetetrazole (*sc*PTZ) and the kindling model. From these tests the MES and *sc*PTZ screens are recognized as the 'gold standards' in the early stages of testing.[27]

The profile of anticonvulsant activity of compounds **5a**–**h** and **7a**–**h** was established in the maximal electroshock (MES) and subcutaneous pentylenetetrazole (*sc*PTZ) tests, after intraperitoneal injection into mice at doses of 30, 100, and 300 mg/kg. An observation was carried out at two different time intervals—0.5 and 4 h. The acute neurological toxicity (NT) was determined by use of the minimal motor impairment-rotarod screen (NT). The results are shown in Table 1.

The initial anticonvulsant evaluation of spirosuccinimides (**5a**–**h**) showed, that excluding **5a** and **5b**, devoid of anticonvulsant activity, all other derivatives were effective in MES test (**5c**–**h**) and several of them, namely **5c**, **5g**, and **5h** were also active in the *sc*PTZ screen. Compounds that revealed protection in the MES



Scheme 1. Synthetic protocol of the target compounds **5a**–**h** and **7a**–**h**. Reagents and conditions: (a) 25% NH_4OH, 190 °C, 2 h, (b) 4-substituted piperazine derivatives, formaldehyde, 96% ethyl alcohol, 12 h room temperature, and (c) morpholine, formaldehyde, 96% ethyl alcohol, 12 h room temperature.

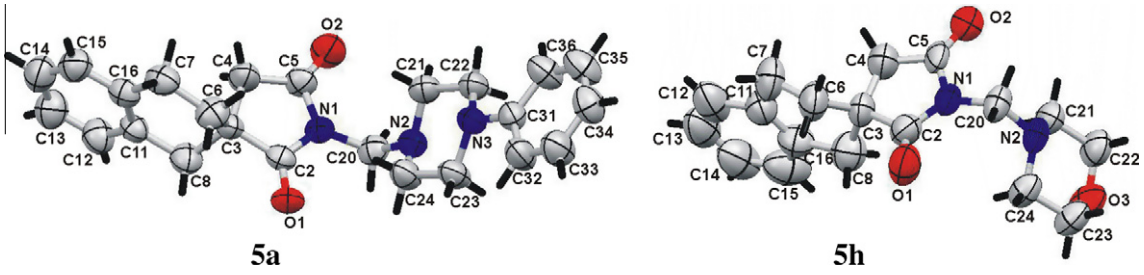


Figure 2. ORTHEP drawing of the **5a** and **5h** molecules.

test, indicative the ability of substance to prevent seizure spread, at a dose of 100 mg/kg included **5c**–**e**, **5g**, and **5h**, whereas molecule **5f** provided anti-MES protection at a dose of 300 mg/kg. Except of compound **5c**, which was active only at 0.5 h after ip administration, the other derivatives **5d**–**h** showed activity at 4 h, indicating long duration of anticonvulsant activity. Apart from the anti-MES protection, compounds **5c**, **5g**, and **5h** were found to be active in the *sc*PTZ test, which identify substances elevating seizure threshold. Among these molecules, **5c** and **5h** showed anti-*sc*PTZ activity at the dose 100 mg/kg that was equivalent to ethosuximide used as reference anticonvulsant. In the neurotoxicity screen (NT), only **5f** did not show neurotoxicity in the maximum dose administered (300 mg/kg). The other derivatives **5c**–**e**, **5g**, and **5h** showed neurotoxicity at a dose of 300 mg/kg. At the same dose mice were unable to grasp rotarod after administration of **5g** and **5h**.

The change of core fragment from [7,8-*f*]benzo-2-aza-spiro-[4.5]decane-1,3-dione (spirosuccinimide) to [7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (spirohydantoin) yielded in more potent, however at the same time more neurotoxic compounds **7a**–**h**. In this series only **7c** was inactive that was in contrary to its succinimide analogue **5c**, active both in the MES and *sc*PTZ test. The other derivatives inhibited seizures only in the MES—**7a**, **7b**, **7d**, **7e**, and **7f** or in both MES and *sc*PTZ screens—**7g** and **7h**. In this series, the most active was **7h** which showed anti-MES protection

Table 1
Anticonvulsant screening project (ASP): phase I results for compounds **5a**–**h** and **7a**–**h**

Compd	Intraperitoneal injection in mice[a]						ASP class[e]
	MES[b]		*sc*PTZ[c]		NT[d]		
	0.5 h	4 h	0.5 h	4 h	0.5 h	4 h	
5a	—	—	—	—	100	300	3
5b	—	—	—	—	300	—	3
5c	100	—	100[25]	—	300	300	1
5d	300	100	—	—	300	—	1
5e	—	100	—	—	—	300	1
5f	300	300	—	—	—	—	2
5g	100	100	300	—	300[14]	—	1
5h	100	300	100	—	300[14]	—	1
7a	—	30	—	—	300	300	1
7b	—	100	—	—	300[33]	300[12,34]	1
7c	—	—	—	—	30	30	4
7d	—	100	—	—	30	30	4
7e	300	30	—	—	100	30	4
7f	100	100	—	—	300[1]	—	1
7g	100	300	100	300	100[14]	—	1
7h	30	30	100	100	100	—	1
Phenytoin[f]	30	30	—	—	100	100	
Ethosuximide[f]	—	—	100	300	—	—	

Response comments: [1]death, [12]ataxia, [14]unable to grasp rotarod, [25]myoclonic jerks, [33]tremors, [34]muscle spasms.

[a] Doses of 30, 100, and 300 mg/kg were administered. The data indicate the minimum dose whereby anticonvulsant activity or neurotoxicity was demonstrated in half or more animals. The animals were examined at 0.5 and 4 h. A dash indicates the absence of anticonvulsant activity and neurotoxicity at the maximum dose administered (300 mg/kg).

[b] Maximal electroshock test.

[c] Subcutaneous pentylenetetrazole test.

[d] Neurotoxicity screening—rotarod test.

[e] The ASP classification is as follows: 1—anticonvulsant activity at doses of 100 mg/kg or less; 2—anticonvulsant activity at doses of 300 mg/kg; 3—compound inactive at doses of 300 mg/kg; 4—compound active but toxic at a dose of 30 mg/kg or only toxic at the same dose.

[f] Reference drugs, data for phenytoin and ethosuximide Ref. 39.

at a dose of 30 mg/kg in both time intervals that was equal to activity of phenytoin used as reference drug. Additionally, it was also effective in the *sc*PTZ screen at a dose of 100 mg/kg at 0.5 and 4 h. Furthermore, the anti-MES activity at doses of 30 mg/kg was observed for **7a** and **7e**, however the letter was neurotoxic at the same dose unfortunately. The 2-fluoro derivative **7b** was active at a dose of 100 mg/kg but at a dose of 300 mg/kg provoked muscle spasms, tremors, and ataxia. As presented in Table 1, compounds **7d** and **7f** inhibited seizure at a dose of 100 mg/kg (MES). The wider activity was observed for **7g**, which showed anti-MES and anti-*sc*PTZ protection at doses of 100 mg/kg (0.5 h) and 300 mg/kg (4 h), respectively. This derivative exhibited neurotoxicity at a dose of 100 mg/kg—mice were unable to grasp rotarod. It is worthy of note that compounds active both in MES and *sc*PTZ tests, may be useful in treating not only generalized tonic-clonic epilepsy but also absence seizures.

On the basis of data obtained in mice and according to Anticonvulsant Screening Project (ASP) disposition, nine compounds **5c**–**e**, **5g**, **5h**, **7a**, **7f**, **7g**, and **7h** were selected and examined for their anticonvulsant activity (MES screen) and neurotoxicity after po administration into rats at a dose of 30 mg/kg. The results obtained are presented in Table 2.

As can be seen from these data, in the series of spirosuccinimides the most active was **5e** that showed one peak of 100% protection at 4 h and 75% protection at time intervals 1 and 2 h. The other derivatives (**5d**, **5g**, and **5h**) were less active and protected at most of 50% animals. Much higher activity was observed for spirohydantoin derivatives. In this series the most active were **7a** and **7g** that protected 100% of animals at 2 and 4 h. Compound **7a** protected also 50% of rats (0.5 h, 1 h) and 25% (0.25 h), whereas **7g**—50% (1 h) and 25% (0.5 h). A little less activity revealed **7h**, namely

Table 2
Anticonvulsant activity (MES test) of selected compounds administrated orally to rats (30 mg/kg)

Compd	Oral administration to rats[a]				
	0.25 h	0.5 h	1 h	2 h	4 h
5c	0	0	0	1	1
5d	0	0	1	2	2
5e	0	0	3	3	4
5g	1	1	0	1	2
5h	2	1	1	1	2
7a	1	2	2	4	4
7f	1	2	3	2	2
7g	0	1	2	4	4
7h	0	1	2	3	4
Phenytoin[b]	1	4	3	3	3

[a] The data in the oral MES screen indicate the number of rats of four that were protected at a dose of 30 mg/kg.

[b] Reference drug, data from Ref. 40.

100% of animals at 4 h, 75% at 2 h, 50% at 1 h, and 25% at 0.5 h. Finally **7f** showed one peak of 75% protection at 1 h and was also effective in 50% of animals at 0.5, 2, and 4 h. As can be seen from the data above, the compounds tested were characterized by delayed onset and long time of anticonvulsant action. Furthermore, several molecules showed activity comparable to observed for phenytoin. When given orally they were non-neurotoxic (data not indicated in Table 2).

Sprosuccinimides **5c** and **5h**, active in both MES and *sc*PTZ screens in mice, were evaluated in the subcutaneous pentylenetetrazole test after oral administration into rats. Initially a dose of 50 mg/kg was employed and the anticonvulsant activity was assessed in five time points (Table 3).

As can be seen, **5c** protected 25% of animals at 2 and 4 h, whereas **5h** showed 25% protection time intervals from 0.5 to 4 h. These molecules were less active than ethosuximide, which is the model substance effective in the pentylenetetrazole seizures. These molecules did not exhibit neurotoxicity at the dose applied.

Compounds **5d**, **5e**, **5h**, **7a**, **7g**, and **7h** were chosen for quantification of the pharmacological parameters (ED_{50} and TD_{50}) after oral administration into rats. The quantitative evaluation of the MES and *sc*PTZ median effective dose (ED_{50}) and toxic dose (TD_{50}) were performed at previously estimated time of peak effect (TPE). Results of the quantitative tests along with the data for the standard drugs phenytoin and ethosuximide are shown in Table 4.

The analysis of quantitative data revealed that except of **5h** the other compounds were more potent than phenytoin in the MES test. In the applied range of doses none of them was enough effective to determine the ED_{50} in the pentylenetetrazole seizures. In this group the most active was **7a** with ED_{50} value of 1.88 mg/kg and TD_{50} of >500 mg/kg, that resulted in very promising protection index (TD_{50}/ED_{50}) of 265.78, especially when comparing to 35.58 of phenytoin. It should be pointed out that calculated PI values have only an approximate character because in general low neurotox-

Table 3
Anticonvulsant activity (*sc*PTZ test) of selected compounds administrated orally to rats (50 mg/kg)

Compd	Oral administration to rats[a]				
	0.25 h	0.5 h	1 h	2 h	4 h
5c	0	0	0	1	1
5h	0	1	1	1	1
Ethosuximide[b]	0	2	1	1	0

[a] Dose of 50 mg/kg was administrated. The data in the oral *sc*PTZ screen indicate the number of rats of four that were protected.

[b] Reference drug, data from Ref. 39.

Table 4
Quantitative anticonvulsant data in rats dosed orally for compounds **5d**, **5e**, **5h**, **7a**, **7g**, and **7h**

Compd	TPE (h)[a]	ED_{50}[b] MES (mg/kg)	ED_{50}[b] *sc*PTZ (mg/kg)	TD_{50}[b] (mg/kg)	PI[c] MES (TD_{50}/ED_{50})
5d	6.0	19.57 (9.23–34.80)	ND	>500	25.55
5e	2.0	9.00 (4.81–14.55)	ND	>250	>27.78
5h	0.5	>160	>150	>500	—
7a	6.0	1.88 (0.93–3.43)	ND	>500	>265.78
7g	4.0	14.93 (10.73–18.77)	>250	>300	>20.09
7h	6.0	10.65 (6.86–16.09)	>200	400	37.55
Phenytoin[d]	1.0	28.1 (27.7–35.20)	>500	>100	>3.6
Etosuximide[d]		>500	167 (116–237)	>500	>2.99

ND—no data.
[a] Time to peak effect.
[b] Results are represented as mean ± SEM at 95% confidence limit (MES-maximal electroshock test; *sc*PTZ—subcutaneous pentylenetetrazole test; neurotoxicity).
[c] Protection index (TD_{50}/ED_{50}).
[d] Reference drug, data from Ref. 41.

icity of all substances, including standard drugs, made impossible the precise estimation of TD_{50} parameters. However it is justified to claim that **5d**, **5e**, **7a**, **7g**, and **7h** were equal or more potent than reference phenytoin.

Selected compounds **5d**, **5e**, and **5f** were chosen for the evaluation of anticonvulsant activity in the 6-Hz test. The selection was made randomly as a part of the search of molecules providing anti 6-Hz protection among chemically diversified compounds pursued in the NIH/NINDS. The 6-Hz screen has been validated recently as a model of therapy-resistant epilepsy. It was not used widely because of its lack clinical validity since the hydantoins such as phenytoin failed to show protective activity. Nevertheless, the clinically effective antiepileptic drug levetiracetam, which is not active in the conventional MES and *sc*PTZ tests, does exhibit protective activity in the 6-Hz model. This suggested that the 6-Hz model might be capable for identifying anti-seizure agents with a novel spectrum of activity and unknown mechanism of anticonvulsant action.[28] The results obtained are shown in Table 5.

As can be seen compounds **5d**, **5e**, and **5f** administrated intraperitoneally at a dose of 100 mg/kg revealed marginal activity and protected 50% mice (**5d** at 4 h, **5e** at 2 h) and 25% at 1 h (**5e**, **5f**).

The ASP has employed recently a series of models designed to arrest benzodiazepine resistant *status epilepticus* including: pilocarpine induced status, neuroprotection against excitotoxins such as kainic acid (KA) or *N*-methyl-D-aspartate (NMDA) in in vitro hippocampal slice culture model, non-convulsive electrophysiology monitoring and isobolographic studies. These models were developed and incorporated into the ASP screening capabilities with the intent to find compounds useful against both convulsive and non-convulsive status.

According to the ASP dispositions, compounds **5c**–**e** were assessed for potential activity against nerve agents using the Pilocarpine Induced Status Prevention (PISP) model. This model shares many characteristics with nerve agent induced seizures since both initiation and early expression of nerve agent induced seizures are cholinergic followed by the recruitment of other neurotransmitter systems that serve to reinforce recurring seizure activity progressing to *status epilepticus*. To determine if the tested substances **5c**–**e** can prevent acute pilocarpine induced status in the Pilocarpine Induced Status Prevention (PISP) model an initial qualitative efficacy screens were performed. These molecules were given to rats via the ip route of administration. Then a challenge dose of pilocarpine was administrated observing for treatment-effects of the substance. The outcome measures are determination of 'protection' or 'no-protection'. The seizure severity is determined using the well known Racine scale,[29] as follow: (I) immobility, eye closure, twitching of vibrissae, sniffing, and facial clonus; (II) head nodding associated with more severe facial clonus; (III) clonus of one of the fore limbs; (IV) rearing often accomplished by bilateral forelimb clonus; and (V) all of the above plus loss of balance and falling, accomplished by generalized clonic seizures. The anticonvulsant activity of compounds **5c**–**e** was assessed at time zero, namely the time from the first stage III seizures. The results are shown in Table 6.

In the PISP screen, all molecules were found to be inactive.

During further studies compounds, **5d** and **5h** were examined in the in vitro hippocampal slice culture neuroprotection assay, however did not exhibit neuroprotection against KA and NMDA.

2.3. In vitro sodium channels binding assays

Intensive studies into the physiological and biochemical events taking place during epileptic seizures have provided insight into the molecular mechanisms by which these might be controlled. Inhibition of neuronal conductance via block of voltage-gated sodium channels is proven, however not only mechanism, by which anticonvulsants such phenytoin, carbamazepine or lamotrigine act to control seizures. To search for possible mechanism of action of chosen, active derivatives **5d**, **5e**, **7a**, and **7h**, their affinities for sodium channels were assessed in vitro. The sodium channels were

Table 5
Results of anticonvulsant activity by psychomotor seizure test (6-Hz, dose of 100 mg/kg)

Compd	Intraperitoneal injection into mice[a]				
	0.25 h	0.5 h	1 h	2 h	4 h
5d	0	0	0	0	2
5e	0	0	1	2[15]	0
5f	0	0	1	0	0

Response comments: [15]minimal motor impairment.
[a] Dose of 100 mg/kg was administrated. The data indicate the number of mice of four that were protected.

Table 6
Pilocarpine Induced Status Prevention (PISP) model. Results for compounds **5c**–**e** after ip administration (Test 71)

Compd	Dose (mg/kg)	Time (h)[a]	Protected rats	Non-protected rats	Average weight change ± SEM[b] (g)	
					Protected rats	Non-protected rats
5c	600	0	0	6	0	20 ± 1.1
5d	600	0	0	8	0	20.6 ± 1.1
5e	600	0	0	7	0	15.0 ± 0.0

[a] Post first stage III seizure.
[b] Weight change 24 h Post first stage III seizure.

chosen from many molecular targets for anticonvulsants on the basis of in vivo data that showed high activity of compounds tested in the maximal electroshock test. These studies were based on the ability of compound to displace [^{3}H]batrachotoxin from neurotoxin site 2 of voltage-gated sodium channels in rat forebrain tissue. The IC_{50} values of **5d**, **5e**, **7a**, and **7h** together with phenytoin used as standard drug effective in the electrically induced seizures are listed in Table 7.

As shown in Table 7 compounds **5d**, **5e**, and **7a** revealed higher affinities than phenytoin whereas **7h** did not bind to the voltage-gated sodium channels. The radioligand binding experiments suggest that anticonvulsant activity of compounds tested may be partially mediated by influence on the sodium channels. It should be emphasized that it is one but not only mechanism of action.

2.4. Structure–activity relationships

The results of the preliminary anticonvulsant screening of spirosuccinimides revealed that the most active were compounds with 3-chloro atom (**5d**) and 3-CF_3 (**5e**) group at the 4-arylpiperazine fragment that showed ED_{50} values of 19.23 and 9.00 mg/kg, respectively. It was in agreement with our previous studies in which we have demonstrated that presence of electron-attracting substituents, especially CF_3 groups, increases anticonvulsant activity.[10,12–14] The removal or replacement of the above substituents decreased anticonvulsant activity (**5c**) or made compounds inactive (**5a**, **5b**). The similar effect was observed in respect to the benzyl-piperazine analogue **5f**. The change of phenyl ring at position-4 of piperazine into hydroxyethyl chain (**5g**) has extended the profile of anticonvulsant activity and made compound active in both MES and *sc*PTZ seizures. Further data revealed that introduction of the spirohydantoin moiety as core fragment yielded in more potent, however at the same time more neurotoxic, derivatives. In this series the most active were unsubstituted compound **7a**, and derivatives with 2-hydroxyethyl-piperazine- (**7g**) and morpholine (**7h**) with an ED_{50} values of 1.88 mg/kg (**7a**), 14.93 mg/kg (**7g**) and 10.65 mg/kg (**7h**) in the MES test. It should be pointed out that **7d** and **7e**, obtained as analogues of the most effective spirosuccinimides (**5d** and **5e**), showed high activity, however due to the strong neurotoxicity were excluded from further testing.

3. Conclusion

The results obtained revealed that majority of compounds synthesized exhibited high anticonvulsant activity, especially in the maximal electroshock test, which is the animal model of human generalized tonic-clonic seizures. The ED_{50} values for the most active compounds were in range of 1.88–19.57 mg/kg. Several molecules were more potent and safer than phenytoin used as reference antiepileptic drug. In summary, it is justified to claim that the spirosuccinimide and spirohydantoin moieties enable the design of new prototypic molecules, their consequent structural modifications and for that reason they play an essential role in the search for novel anticonvulsants.

Table 7
Results of in vitro sodium channels binding assays

Compd	IC_{50} (μM)
5d	2.45
5e	9.28
7a	9.35
7h	No affinity
Phenytoin[a]	40.0

[a] Reference drug, data from Ref. 42.

4. Experimental part

4.1. Chemistry

All the chemicals and solvents were purchased from Merck (Darmstadt, Germany) 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 with TLC. The thin-layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} aluminum sheets (Merck; Darmstadt, Germany), using developing systems consisted of S_1 chloroform/acetone (9:1, v/v), S_2—benzene/ethyl acetate/acetone (10:5:1, v/v/v), S_3—methanol/25% ammonia (100:1.5, v/v).

Spots were detected by their absorption under UV light (λ = 254 nm) and by visualization with 0.05 mol I_2 in 10% HCl. 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 spectra were obtained in a Varian Mercury spectrometer (Varian Inc., Palo Alto, CA, USA), in $CDCl_3$, operating at 300 MHz. 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), t (triplet), dd (double doublet), q (quartet), m (multiplet).

4.1.1. 2-Carboxy-1,2,3,4-tetrahydronaphtalene-2-carboxylic acid (3), [7,8-*f*]benzo-2-azaspiro[4.5]decane-1,3-dione (4), [7,8-*f*]benzo-1,3-diaza-spiro-decane-2,4-dione (6)

The starting materials 2-carboxy-1,2,3,4-tetrahydronaphtalene-2-carboxylic acid (**3**) or [7,8-*f*]benzo-2-azaspiro[4.5]decane-1,3-dione (**4**) were prepared using methods reported by Faust et al.[21] The β-tetralonehydantoin ([7,8-*f*]benzo-1,3-diaza-spiro-decane-2,4-dione) (**6**) was prepared from the 3,4-dihydronaphtalen-2-on (β-tetralone) by the Bücherer–Berg reaction with modifications described by Goodson et al.[22]

4.1.2. General procedure for the synthesis of compounds 5a–h and 7a–h

The mixture of [7,8-*f*]benzo-2-aza-spiro[4.5]decane-1,3-dione (0.01 mol) (**4**), or [7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (0.01 mol) (**6**), 40% formaldehyde solution (0.01 mol) and corresponding 4-substituted piperazine (0.01 mol) or morpholine (0.01 mol) in 96% ethanol (40 ml) was left for ca. 12 h and then refrigerated ca. −10 °C for 24 h. The products were washed with cold ethanol and the solid products (**5a**–**h** and **7a**–**h**) were separated by filtration and recrystallized from 96% ethanol.

4.1.2.1. *N*-[(4-Phenylpiperazin-1-yl)-methyl]-[7,8-*f*]benzo-2-aza-spiro[4,5]decane-1,3-dione (5a). White powdery crystals. Yield: 70%; mp 145–147 °C; TLC: R_f = 0.66 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.91 (br s, 1H cyclohexane), 2.18–2.26 (m, 1H cyclohexane), 2.52–2.99 (m, 7H, 1H cyclohexane, 2H imide, 4H piperazine), 3.00–3.06 (m, 2H cyclohexane), 3.22 (br s, 4H piperazine), 3.30 (d, 1H cyclohexane, *J* = 16.40 Hz), 4.60 (s, 2H, –CH_2–), 6.91–7.10 (m, 2H, ArH), 7.13–7.20 (m, 4H, ArH), 7.27–7.34 (m, 3H, ArH); $C_{24}H_{27}N_3O_2$ (389.50).

4.1.2.2. *N*-[{4-(2-Fluorophenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]-benzo-2-aza-spiro[4.5]decane-1,3-dione (5b). White powdery crystals. Yield: 69%; mp 115–117 °C; TLC: R_f = 0.86 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.88 (t, 1H cyclohexane, *J* = 7.43 Hz), 2.19–

2.30 (m, 1H cyclohexane), 2.52 (d, 1H cyclohexane, J = 18.20 Hz) 2.68 (q, 2H imide, J = 8.46 Hz), 2.84–99 (m, 5H, 1H cyclohexane, 4H piperazine), 3.00–3.10 (m, 1H cyclohexane), 3.22 (br s, 4H piperazine), 3.30 (d, 1H cyclohexane, J = 16.40 Hz), 4.60 (s, 2H, $-CH_2-$), 6.94–6.99 (m, 2H, ArH), 7.02–7.16 (m, 3H, ArH), 7.20–7.24 (m, 3H, ArH); $C_{24}H_{26}FN_3O_2$ (407.49).

4.1.2.3. *N*-[{4-(4-Fluorophenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]-benzo-2-aza-spiro[4.5]decane-1,3-dione (5c). White powdery crystals. Yield: 77%; mp 173–175 °C; TLC: R_f = 0.45 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.88 (br s, 1H cyclohexane), 2.15–2.30 (m, 1H cyclohexane), 2.51 (d, 1H cyclohexane, J = 18.46), 2.66 (q, 2H, imide, J = 8.63 Hz), 2.74–2.99 (m, 5H, 1H cyclohexane, 4H piperazine), 3.00–3.11 (m, 5H, 1H cyclohexane, 4H piperazine), 3.33 (d, 1H cyclohexane, J = 16.15 Hz), 4.59 (t, 2H $-CH_2-$, J = 13.59 Hz), 6.88–7.20 (m, 8H, ArH); $C_{24}H_{26}FN_3O_2$ (407.49).

4.1.2.4. *N*-[{4-(3-Chlorophenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]-benzo-2-aza-spiro[4.5]decane-1,3-dione (5d). White powdery crystals. Yield: 79%; mp 152–153 °C; TLC: R_f = 0.71 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.88 (br s, 1H cyclohexane), 2.16–2.30 (m, 1H cyclohexane), 2.47–2.55 (m, 1H cyclohexane), 2.62–2.99 (m, 7H, 1H cyclohexane, 2H imide, 4H piperazine), 3.00–3.06 (m, 1H cyclohexane), 3.21–3.35 (m, 4H piperazine), 3.33 (d, 1H cyclohexane, J = 16.16 Hz), 4.59 (s, 2H $-CH_2-$), 6.79–6.99 (m, 3H, ArH), 7.06–7.20 (m, 5H, ArH); $C_{24}H_{26}ClN_3O_2$ (423.94).

4.1.2.5. *N*-[{4-(3-Trifluoromethylphenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]benzo-2-aza-spiro[4.5]decane-1,3-dione (5e). White powdery crystals. Yield: 75%; mp 140–142 °C; TLC: R_f = 0.67 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.83–1.90 (m, 1H cyclohexane), 2.19–2.30 (m, 1H cyclohexane), 2.52 (d, 1H cyclohexane, J = 18.46 Hz), 2.68 (d, 2H imide, J = 18.72 Hz), 2.85–2.93 (m, 5H, 1H cyclohexane, 4H piperazine), 3.02 (dd, 1H cyclohexane, J = 17.95 Hz) 3.16–3.36 (m, 5H, 1H cyclohexane, 4H piperazine), 4.61 (s, 2H, $-CH_2-$), 7.06–7.20 (m, 7H, ArH), 7.36 (t, 1H, ArH, J = 8.08 Hz); $C_{25}H_{26}F_3N_3O_2$ (457.51).

4.1.2.6. *N*-[(4-Benzylpiperazin-1-yl)-methyl]-[7,8-*f*]benzo-2-aza-spiro[4.5]decane-1,3-dione (5f). White powdery crystals. Yield: 78%; mp 132–134 °C; TLC: R_f = 0.42 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.86 (br s, 1H cyclohexane), 2.17–2.28 (m, 1H cyclohexane), 2.44–2.60 (m, 4H, piperazine), 2.63–2.66 (m, 6H, 2H imide, 4H piperazine), 2.80–2.90 (m, 2H cyclohexane), 2.92–3.05 (m, 1H cyclohexane), 3.31 (d, 1H cyclohexane, J = 16.41 Hz), 3.51 (s, 2H, $-CH_2-$), 4.51 (s, 2H, $-CH_2-$), 7.09–7.19 (m, 4H, ArH), 7.31–7.54 (m, 5H, ArH); $C_{25}H_{29}N_3O_2$ (403.53).

4.1.2.7. *N*-[{4-(2-Hydroxyethyl)-piperazin-1-yl}-methyl]-[7,8-*f*]benzo-2-aza-spiro[4.5]decane-1,3-dione (5g). White powdery crystals. Yield: 80%; mp 46–48 °C; TLC: R_f = 0.66 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.85–1.91 (m, 1H cyclohexane), 2.08 (d, 1H OH, J = 16.67 Hz), 2.18–2.27 (m, 1H cyclohexane), 2.51 (d, 1H cyclohexane, J = 18.21 Hz), 2.63–2.99 (m, 12H, 8H piperazine, 2H imide, 2H $-CH_2-$), 3.00–3.15 (m, 1H cyclohexane), 3.32 (d, 1H cyclohexane, J = 16.41 Hz), 3.49 (s, 1H cyclohexane), 3.71–3.78 (m, 2H, $-CH_2-$), 4.52 (t, 2H, $-CH_2-$, J = 13.59 Hz), 7.08–7.19 (m, 4H, ArH); $C_{20}H_{27}N_3O_3$ (357.46).

4.1.2.8. *N*-(Morpholin-1-yl)-methyl-[7,8-*f*]benzo-2-aza-spiro-[4.5]decane-1,3-dione (5h). White powdery crystals. Yield: 70%; mp 148–150 °C; TLC: R_f = 0.69 (S_1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.80–1.88 (m, 1H cyclohexane), 2.18–2.29 (m, 1H cyclohexane), 2.50 (d, 1H cyclohexane, J = 18.21 Hz), 2.62–2.70 (m, 6H, 2H imide, 4H morpholine), 2.81–2.99 (m, 1H cyclohexane) 3.00–3.07 (m, 1H cyclohexane), 3.33 (d, 1H cyclohexane, J = 16.16 Hz), 3.70 (t, 4H morpholine, J = 4.49 Hz), 4.50 (s, 2H $-CH_2-$), 7.06–7.20 (m, 4H, ArH); $C_{18}H_{22}N_2O_3$ (314.39).

4.1.2.9. *N*-[(4-Phenylpiperazin-1-yl)-methyl]-[7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (7a). White powdery crystals. Yield: 82%; mp 220–222 °C; TLC: R_f = 0.21 (S_1); R_f = 0.37 (S_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.93–1.96 (m, 1H cyclohexane), 2.17–2.34 (m, 1H, cyclohexane), 2.77–2.92 (m, 6H, 2H cyclohexane, 4H piperazine), 2.96–3.26 (m, 5H, 1H cyclohexane, 4H piperazine), 3.46 (d, 1H cyclohexane, J = 16.37 Hz), 4.58 (s, 2H, $-CH_2-$), 6.02 (br s, 1H, N_1H), 6.90–6.94 (m, 3H, ArH), 7.05–7.31 (m, 6H, ArH); $C_{23}H_{26}O_2N_4$ (389.48).

4.1.2.10. *N*-[{4-(2-Fluorophenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (7b). White powdery crystals. Yield: 78%; mp 148–150 °C; TLC: R_f = 0.37 (S_2), R_f = 0.96 (S_3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.86–1.92 (m, 1H, cyclohexane), 2.18–2.29 (m, 1H, cyclohexane), 2.75 (t, 4H, piperazine, J = 5.05 Hz), 2.81–2.86 (m, 2H cyclohexane), 2.91 (t, 4H piperazine, J = 5.0 Hz), 3.03–3.16 (m, 1H cyclohexane), 3.42 (d, 1H cyclohexane, J = 16.47 Hz), 4.56 (s, 2H, $-CH_2-$), 6.58 (s, 1H, N_1H), 6.82–7.05 (m, 8H, ArH); $C_{23}H_{25}O_2N_4F$ (408.48).

4.1.2.11. *N*-[{4-(4-Fluorophenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (7c). White powdery crystals. Yield: 81%; mp 211–213 °C; TLC: R_f = 0.36 (S_2), R_f = 0.94 (S_3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.85–1.91 (m, 1H cyclohexane), 2.16–2.27 (m, 1H cyclohexane), 2.75 (t, 4H piperazine, J = 4.95 Hz,), 2.80–2.85 (m, 2H cyclohexane), 2.91 (t, 4H piperazine, J = 5.0 Hz), 3.02–3.15 (m, 1H cyclohexane), 3.42 (d, 1H cyclohexane, J = 16.45 Hz), 4.55 (s, 2H, $-CH_2-$), 6.58 (s, 1H, N_1H), 6.81–7.18 (m, 8H, ArH); $C_{23}H_{25}O_2N_4F$ (408.48).

4.1.2.12. *N*-[{4-(3-Chlorophenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (7d). White powdery crystals. Yield: 86%; mp 190–191 °C; TLC: R_f = 0.39 (S_2), R_f = 0.39 (S_3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.84–1.94 (m, 1H cyclohexane), 2.16–2.32 (m, 1H cyclohexane), 2.75 (t, 4H piperazine, J = 4.95 Hz), 2.80–2.96 (m, 2H cyclohexane), 3.00–3.24 (m, 5H, 4H piperazine, 1H cyclohexane), 3.42 (d, 1H cyclohexane, J = 16.50 Hz) 4.58 (s, 2H, $-CH_2-$), 6.02 (br s, 1H, N_1H), 6.86–6.98 (m, 3H, ArH), 7.05–7.35 (m, 5H, ArH); $C_{23}H_{25}ClO_2N_4$ (424.93).

4.1.2.13. *N*-[{4-(3-Trifluoromethylphenyl)-piperazin-1-yl}-methyl]-[7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (7e). White powdery crystals. Yield: 67%; mp 173–174 °C; TLC: R_f = 0.33 (S_2), R_f = 0.85 (S_3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.88–1.95 (m, 1H cyclohexane), 2.17–2.32 (m, 1H cyclohexane), 2.76 (t, 4H piperazine, J = 5.05 Hz), 2.82–3.06 (m, 3H cyclohexane), 3.12–3.24 (m, 4H piperazine), 3.40 (d, 1H cyclohexane, J = 16.49 Hz), 4.60 (s, 2H, $-CH_2-$), 6.26 (s, 1H, N_1H), 7.04–7.27 (m, 7H, ArH), 7.35 (t, 1H, ArH, J = 7.95 Hz); $C_{24}H_{25}O_2N_4F_3$ (458.49).

4.1.2.14. *N*-[(4-Benzylpiperazin-1-yl)-methyl]-[7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (7f). White powdery crystals. Yield: 82%; mp 81–83 °C; TLC: = 0.21 (S_1); R_f = 0.37 (S_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.91–1.98 (m, 1H cyclohexane), 2.21–2.31 (m, 1H cyclohexane), 2.28–2.54 (m, 4H piperazine), 2.69–2.80 (m, 5H, 1H cyclohexane, 4H piperazine), 2.86–3.01 (m, 2H cyclohexane), 3.38 (d, 1H cyclohexane, J = 16.41 Hz) 3.49 (s, 2H, $-CH_2-$), 4.52 (s, 2H, $-CH_2-$), 5.64 (br s, 1H, N_1H), 7.01–7.19 (m, 5H, ArH), 7.26–7.33 (m, 4H, ArH); $C_{24}H_{28}O_2N_4$ (404.52).

4.1.2.15. *N*-[{4-(2-Hydroxyethyl)-piperazin-1-yl}-methyl]-[7,8-*f*]benzo-1,3-diaza-spiro[4.5]decane-2,4-dione (7g). White powdery crystals. Yield: 62%; mp 143–145 °C; TLC: R_f = 0.29 (S_1);

R_f = 0.37 (S_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.89–1.96 (m, 1H cyclohexane), 2.20–2.32 (m, 1H cyclohexane), 2.47–2.52 (m, 6H, 4H piperazine, 2H $-CH_2-$), 2.62–2.80 (m, 6H, 4H piperazine, 1H cyclohexane, 1H OH), 2.95–3.10 (m, 2H cyclohexane), 3.40 (d, 1H cyclohexane, J = 16.36 Hz), 3.60 (t, 2H, $-CH_2-$, J = 5.30 Hz), 4.48 (s, 2H, $-CH_2-$), 6.32 (br s, 1H, N_1H), 7.05–7.18 (m, 4H, ArH); $C_{17}H_{26}O_3N_4$ (358.44).

4.1.2.16. *N*-(Morpholin-1-yl)-methyl-[7,8-*f*]benzo-1,3-diaza-spiro-[4.5]decane-2,4-dione (7h). White powdery crystals. Yield: 88%; mp 185–187 °C; TLC: R_f = 0.61 (S_1), R_f = 0.28 (S_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.87–1.94 (m, 1H, cyclohexane), 2.18–2.30 (m, 1H cyclohexane), 2.56 (t, 4H morfoline, J = 4.65 Hz), 2.77 (d, 1H cyclohexane, J = 18.20 Hz), 2.92–3.06 (m, 2H cyclohexane), 3.40 (d, 1H cyclohexane, J = 16.18 Hz), 3.62 (t, 4H morpholine, J = 4.50 Hz), 4.41 (s, 2H, $-CH_2-$), 6.54 (s, 1H, N_1H), 7.04–7.14 (m, 4H, ArH); $C_{15}H_{21}O_3N_3$ (315.38).

4.2. Pharmacology

The initial anticonvulsant evaluation was performed within the Antiepileptic Drug Development (ADD) Program in Epilepsy Branch, National Institutes of Health, National Institute of Neurological Disorders and Stroke (NIH/NINDS), Rockville, MD, USA, by using procedures described elsewhere.[30,31]

Male albino mice (CF-1 strain) and male albino rats (Sprague–Dawley) were used as experimental animals. The animals were housed in metabolic cages and allowed free access to food and water. The compounds were suspended in 0.5% methylcellulose/water mixture.

The ASP initially evaluates anticonvulsant activity for newly submitted compounds following intraperitoneal (ip) administration in mice and oral administration in rats. Groups of eight mice or four rats are employed. Phase I studies in mice involved two convulsant tests: maximal electroshock seizure test (MES), subcutaneous pentylenetetrazole seizure test (*sc*PTZ) and rotarod test for neurological toxicity (NT).

4.2.1. The maximal electroshock test (MES)

In the MES screen, an electrical stimulus of 0.2 s in duration (50 mA in mice and 150 mA in rat at 60 Hz) is delivered via corneal electrodes primed with an electrolyte solution containing an anesthetic agent.

4.2.2. The subcutaneous pentylenetetrazole seizure test (*sc*PTZ)

This screen utilizes a dose of pentylenetetrazole (85 mg/kg in mice and 70 mg/kg in rats) that produces clonic seizures lasting for a period of at least five seconds in 97% (CD_{97}) of animals tested. At the anticipated time of testing the convulsant is administered subcutaneously.

All the compounds were injected intraperitoneally into mice at the dose levels of 30, 100, and 300 mg/kg with anticonvulsant activity and neurotoxicity assessment at 0.5 and 4 h after administration. The results are presented in Table 1.

Selected derivatives were administrated orally into rats using four animals at a fixed dose of 30 mg/kg (MES test) and 50 mg/kg (*sc*PTZ test) (Phase VIa). This screen discloses the time of onset, the approximate time of peak effect (TPE) and the duration of anticonvulsant activity. For both doses the motor impairment was studied in parallel. Rats were tested at five time periods ranging from one quarter to 4 h post substance administration. The results are shown in Tables 2 and 3.

4.2.3. The neurological toxicity (NT)

The neurological toxicity (NT) induced by compound was detected in mice or rats using standardized rotarod test.[32] Untreated control mice or rats, when placed on the rod, can maintain their equilibrium for a prolonged time period. The acute motor impairment can be demonstrated by the inability of animal to maintain equilibrium for given time.

4.2.4. Quantification studies

The quantitative determination of ED_{50} and TD_{50} values was performed at previously estimated time of peak effect after oral administration into rats. Groups of eight rats received various doses of the compound until at least three points were established in the range of 10–90% seizure protection or minimal neurotoxicity. From the plot of the data obtained, the respective ED_{50} and TD_{50} values, 95% confidence intervals, slope of the regression line, and standard error of the slope were calculated by means of a computer program written at NINDS/NIH. The results are shown in Table 4.

4.2.5. The 6-Hz model

This screen was carried out according to the protocol originally described by Brown et al.[33] and more recently by Barton et al.[28] and Kaminski et al.[34] It is an alternative electroshock paradigm that uses low-frequency (6 Hz), long-duration (3 s) electrical stimulation. Corneal stimulation (0.2 ms-duration monopolar rectangular pulses at 6 Hz for 3 s) was delivered by a constant-current device. During the stimulation, mice were manually restrained and released into the observation cage immediately after the current application. The seizures manifest in 'stunned' posture associated with rearing, forelimb, automatic movements and clonus, twitching of the vibrissae and Straub-tail. The duration of the seizure activity ranges from 60 to 120 s in untreated animals. At the end of the seizure, animals resume their normal exploratory behavior. The experimental end point is protection against the seizure. The animal is considered to be protected if it resumes its normal exploratory behavior within 10 s from the stimulation[34] (Table 5).

4.2.6. The Pilocarpine Induced Status Prevention (PISP) model

The male albino rats (Sprague–Dawley, 150–180 g) were used as experimental animals. The compounds were administrated via the ip rout of administration. Then a challenge dose of pilocarpine is given observing for treatment-effects of the substance tested. The seizure severity is determined using the well known Racine scale,[29] as follow: (I) immobility, eye closure, twitching of vibrissae, sniffing, and facial clonus; (II) head nodding associated with more severe facial clonus; (III) clonus of one of the fore limbs; (IV) rearing often accomplished by bilateral forelimb clonus; and (V) all of the above plus loss of balance and falling, accomplished by generalized clonic seizures. The anticonvulsant activity of tested compounds was assessed at time zero, namely the time from the first stage III seizures (Test 71). The outcome measures are determination of 'protection' or 'no protection'. The results are given in Table 6.

4.2.7. In vitro hippocampal slice culture neuroprotection assay

Organotypic hippocampal brain slice cultures have been gaining importance as an early stage drug screening tool in the neuroprotection arena.[35] It is possible, with the use of propidium iodide (PI), to quantitatively assess neuronal cell death in the slice cultures following treatment with the excitotoxic agents, kainic acid (KA) or *N*-methyl-D-aspartate (NMDA). PI binds to the DNA of dead and dying cells and fluoresces. This assay enabled evaluation the ability of novel compounds to prevent such excitotoxic cell death when these compounds are added either in conjunction with, or following, treatment with agents that induce excitotoxic cell death. When organotypic slice are treated for 6 h with either KA (10 μm) or NMDA (10 μm), robust cell death occurs in the granule

cell layer of the dentategyrus and in the CA3 and CA1 pyramidal cell layers of the organotypic hippocampal slice preparation and this cell death can be quantified with PI. Treatment with agents known to prevent excitotoxic cell death can significantly reduce the percentage of total PI uptake in a dose-dependent manner. Therefore, if new compounds can prevent cell death, the amount of total PI will be significantly reduced. It is important to bear in mind that these experiments are performed in an in vitro cell culture system. Thus, until confirmed in other experiments, it would be premature to conclude that compounds effective in this system would exhibit neuroprotection against excitotoxic agents in vivo. The in vitro Hippocampal Slice Culture Neuroprotection Assay is described in details in separate literature.[35,36]

4.3. In vitro sodium channels binding assays

The radioligand binding studies were performed commercially by Caliper Life Sciences (Hopkinton, MA, USA). The methodology applied is precisely described elsewhere.[37]

4.4. X-ray structure analysis

Crystal data for **5a**: $C_{24}H_{27}N_3O_2$, $M = 389.49$, monoclinic, space group $P2_1/n$, $a = 17.284(3)$ Å, $b = 6.0973(8)$ Å, $c = 20.714(3)$ Å, $\beta = 111.157(4)^\circ$, $V = 2035.8(5)$ Å^3, $Z = 4$, $D_x = 1.271$ g cm^{-3}, $T = 293$ K, $\mu = 0.082$ mm^{-1}, $\lambda = 0.71073$ Å, data/parameters = 2813/263; final $R_1 = 0.0513$.

Crystal data for **5h**: $C_{18}H_{22}N_2O_3$, $M = 314.38$, monoclinic, space group $P2_1/c$, $a = 7.6388(6)$ Å, $b = 9.9055(9)$ Å, $c = 21.960(2)$ Å, $\beta = 93.675(8)^\circ$, $V = 1658.2(3)$ Å^3, $Z = 4$, $D_x = 1.259$ g cm^{-3}, $T = 293$ K, $\mu = 0.086$ mm^{-1}, $\lambda = 0.71073$ Å, data/parameters = 2877/209; final $R_1 = 0.0688$.

The crystals of **5a** and **5h** were obtained by slow evaporation from methanol and propanol (1:1) solutions. The measurements of the crystals were performed on a SMART diffractometer with graphite-monochromated MoKα radiation ($\lambda = 0.71073$ Å) at room temperature. The structures were solved by direct method and refine with SHELXTL.[38] E-maps provided positions for all non-H-atoms. The full-matrix least-squares refinement was carried out on F^2's using anisotropic temperature factors for all non-H-atoms. All C-bound H atoms were placed in idealized locations and refined using a riding model, with C–H = 0.93 Å and $U_{iso}(H) = 1.2\ U_{eq}(C)$.

Crystallographic data (excluding structural factors) for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Centre and allocated with the deposition numbers: CCDC 749459 and 749460 for compounds **5a** and **5h**, respectively. Copies of data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EW, UK (Fax: Int. code (1223) 336-033; E-mail: deposit@ccdc.cam.ac.uk).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.064. These data include MOL files and InChiKeys of the most important compounds described in this article.

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