European Journal of Medicinal Chemistry 48 (2012) 347–353

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Synthesis and anticonvulsant activity of novel 2,6-diketopiperazine derivatives. Part 2: Perhydropyrido[1,2-*a*]pyrazines

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ARTICLE INFO

Article history:
Received 24 August 2011
Received in revised form
14 November 2011
Accepted 20 November 2011
Available online 26 November 2011

Keywords:
2,6-diketopiperazine
2,6-piperazinedione
Pyrido[1,2-*a*]pyrazine
Anticonvulsant activity
Chirality

ABSTRACT

A new series of chiral pyrido[1,2-*a*]pyrazine derivatives was synthesised and evaluated in *in vivo* animal models of epilepsy. A significant influence of the stereochemistry of the pyrido[1,2-*a*]pyrazine framework on the pharmacological activity was observed. Compounds with (4*R*,9a*S*) absolute configuration proved inactive, whereas other stereoisomers exhibited markedly dissimilar spectra of anti-seizure efficacy in the maximal electroshock seizure (MES), subcutaneous Metrazol seizure (scMET) and Pilocarpine-induced status prevention (PISP) tests. Importantly, the investigated agents revealed high potency in the 6 Hz model, with the ED_{50} values comparable to the reference drug Levetiracetam. Derivatives **(4*S*,9a*R*)-6** and **(4*R*,9a*R*)-6** emerged as promising new lead structures, the former having a broad spectrum of anticonvulsant activity and the latter showing high potency in 6 Hz and PISP models.

1. Introduction

Epilepsy is one of the most common neurological disorders, characterised by excessive abnormal bioelectrical functions of the brain. The occurrence of the disorder is estimated to be about 1% of the global population. Currently, the main treatment for epilepsy is continuous administration of antiepileptic drugs (AEDs). Since approximately 30% of the patients fail to achieve adequate seizure control, there is a need for laborious research towards novel therapeutics with improved efficacy and fewer side-effects [1,2].

In our recent report, we developed a structurally novel class of anticonvulsant agents, derivatives of perhydropyrrole[1,2-*a*]pyrazine with a broad activity in animal models of epilepsy [3]. Importantly, we observed a very pronounced impact of stereochemistry and conformational preferences on the *in vivo* biological properties of these compounds. From the two investigated diastereomeric series, the derivatives with (4*R*,8a*S*) absolute configuration proved inactive, whereas the (4*S*,8a*S*) isomers revealed significant seizure protection. Among the active (4*S*,8a*S*) diastereoisomers, compounds **(4*S*,8a*S*)-1**, **-2**, and **-3** (Fig. 1) proved most potent, with the ED_{50} values in maximal electroshock seizure (MES), subcutaneous Metrazol (scMET) and 6 Hz tests comparable to the reference AEDs.

As an extension of this study, we report synthesis and biological evaluation of novel perhydropyrido[1,2-*a*]pyrazines, the homologues of perhydropyrrole[1,2-*a*]pyrazine. Considering the fact that the *in vivo* activity of the parent group was highly stereoselective, we decided to primarily investigate the pharmacological differences between all four possible stereoisomers of 4-phenylperhydropyrido[1,2-*a*]pyrazine-1,3-dione, the model compound in the new series. The most potent isomers were further subjected to structural modifications (i.e. *N*-alkylation and phenyl deletion). The collected pharmacological data was compared with the reference AEDs.

2. Results and discussion

2.1. Chemistry

The synthesis of target compounds was accomplished as depicted in Scheme 1.

As substrates for the synthesis, enantiopure (*R*)- or (*S*)-2-piperidinecarboxylic acids were used. The esterification of the amino acids, followed by the ammonolysis of the resulting methyl esters yielded enantiomerically pure (*R*)- and (*S*)-2-piperidinecarboxamides **(2*S*)-4** and **(2*R*)-4**, respectively. The obtained amino acid amides were used as the starting materials for the subsequent reactions.

Stereochemically pure **(4*S*,9a*S*)-6**, **(4*R*,9a*S*)-6**, **(4*S*,9a*R*)-6** and **(4*R*,9a*R*)-6** were synthesised from the corresponding **(2*S*)-4** or **(2*R*)-4** and (*S*)- or (*R*)-2-(4-toluenesulfonyloxy)-phenylacetic acid methyl esters, by the methods described previously [4].

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doi:10.1016/j.ejmech.2011.11.032

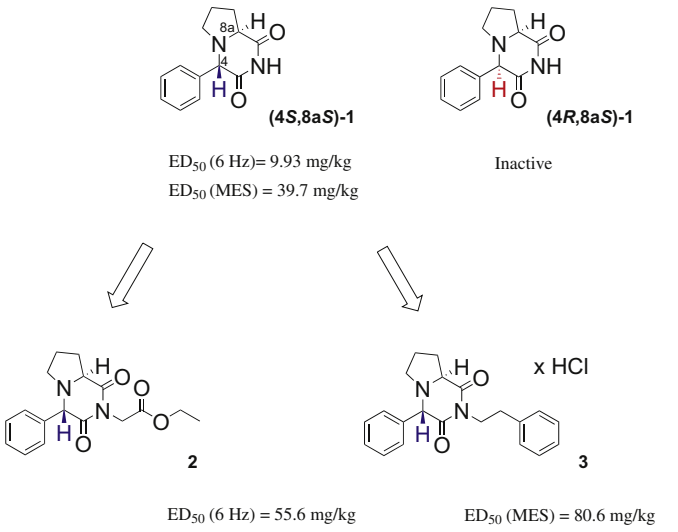
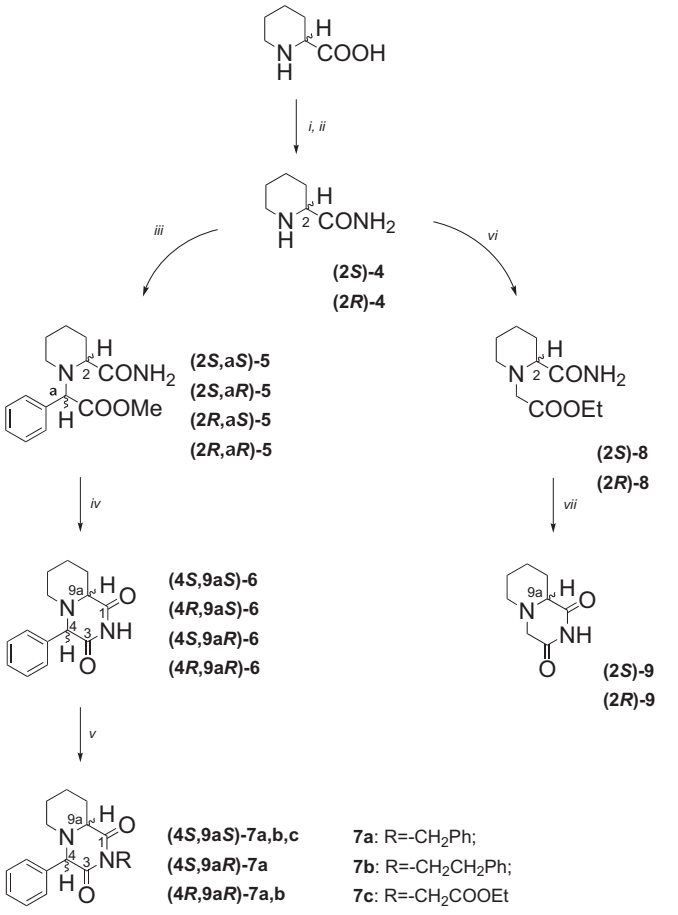


Fig. 1. Structures and pharmacological data of example perhydropyrrole[1,2-*a*]pyrazine derivatives **(4*R*,8a*S*)-1**, **(4*S*,8a*S*)-1**, **2** and **3**.

N-alkylation of the imide nitrogen of **(4*S*,9a*S*)-6**, **(4*S*,9a*R*)-6** or **(4*R*,9a*R*)-6** with appropriate alkyl bromides was performed under solid–liquid phase transfer catalysis (S-L PTC) conditions [5], to furnish **(4*S*,9a*S*)-7a,b,c**, **(4*S*,9a*R*)-7a** and **(4*R*,9a*R*)-7a,b**. The reactions were accompanied by a partial inversion at the stereogenic centres C-4 of the products, as judged by the TLC analysis of unpurified reaction mixtures. Nonetheless, the resulting small amounts of the unwanted opposite epimers could be removed by means of column chromatography.



Scheme 1. Synthesis of compounds **4**–**9**. Reagents and conditions: *i*. $SOCl_2$, MeOH, reflux, 2 h, then AcOEt, Et_3N, rt; *ii*. NH_3, MeOH, rt, 72 h; *iii*. (*R*)- or (*S*)-2-(4-toluenesulfonyloxy)-phenylacetic acid methyl ester, Py, MeCN, reflux, 2 h; *iv*. NaOH, EtOH, rt, 10 min; *v*. RBr, K_2CO_3, TBAB, acetone, reflux, 30–45 min.; *vi*. $BrCH_2COOEt$, *N*,*N*-diisopropyl-*N*-ethylamine, MeCN, rt, 17 h; *vii*. NaOH, EtOH, rt, 10 min.

Reaction of **(2*S*)-4** or **(2*R*)-4** with ethyl bromoacetate in the presence of Hunig's base furnished amidoesters **(2*S*)-8** or **(2*R*)-8**, respectively. The subsequent intramolecular cyclocondensations afforded the corresponding 2,6-DKP derivatives **(9a*S*)-9** and **(9a*R*)-9**.

All the new compounds were characterised by physical constants, IR, NMR spectroscopy and elemental analysis or mass spectrometry (see Experimental section).

2.2. Biological evaluation

The synthesised compounds were screened in animal models of epilepsy under the auspices of the Anticonvulsant Screening Project (ASP) of National Institutes of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH), Bethesda, USA [6]. Primary anticonvulsant studies involved two tests: maximal electroshock seizure (MES) and subcutaneous Metrazol (scMET), in mice. It has been shown that nearly all clinically significant AEDs are protective in at least one of these two models. Additionally, the neurological toxicity was assessed in a neurological impairment (TOX) test. The active compounds were further screened orally in rats. The most promising derivatives were subjected to the quantitative determination of the median effective dose (ED_{50}) and toxic dose (TD_{50}) at previously estimated time of peak effect (TPE). Selected agents were tested in the minimal clonic seizure (6 Hz) model. The results have been summarised in Tables 1–5.

Regarding the broad and stereoselective anti-seizure activity of the prototype compound **(4*S*,8a*S*)-1**, four stereoisomers of homologous 4-phenylperhydropyrido[1,2-*a*]pyrazine-1,3-dione **(4*R*,9a*S*)-6**, **(4*S*,9a*S*)-6**, **(4*S*,9a*R*)-6** and **(4*R*,9a*R*)-6** were selected as model structures. The results of the *in vivo* evaluation revealed very pronounced differences of the pharmacological action within this initial set of compounds.

In the primary tests in mice (see Table 1), stereoisomer **(4*S*,9a*S*)-6** was most potent in MES and scMET models (3/3 and 1/1, respectively, at 100 mg/kg, at 0.5 h). However, the compound produced a very pronounced neurotoxic effect (8/8 at 100 mg/kg, at 0.5 h) and was not subjected to quantitative evaluations. Isomer **(4*S*,9a*R*)-6** proved less active (4/5 and 1/1, respectively, at 300 mg/kg, at 0.5 h) and revealed a moderate neurotoxicity (4/4 at 300 mg/kg and 3/8 at 100 mg/kg, at 0.5 h). When evaluated quantitatively, it displayed ED_{50} and TD_{50} values comparable to homologous **(4*S*,8a*S*)-1** and **3** with (4*S*,8a*S*) stereochemistry (see Table 2). The other isomer, **(4*R*,9a*S*)-6**, was devoid of pharmacological effects, similar to the previously studied homologue, **(4*R*,8a*S*)-1**. Interestingly, the lack of activity in the primary testing in mice was also observed for diastereoisomer **(4*R*,9a*R*)-6**, which suggested a possible unfavourable influence of the *R* stereochemistry at carbon C-4.

Several attempts were made to enhance the anti-seizure potency of the model compounds. Substitution of the imide nitrogen of **(4*S*,9a*S*)-6**, **(4*S*,9a*R*)-6** or **(4*R*,9a*R*)-6** with benzyl (**(4*S*,9a*S*)-7a**, **(4*S*,9a*R*)-7a** and **(4*R*,9a*R*)-7a**), phenylethyl (**(4*S*,9a*S*)-7b** and **(4*R*,9a*R*)-7a**) or ethoxycarbonylmethyl (**(4*S*,9a*S*)-7c**) groups did not improve the pharmacological effects in the initial mice testing (see Table 1). In fact, these structural modifications furnished inactive derivatives of **(4*S*,9a*S*)-6** and **(4*S*,9a*R*)-6**, whereas only a slight improvement was observed in the MES efficacy of the benzyl derivative of **(4*R*,9a*R*)-6** (**(4*R*,9a*R*)-7a**, 1/1 at 300 mg/kg at 0.5 h and 4.0 h). The compound produced significant neurotoxicity at the same dose at which the anti-seizure effect was present (2/4

Table 1
Anticonvulsant activity and neurotoxicity of compounds in the MES and scMET models following intraperitoneal (ip.) administration in mice.

Compound	Dose (mg/kg)	MES[a]		scMET[b]		TOX[c]		Clog*P*[d]
		0.5 h	4.0 h	0.5 h	4.0 h	0.5 h	4.0 h	
(4*R*,9a*S*)-6	30	–[e]	–	–	–	–	–	1.43
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	
(4*S*,9a*S*)-6	30	–	–	–	–	–	–	1.43
	100	3/3	–	1/1	–	8/8	–	
	300	1/1	1/1	1/1	1/1	4/4	1/2[f]	
(4*S*,9a*R*)-6	30	–	–	–	–	–	–	1.43
	100	1/7	–	–	–	3/8	–	
	300	4/5	–	1/1	–	4/4[g]	–	
(4*R*,9a*R*)-6	30	–	–	–	–	–	–	1.43
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	
(9a*S*)-9	30	–	–	–	–	–	–	−0.54
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	
(9a*R*)-9	30	–	–	–	–	–	–	−0.54
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	
(4*S*,9a*S*)-7a[h]	30	–	–	–	–	–	–	3.45
	100[i]	–	–	–	–	–	–	
	300	–	1/1	–	–	–	–	
(4*S*,9a*R*)-7a	30	–	–	–	–	–	–	3.45
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	
(4*R*,9a*R*)-7a[h]	30	–	–	–	–	–	–	3.45
	100	–	–	–	–	–	–	
	300	1/1	1/1	–	–	2/4	–	
(4*S*,9a*S*)-7b[j]	30	–	–	–	–	–	–	3.71
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	
(4*R*,9a*R*)-7b[j]	30	–	–	–	–	–	–	3.71
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	
(4*S*,9a*S*)-7c	30	–	–	–	–	–	–	1.44
	100	–	–	–	–	–	–	
	300	–	–	–	–	–	–	

[a] Maximal electroshock test (number of animals protected/number of animals tested).
[b] Subcutaneous Metrazole test (number of animals protected/number of animals tested).
[c] Neurotoxicity test (number of animals exhibiting neurological toxicity/number of animals tested).
[d] Theoretical log*P* value calculated by a logarithm included in HyperChem 7.5 package [7].
[e] Not active.
[f] Sedated.
[g] Unable to grasp rotorod.
[h] Tested as a hydrochloride salt.
[i] Active also in 2/3 at 2.0 h post administration.
[j] No effect also for hydrochloride salt.

Table 3
Anticonvulsant activity and neurotoxicity of compounds in the MES model following oral (p.o.) administration in rats.

Compound	Test[a]	0.25 h	0.5 h	1.0 h	2.0 h	4.0 h
(4*S*,9a*S*)-6	MES[b]	–	–	–	–	1/4
	TOX[c]	–	–	–	–	–
(4*S*,9a*R*)-6	MES	–	–	–	–	–
	TOX	–	–	–	–	–
(4*R*,9a*R*)-6	MES	–	–	–	–	1/4
	TOX	–	–	–	–	–

[a] At dose 30 mg/kg.
[b] Maximal electroshock test (number of animals protected/number of animals tested).
[c] Neurotoxicity test (number of animals exhibiting neurological toxicity/number of animals tested).

at 300 mg/kg at 0.5 h). No activity was observed for the phenylethyl analogue of **(4*R*,9a*R*)-6** (**(4*R*,9a*R*)-7b**), either in the form of a free base or a hydrochloride salt.

The structural modification consisting of phenyl deletion at carbon C-4 produced **(9a*S*)-9** and **(9a*R*)-9**, which were devoid of pharmacological effects in MES, scMET and TOX screening.

None of the investigated agents revealed seizure protection in the initial studies in rats (see Table 3).

The moderate activity of the synthesised compounds in the primary testing suggested their rather limited value as potential lead structures for novel AEDs. In this context, the outcome of the alternative 6 Hz screening proved more encouraging [8]. The results are summarised in Tables 4 and 5.

Except the inactive **(4*R*,9a*S*)-6**, all isomers of the model 4-phenylperhydropyrido[1,2-*a*]pyrazine-1,3-dione **(4*S*,9a*S*)-6**, **(4*S*,9a*R*)-6** and **(4*R*,9a*R*)-6**, were almost equally potent in 6 Hz model. Their ED_{50} values were comparable to the reference AED Levetiracetam (see Table 5). Surprisingly, stereoisomer **(4*R*,9a*R*)-6**, which was inactive in the primary MES and scMET screening, revealed an excellent result of the quantitative 6 Hz test ($ED_{50} = 38.2$ mg/kg at 0.25 h). Therefore it seems probable that the 4*S* configuration is required for the pharmacological effects in conventional models (i.e. MES, scMET), but is not the prerequisite of 6 Hz activity. No neurotoxicity was detected for the compound, which is regarded a desirable feature of a candidate anticonvulsant. Good level of seizure protection was determined for the hydrochloride of the *N*-phenylethyl analogue of **(4*R*,9a*R*)-6** (**(4*R*,9a*R*)-7b**, $ED_{50} = 78.5$ mg/kg at 0.25 h). Notably, the free base of this compound was inactive, suggesting the poor bioavailability after intraperitoneal administration in mice. The results of the quantitative 6 Hz test performed for the *N*-ethoxycarbonylmethyl derivative of **(4*S*,9a*S*)-6** (**(4*S*,9a*S*)-7c**)

Table 2
Quantification studies of **(4*S*,9a*R*)-6** in the MES, scMET and neurotoxicity tests following intraperitoneal (ip.) administration in mice.

Compound	ED_{50} MES[a] (mg/kg)	ED_{50} scMET[b] (mg/kg)	TD_{50} (mg/kg)[c]	PI[d] MES	TPE[e] MES (h)
(4*S*,9a*R*)-6	95.72 (78.37–109.67)	99.16 (93.16–106.27)	151.21 (133.73–176.43)[f,g]	nd	0.5
1[h]	69.5 (60.9–77.9)	87.9 (72.2–100.4)	121.5 (104.9–142.8)	1.7	0.25
3[h]	80.6 (71.1–92.8)	>200	178.1 (121.0–220.3)	2.2	0.25
Phenytoin[i]	5.64 (4.74–6.45)	>50	41.0 (39.4–43.0)	7.3	1.0

Values in parentheses are 95% confidence intervals determined by probit analysis.
[a] Maximal electroshock test.
[b] Subcutaneous Metrazole test.
[c] ED_{50} for neurotoxicity test.
[d] Protective index (TD_{50}/ED_{50} MES).
[e] Time to peak effect.
[f] Response comments: unable to grasp rotorod.
[g] At 0.25 h.
[h] Data from Ref. [3].
[i] Data from Ref. [6].

Table 4
Anticonvulsant activity and neurotoxicity of compounds in the 6 Hz model following intraperitoneal (ip.) administration in mice.

Compound	Test[a]	0.25 h	0.5 h	1.0 h	2.0 h	4.0 h
(4*R*,9a*S*)-6	6 Hz[b]	–	–	–	–	–
	TOX[c]	–	–	–	–	–
(4*S*,9a*S*)-6	6 Hz	4/4	4/4	3/4	2/4	–
	TOX	4/4[d]	4/4[d]	–	–	–
(4*S*,9a*R*)-6	6 Hz	4/4	4/4	–	–	–
	TOX	–	–	–	–	–
(4*R*,9a*R*)-6	6 Hz	4/4	3/4	2/4	1/4	–
	TOX	–	–	–	–	–
(9a*S*)-9	6 Hz	1/4	–	2/4	–	–
	TOX	–	–	–	–	–
(9a*R*)-9	6 Hz	–	2/4	1/4	–	–
	TOX	–	–	–	–	–
(4*S*,9a*R*)-7a	6 Hz	–	–	–	–	–
	TOX	–	–	–	–	–
(4*R*,9a*R*)-7b	6 Hz	–	–	–	–	–
	TOX	–	–	–	–	–
(4*S*,9a*S*)-7b[e]	6 Hz	1/4	2/4	–	–	–
	TOX	–	–	–	–	–
(4*R*,9a*R*)-7b[e]	6 Hz	4/4	2/4	1/4	2/4	–
	TOX	–	–	–	–	–
(4*S*,9a*S*)-7c	6 Hz	4/4	2/4	1/4	–	–
	TOX	–	–	–	–	–

[a] At dose 100 mg/kg.
[b] 6 Hz test, 32 mA (number of animals protected/number of animals tested).
[c] Neurotoxicity test (number of animals exhibiting neurological toxicity/number of animals tested).
[d] Unable to grasp rotorod.
[e] Tested as a hydrochloride salt.

revealed its high potency ($ED_{50} = 55.3$ mg/kg at 0.25 h). It should be noted that also this compound was devoid of pharmacological activity in the conventional MES, scMET and TOX screens.

Other investigated agents revealed weaker activities in the 6 Hz model (see Table 4) and therefore they were not subjected to secondary quantitative studies. Worth taking note of is that the compounds lacking phenyl substituent in the C-4 position (**(9a*S*)-9** and **(9a*R*)-9**) proved active in this screen. No significant differences between these stereoisomers were observed.

Compounds **(4*S*,9a*R*)-6** and **(4*R*,9a*R*)-6** were additionally assessed for the potential efficacy against the nerve agents using the Pilocarpine-induced status prevention (PISP) screen, which is one of the animal models of pharmacoresistant status epilepticus [9]. The results are summarised in Table 6.

Both compounds exhibited similar high activities. **(4*S*,9a*R*)-6** and **(4*R*,9a*R*)-6** were found to produce full protection (8/8 rats) at time-zero using doses of 600 mg/kg and 450 mg/kg, respectively. In the case of **(4*S*,9a*R*)-6**, 2 of the 8 animals were protected at 30 min after post-first stage III seizure, at dose 600 mg/kg. The **(4*R*,9a*R*)-6** stereoisomer evoked fair protection (6/8 rats) using a higher dose of 900 mg/kg.

Table 5
Quantification studies of **(4*S*,9a*S*)-6**, **(4*S*,9a*R*)-6**, **(4*R*,9a*R*)-6**, **(4*R*,9a*R*)-7b** and **(4*S*,9a*S*)-7c** in the 6 Hz model following intraperitoneal (ip.) administration in mice.

Compound	ED_{50} 6 Hz (mg/kg)	TPE[a] (h)
(4*S*,9a*S*)-6	27.2 (22.5–38.8)	0.25
(4*S*,9a*R*)-6	32.3 (22.3–43.4)	0.25
(4*R*,9a*R*)-6	38.2 (29.8–54.1)	0.25
(4*R*,9a*R*)-7b[b]	78.5 (62.9–98.8)	0.25
(4*S*,9a*S*)-7c[c]	55.3 (34.7–91.3)	0.25
1[d]	9.93 (5.97–14.55)	0.25
2[d]	55.6 (42.7–74.9)	0.25
Levetiracetam[e]	19.4 (9.90–36.0)	1.0

Values in parentheses are 95% confidence intervals determined by probit analysis.
[a] Time to peak effect.
[b] Tested as a hydrochloride salt.
[c] TD_{50} (mg/kg) was estimated as 186.6 (120.2–266.4) mg/kg at 0.5 h post administration.
[d] Data from Ref. [3].
[e] Data from Ref. [8].

3. Conclusions

A series of novel, chiral derivatives of pyrido[1,2-*a*]pyrazine was synthesised and evaluated in *in vivo* animal models of epilepsy. Similar to the previously investigated homologous perhydropyrrole [1,2-*a*]pyrazines, the very pronounced impact of the absolute configuration of the stereogenic centres on the biological activity was observed. The most important differences in pharmacological properties of the stereoisomers of the model 4-phenylperhydropyrido [1,2-*a*]pyrazine-1,3-dione are summarised in Fig. 2.

Compounds **(4*S*,9a*R*)-6** and **(4*R*,9a*R*)-6** emerged as the most promising agents from the group. The first revealed a broad spectrum of anticonvulsant activity, with good ED_{50} values in MES, scMET and 6 Hz models and fair therapeutic indices. The second isomer showed a different, 'levetiracetam-like' profile, with high efficacy in 6 Hz screen and without concomitant neurological toxicity. Both compounds displayed similar protection levels in suppressing Pilocarpine-induced status epilepticus.

4. Experimental section

4.1. Chemistry

Melting points were determined on an Electrothermal 9100 apparatus in open capillary tubes and were uncorrected. The IR spectra (thin film on KBr pellets) were recorded on a Shimadzu FTIR-8300 instrument. The NMR spectra were obtained on a Varian Inova 500 MHz or Varian Unity Plus 200 MHz spectrometer. Chemical shifts (δ) were expressed in parts per million (ppm) relative to tetramethylsilane (TMS) or solvent used as the internal reference. The following abbreviations were used to describe the peak patterns: s (singlet), d (doublet), t (triplet), q (quartet), qt (quintet), m (multiplet), p (pseudo-) and b (broad-). Coupling constants (J) were in hertz (Hz). The electrospray ionisation high resolution mass spectra (ESI-HRMS) were recorded on a LCT TOF (Micromass) instrument. Optical rotations were measured with a Perkin–Elmer 241 polarimeter at 20 °C, using a sodium lamp (589 nm). Elemental analyses were performed on a Perkin–Elmer 2400 analyser and the results were within ±0.4% of the theoretical values. Thin-layer chromatography (TLC) was run on Merck Silica gel-60 F_{254} plates. The spots were visualised by ultraviolet light (254 nm) or iodine vapours. Flash column chromatography (FC) was carried out on Merck Silica gel-60 (particle size: 0.040–0.063 mm). Solvents were dried and purified by standard methods. Petroleum ether referred to the fraction boiling at 40–60 °C. Starting (*S*)- and (*R*)-2-piperidinecarboxylic acid amides **(*S*)-4** and **(*R*)-4** were obtained by from (*S*)- and (*R*)-2-piperidinecarboxylic acids [10], respectively, by methods described previously [4]. All other reagents were purchased from commercial sources and used as received. The chemical yields were calculated for pure (dr ≥ 99/1) diastereoisomers.

4.1.1. Synthesis of compounds *(4R,9aS)-6*, *(4S,9aS)-6*, *(4S,9aR)-6* and *(4R,9aR)-6* under S-L PTC conditions

General method: A mixture of appropriate 2,6-DKP (1 equivalent), potassium carbonate (1 equivalent), tetrabutylammonium bromide (single crystal), alkylating agent (7 equivalents) and acetone (2 mL per 1 mmol of 2,6-DKP) was stirred vigorously under reflux until TLC showed no future reaction (30–60 min). The

Table 6
Anticonvulsant activity of **(4*R*,9a*R*)-6** and **(4*S*,9a*R*)-6** in the Pilocarpine-induced status prevention model following intraperitoneal (ip.) administration in rats.

Compound	Dose (mg/kg)	Time (h)[a]	Number of animals protected/Number of animals tested	Average weight change (g) ±S.E.M[b]	
				Protected rats	Non-protected rats
(4*S*,9a*R*)-6	600	0	8/8[c]	+4.4 ± 1.1	–
	600	0.5	2/8[d]	−30.0 ± 0.0	−31.0 ± 2.6
(4*R*,9a*R*)-6	450	0	8/8	+3.1 ± 1.6	–
	900	0.5	6/8[c,e]	−17.5 ± 3.7	−20.0 ± 5.0

[a] Post-first stage III seizure.
[b] Weight change 24 h post-first stage III seizure.
[c] Sedated.
[d] One animal died.
[e] Two animals died.

resulting suspension was cooled, filtered and the solvent was evaporated *in vacuo*. The residue was purified by FC [11].

*4.1.1.1. (4S,9aS)-2-Benzyl-4-phenyl-perhydropyrido[1,2-a]pyrazine-1,3-dione hydrochloride **(4S,9aS)-7a***. From **(4S,9aS)-6** (0.40 g, 1.64 mmol) and benzyl bromide (1.4 mL, 11.47 mmol); FC (gradient: petroleum ether/ethyl acetate 9:1–6:1): yield 0.46 g (76%).

White crystals; mp 116–119 °C; $[\alpha]_D = +27.5$ (*c* 1, $CHCl_3$); IR: 737, 1204, 1354, 1497, 1670, 1732, 2858, 2939, 3063; TLC (free base, petroleum ether/ethyl acetate 5:1): $R_f = 0.57$; ^{1}H NMR (free base, $CDCl_3$, 500 MHz): δ 1.42 (m, 2H, H′-8, H-8), 1.61 (m, 2H, H′-7, H-7), 1.88 (m, 1H, H-9), 1.96 (m, 1H, H′-9), 2.53 (q, $^2J = 11.0$, $^3J = 5.0$, 1H, H-6), 2.67 (q, $^2J = 11.0$, $^3J = 6.0$, 1H, H′-6), 3.41 (pt, 1H, H-9a), 4.67 (s, 1H, H-4), 5.03 (d, $^2J = 13.5$, 1H, H-α), 5.10 (d, $^2J = 13.5$, 1H, H′-α), 7.16–7.44 (m, 10H, H-Ar); ^{13}C NMR (free base, $CDCl_3$, 125 MHz): δ 22.2 (C-8), 25.1 (C-7), 26.7 (C-9), 42.7 (C-α), 50.9 (C-6), 56.2 (C-9a), 69.5 (C-4), 127.8, 128.4, 128.6, 128.7, 128.9, 129.3, 133.2, 137.2 (C-Ar), 171.1 (C-3), 172.6 (C-1); Elemental analysis: calculated for $C_{21}H_{22}N_2O_2 \times$ HCl: C 68.01%, H 6.25%, N 7.55%; found: C 67.73%, H 6.01%, N 7.22%.

*4.1.1.2. (4R,9aR)-2-Benzyl-4-phenyl-perhydropyrido[1,2-a]pyrazine-1,3-dione hydrochloride **(4R,9aR)-7a***. From **(4R,9aR)-6** (0.40 g, 1.64 mmol) and benzyl bromide (1.4 mL, 11.47 mmol); FC (gradient: petroleum ether/ethyl acetate 9:1–6:1): yield 0.52 g (86%).

White powder; mp 102–104 °C; $[\alpha]_D = -24.0$ (*c* 1, $CHCl_3$); Elemental analysis: calculated for $C_{21}H_{22}N_2O_2 \times$ HCl: C 68.01%, H 6.25%, N 7.55%; found: C 67.89%, H 6.34%, N 7.28%.

*4.1.1.3. (4S,9aR)-2-Benzyl-4-phenyl-perhydropyrido[1,2-a]pyrazine-1,3-dione **(4S,9aR)-7a***. From **(4S,9aR)-6** (0.40 g, 1.64 mmol) and benzyl bromide (1.4 mL, 11.47 mmol); FC (gradient: petroleum ether/ethyl acetate 9:1–6:1): yield 0.46 g (84%).

White powder; mp 163–164 °C; $[\alpha]_D = +120.0$ (*c* 1, $CHCl_3$); IR: 748, 1176, 1292, 1431, 1728, 2812, 2858, 2931; TLC (petroleum ether/ethyl acetate 6:1): $R_f = 0.53$; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.31 (4 t, $^2J = {}^3J_1 = 13.0$, $^3J_2 = 4.0$, 1H, H-8), 1.40 (4 t, $^2J = {}^3J_1 = 12.0$, $^3J_2 = 3.5$, 1H, H-7), 1.51 (m, 1H, H′-7), 1.61 (m, 1H, H-9), 1.76 (td, $^2J = {}^3J_1 = 12.5$, $^3J_2 = 2.5$, 1H, H-6), 1.86 (m, 1H, H′-8), 2.43 (dt, $^2J = 13.5$, $^3J = 2.5$, 1H, H′-9), 2.72 (pd, 1H, H′-6), 2.96 (dd, $^3J_1 = 11.0$, $^3J_2 = 2.5$, 1H, H-9a), 3.92 (s, 1H, H-4), 4.89 (d, $^2J = 13.5$, 1H, H-α), 4.98 (d, $^2J = 13.5$, 1H, H′-α), 7.20–7.39 (m, 10H, H-Ar); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.9 (C-8), 25.1 (C-7), 27.8 (C-9), 43.5 (C-α), 53.8 (C-6), 64.1 (C-9a), 73.2 (C-4), 127.7, 128.6, 128.7, 128.9, 129.2, 129.4, 137.0, 137.2 (C-Ar), 171.2 (C-3), 171.5 (C-1); Elemental analysis: calculated for $C_{21}H_{22}N_2O_2$: C 75.42%, H 6.63%, N 8.38%; found: C 75.23%, H 6.71%, N 8.72%.

*4.1.1.4. (4R,9aS)-2-(2-Phenylethyl)-4-phenyl-perhydropyrido[1,2-a]pyrazine-1,3-dione **(4R,9aS)-7b***. From **(4R,9aS)-6** (0.40 g, 1.64 mmol) and 2-bromoethylbenzene (1.6 mL, 11.74 mmol); FC (gradient: petroleum ether/ethyl acetate 9:1–6:1): yield 0.32 g (56%).

White crystals; mp 97–98 °C; $[\alpha]_D = -30.1$ (*c* 1, $CHCl_3$); IR: 744, 1169, 1358, 1450, 1678, 1732, 2939; TLC (petroleum ether/ethyl acetate 5:1): $R_f = 0.45$; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.42 (m, 2H, H′-8, H-8), 1.54 (m, 2H, H′-7, H-7), 1.67–1.90 (m, 2H, H-9, H′-9), 2.41 (m, 1H, H-6), 2.73 (m, 1H, H′-6), 2.84 (t, $^3J = 8.0$, 2H, H-β, H′-β), 2.91 (dd, $^3J_1 = 8.0$, $^3J_2 = 8.0$, 1H, H-9a), 3.89 (s, 1H, H-4), 4.02 (m, 2H, H-α, H′-α), 7.19–7.40 (m, 10H, H-Ar); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 23.9 (C-8), 25.2 (C-7), 27.9 (C-9), 34.1 (C-β), 41.4 (C-α), 53.7 (C-6), 64.0 (C-9a), 73.0 (C-4), 126.7, 128.7, 128.9, 129.3, 129.4, 137.3, 138.5 (C-Ar), 171.1 (C-3), 171.4 (C-1); Elemental analysis: calculated for $C_{22}H_{24}N_2O_2$: C 75.83%, H 6.94%, N 8.04%; found: C 75.42%, H 6.41%, N 7.90%.

*4.1.1.5. (4S,9aS)-2-(2-Phenylethyl)-4-phenyl-perhydropyrido[1,2-a]pyrazine-1,3-dione **(4S,9aS)-7b***. From **(4S,9aS)-6** (0.50 g, 2.05 mmol) and 2-bromoethylbenzene (2.0 mL, 14.34 mmol); FC (gradient: petroleum ether/ethyl acetate 9:1–7:1): yield 0.50 g (70%).

White powder; mp 120–122 °C; $[\alpha]_D = -92.2$ (*c* 1, $CHCl_3$); IR: 744, 1180, 1358, 1450, 1678, 1728, 2816, 2928; TLC (petroleum ether/ethyl acetate 5:1): $R_f = 0.48$; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.41 (m, 2H, H-8, H′-8), 1.59 (m, 2H, H-7, H′-7), 1.84 (m, 1H, H-9), 1.92 (m, 1H, H′-9), 2.51 (q, $^2J = 11.0$, $^3J = 5.5$, 1H, H-6), 2.61 (q, $^2J = 11.0$, $^3J = 5.5$, 1H, H′-6), 2.94 (t, $^3J = 7.5$, 2H, H-β, H′-β), 3.38 (dd, $^3J_1 = 6.0$, $^3J_2 = 5.0$, 1H, H-9a), 4.07 (dt, $^2J = 12.5$, $^3J = 7.5$, 1H, H-α), 4.21 (dt, $^2J = 12.5$, $^3J = 7.5$, 1H, H′-α), 4.63 (s, 1H, H-4), 7.19–7.37 (m, 10H, H-Ar); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 22.1 (C-8), 25.1 (C-7), 26.6 (C-9), 34.2 (C-

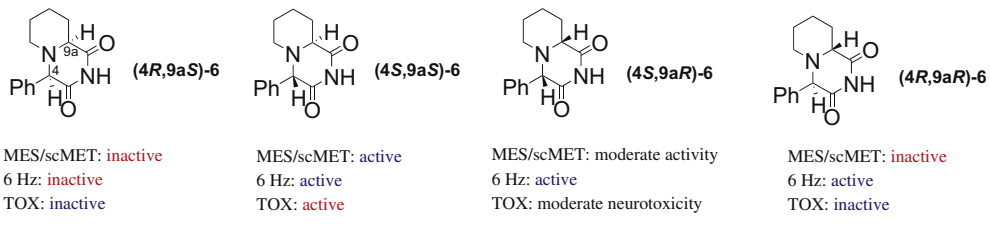


Fig. 2. The differences in the MES, scMET, 6 Hz and TOX activities of the four stereoisomers of 4-phenylperhydropyrido[1,2-*a*]pyrazine-1,3-dione.

β), 40.6 (C-α), 50.8 (C-6), 56.0 (C-9a), 69.6 (C-4), 126.7, 128.3, 128.6, 129.0, 129.3, 133.3, 138.4 (C-Ar), 171.0 (C-3), 172.5 (C-1); Hydrochloride: white powder; mp 196–199 °C; Elemental analysis: calculated for $C_{22}H_{24}N_2O_2 \times HCl$: C 68.65%, H 6.55%, N 7.28%; found: C 69.09%, H 6.31%, N 7.40%.

4.1.1.6. (4R,9aR)-2-(2-Phenylethyl)-4-phenyl-perhydropyrido[1,2-a] pyrazine-1,3-dione ***(4R,9aR)-7b***. From **(4*R*,9a*R*)-6** (0.50 g, 2.05 mmol) and 2-bromoethylobenzene (2.0 mL, 14.34 mmol); FC (gradient: petroleum ether/ethyl acetate 9:1–7:1): yield 0.53 g (75%).

Colourless crystals; mp 121–122 °C; $[\alpha]_D = +96.3$ (*c* 1, $CHCl_3$); Hydrochloride: white powder; mp 201–203 °C; Elemental analysis: calculated for $C_{22}H_{24}N_2O_2 \times HCl$: C 68.65%, H 6.55%, N 7.28%; found: C 68.39%, H 6.50%, N 7.03%.

4.1.1.7. Ethyl (4S,9aS)-α-(1,3-dioxo-4-phenyl-perhydropyrido[1,2-a] pyrazin-2-yl)-acetate ***(4S,9aS)-7c***. From **(4*S*,9a*S*)-6** (0.52 g, 2.13 mmol) and ethyl bromoacetate (1.7 mL, 14.91 mmol); FC (gradient: petroleum ether/ethyl acetate 9:1–4:1): yield 0.64 g (91%).

White powder; mp 82–84 °C; $[\alpha]_D = -68.9$ (*c* 1.1, $CHCl_3$); IR: 707, 746, 1210, 1333, 1377, 1405, 1451, 1690, 1738, 2821, 2859, 2928, 2939; TLC (petroleum ether/ethyl acetate 3:1): $R_f = 0.45$; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.30 (t, $^3J = 7.0$, 3H, OCH_2CH_3), 1.47 (m, 2H, H-8, H′-8), 1.66 (m, 2H, H′-7, H-7), 1.86 (m, 1H, H-9), 2.02 (m, 1H, H′-9), 2.63 (m, 1H, H-6), 2.89 (m, 1H, H′-6), 3.50 (t, $^3J = 5.0$, 1H, H-9a), 4.23 (m, 2H, OCH_2CH_3), 4.59 (d, $^2J = 16.5$, 1H, H-α), 4.65 (d, $^2J = 16.5$, 1H, H′-α), 4.72 (s, 1H, H-4), 7.32–7.44 (m, 5H, H-Ar); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.4 (OCH_2CH_3), 21.9 (C-8), 25.3 (C-7), 26.5 (C-9), 40.5 (C-α), 50.9 (C-6), 55.6 (OCH_2CH_3), 61.9 (C-9a), 69.8 (C-4), 128.6, 128.8, 129.0, 133.2 (C-Ar), 168.1 ($COOCH_2CH_3$), 171.0 (C-3), 172.3 (C-1); Elemental analysis: calculated for $C_{18}H_{22}N_2O_4$: C 65.44%, H 6.71%, N 8.48%; found: C 65.21%, H 6.89%, N 8.55%.

4.1.2. Synthesis of (2S)- and (2R)-α-(2-carbamoylpiperidinyl)-acetic acid ethyl ester ***(2S)-8*** *and* ***(2R)-8***

General method: To a stirred, cooled (0 °C, ice-salt bath) mixture of (*S*)- or (*R*)-2-piperidinecarboxylic acid amide **(*S*)-4** or **(*R*)-4** (1.53 g, 11.94 mmol), *N*,*N*-diisopropyl-*N*-ethylamine (2.08 mL, 11.94 mmol) and acetonitrile (70 mL), ethyl bromoacetate (1.1 mL, 9.95 mmol) was added over 10 min. The mixture was then allowed to warm to room temperature, stirred (20 h) and the solvent was evaporated *in vacuo*. The residue was purified by FC (gradient: petroleum ether/ethyl acetate 1:1 to ethyl acetate, then ethyl acetate/methanol 9.5:0.5).

4.1.2.1. (2S)-α-(2-Carbamoylpiperidinyl)-acetic acid ethyl ester ***(2S)-8***. Yield 1.45 g (73%). White powder; mp 169–170 °C; $[\alpha]_D = -70.2$ (*c* 1, $CHCl_3$); IR: 1184, 1450, 1647, 1728, 2866, 2959, 2194, 3414; TLC (ethyl acetate): $R_f = 0.30$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.27 (t, $^3J = 7.0$, 3H, CH_2CH_3), 1.32 (m, 1H, H-4), 1.51–1.67 (m, 3H, H-3, H-5, H′-5), 1.75 (m, 1H, H′-4), 1.99 (m, 1H, H′-3), 2.26 (td, $^2J = 11.5$, $^3J_1 = 4.5$, $^3J_2 = 3.5$, 1H, H-6), 2.95 (dd, $^3J_1 = 10.5$, $^3J_2 = 3.5$, 1H, H-2), 2.99 (m, $^2J = 11.5$, $^3J = 4.0$, $^4J = 1.0$, 1H, H′-6), 3.08 (d, $^2J = 17.0$, 1H, H-α), 3.38 (d, $^2J = 17.0$, 1H, H′-α), 4.18 (q, $^3J = 7.0$, 2H, CH_2CH_3), 5.34 (bs, 1H, CONH), 6.97 (bs, 1H, CONH′); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.4 (OCH_2CH_3), 23.2 (C-5), 24.9 (C-4), 30.1 (C-3), 52.9 (C-6), 58.2 (C-2), 61.0 (OCH_2CH_3), 66.6 (C-α), 171.3 (CONH), 176.9 ($COOCH_2CH_3$); HRMS (ESI) calculated for $C_{10}H_{18}N_2O_3Na$: 237.1215 $(M + Na)^+$ found 237.1237.

4.1.2.2. (2R)-α-(2-Carbamoylpiperidinyl)-acetic acid ethyl ester ***(2R)-8***. Yield 1.23 g (62%). White powder; mp 168–170 °C; $[\alpha]_D = +73.4$ (*c* 1, $CHCl_3$); HRMS (ESI) calculated for $C_{10}H_{18}N_2O_3Na$: 237.1215 $(M + Na)^+$ found 237.1181.

4.1.3. Synthesis of (9aS)- and (9aR)-perhydropyrido[1,2-a] pyrazine-1,3-dione ***(9aS)-9*** *and* ***(9aR)-9***

General method: To a stirred solution of appropriate amidoester **(2*S*)-8** or **(2*R*)-8** (1 equivalent) in absolute ethanol (10 mL per 1 mmol of amidoester), sodium hydroxide (1 equivalent) pellet was added at room temperature. After dissolution of the hydroxide, the mixture was quenched with saturated aqueous solution of ammonium chloride (100 mL). The resulting cloudy solution was extracted with methylene chloride (2 × 30 mL) and subsequently with a mixture of dichloromethane/methanol 70:30 (v/v) (2 × 30 mL). The combined organic extracts were dried with magnesium sulphate, filtered, and the solvent was evaporated *in vacuo*. The residue was purified by FC (petroleum ether/ethyl acetate 1:3).

4.1.3.1. (9aS)-Perhydropyrido[1,2-a]pyrazine-1,3-dione ***(9aS)-9***. From **(2*S*)-8** (1.10 g, 5.50 mmol); yield 0.75 g (81%).

White powder; mp 96–98 °C; $[\alpha]_D = +14.6$ (*c* 1, $CHCl_3$); IR: 837, 1119, 1284, 1331, 1732, 2858, 2939, 3221; TLC (ethyl acetate): $R_f = 0.62$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.38 (m, 1H, H-8), 1.60 (m, 2H, H-7, H-9), 1.69 (m, 1H, H′-7), 1.83 (m, 1H, H′-8), 2.23 (m, 2H, H′-9, H-6), 2.82 (dd, $^3J_1 = 10.0$, $^3J_2 = 2.5$, 1H, H-9a), 2.91 (dt, $^2J = 11.5$, 1H, H′-6), 3.14 (d, $^2J = 17.0$, 1H, H-4), 3.57 (d, $^2J = 17.0$, 1H, H′-4), 8.33 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.2 (C-8), 25.0 (C-7), 26.5 (C-9), 54.6 (C-6), 58.3 (C-9a), 62.8 (C-4), 170.0 (C-3), 172.3 (C-1); Elemental analysis: calculated for $C_8H_{12}N_2O_2$: C 57.13%, H 7.19%, N 16.66%; found: C 56.92%, H 7.33%, N 16.34%

4.1.3.2. (9aR)-Perhydropyrido[1,2-a]pyrazine-1,3-dione ***(9aR)-9***. From **(2*R*)-8** (1.23 g, 6.15 mmol); yield 0.76 g (74%).

White powder; mp 102–104 °C; $[\alpha]_D = -17.2$ (*c* 1, $CHCl_3$); Elemental analysis: calculated for $C_8H_{12}N_2O_2$: C 57.13%, H 7.19%, N 16.66%; found: C 57.02%, H 7.40%, N 16.82%.

4.2. Pharmacology

The obtained compounds have been submitted for *in vivo* evaluation in the Anticonvulsant Screening Program (ASP) of National Institute of Neurological Disorders and Stroke (NINDS), Bethesda, USA [6]. The experiments were performed in male albino Carworth Farms No. 1 mice (weighing 18–25 g) or albino Sprague-Dawley rats (weighing 100–150 g). The animals had free access to feed and water except during actual testing period. Housing, handling and feeding were all in accordance with recommendations contained in the 'Guide for the Care and Use of Laboratory Animals'. The test compounds were dissolved or suspended in 0.5% (v/v) aqueous solution of methylcellulose.

4.2.1. The maximal electroshock seizure test (MES)

In the MES test, an electrical stimulus of 0.2 s in duration (50 mA in mice and 150 mA in rat at 60 Hz) was delivered via corneal electrodes primed with an electrolyte solution containing an anaesthetic agent (0.5% butacaine hemisulfate in 0.9% sodium chloride). Mice were tested at 30 min and 4 h following intraperitoneal administration of 30, 100 and 300 mg/kg of test compound while rats were tested at time intervals between 0.25 and 4 h following a standard oral dose of 30 mg/kg. Abolition of the hindlimb tonic extensor component indicated the test compound's ability to inhibit MES-induced seizure spread [6].

4.2.2. The subcutaneous Metrazol seizure test (scMET)

This test utilised a dose of Metrazol (pentylenetetrazol, 85 mg/kg in mice and 70 mg/kg in rats). This produced clonic seizures lasting for a period of at least 5 s in 97% (CD_{97}) of animals tested. At the anticipated time of testing the convulsant was administered

subcutaneously. The test compound was administered intraperitoneally in mice and orally in rats and the animals were observed over a 30 min period. Mice were tested at 30 min and 4 h following doses of 30, 100 and 300 mg/kg of test compound. Absence of clonic spasms indicated a compound's ability to abolish the effect of pentylenetetrazol on seizure threshold [6].

4.2.3. The acute neurological impairment (TOX)

Neurological toxicity induced by a compound was detected in mice or rats using the standardised rotorod test [12]. Mice were tested at 30 min and 4 h following intraperitoneal doses of 30, 100 and 300 mg/kg of test compound. Rats were tested at time intervals between 0.25 and 4 h following an oral dose of 30 mg/kg. Neurological impairment was demonstrated by the inability of a mouse or rat to maintain equilibrium on a 6 r.p.m. rotation rod for a given time.

4.2.4. The minimal clonic seizure test (6 Hz)

The 6 Hz screen was carried out according to the protocol originally described by Brown et al. [13] and more recently by Barton et al. [8] and Kaminski et al. [14]. It is an alternative electroshock paradigm that uses low-frequency (6 Hz), long-duration (3 s) electrical stimulation. Mice were tested at time intervals between 0.25 and 4 h following intraperitoneal doses of 100 mg/kg. 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 manifested in 'stunned' posture associated with rearing, forelimb, automatic movements and clonus, twitching of the vibrissae and Straub-tail. The duration of the seizure activity ranged from 60 to 120 s in untreated animals. At the end of the seizure, animals resumed their normal exploratory behaviour. The experimental end point was protection against the seizure. The animal was considered to be protected if it resumed its normal exploratory behaviour within 10 s from the stimulation [14].

4.2.5. Quantification studies

The quantitative determination of the median effective (ED_{50}) and toxic doses (TD_{50}) was conducted at previously calculated time of peak effect (TPE) using the intraperitoneal route in mice. Groups of at least eight animals were tested using different doses of test compound until at least two points were determined between 100 and 0% protection and minimal motor impairment. The dose of the candidate substance required to produce the desired end point in 50% of the animals in each test, and 95% confidence interval were calculated by a computer programme based on methods described by Finney [15].

4.2.6. The Pilocarpine-induced status prevention (PISP)

In this test the investigated compounds were administrated intraperitoneally to the male albino Sprague-Dawley rats (weighing 150–180 g). Then a challenge dose of Pilocarpine was given observing for treatment-effects of the substance tested. The seizure severity was determined using the Racine scale [16] as follows: (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 generalised clonic seizures. The anticonvulsant activity of compounds was assessed at time-zero, namely the time from the first stage III seizures or at 30 min after post-first stage III seizure. The results were expressed as number of animals protected/number of animals tested.

Acknowledgements

The authors would like to thank Dr James Stables, Dr Tracy Chen and Dr Jeff Jiang for providing them pharmacological data through the Anticonvulsant Screening Program (National Institute of Neurological Disorders and Stroke, NINDS Rockville, MD, USA). Financial support was provided by the Committee for Scientific Research, Poland (Grant No. N N405 623138).

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.11.032.

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