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Synthesis and pharmacological characterisation of a conformationally restrained series of indole-2-carboxylates as in vivo potent glycine antagonists

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Abstract

After the identification of GV150526, the indole-2-carboxylate template was further explored in order to identify novel potential anti-stroke agents. In particular, the SAR of the side chain present at the C-3 position of the indole nucleus was widely studied. In this paper, the synthesis and the pharmacological profile of a further class of conformationally restricted analogues of GV150526 as in vitro and in vivo potent glycine antagonists is reported. In particular, a pyrazolidinone derivative was identified as a potent neuroprotective agent in animal models of cerebral ischaemia.

Keywords: Stroke; Neuroprotection; Glycine antagonists; Indole-2-carboxylates

1. Introduction

Since the discovery of the neurotoxic properties of glutamate [1], a significant body of evidence has been gained on the instrumental link between neurotoxicity induced by glutamate and stroke [2–7]. This dramatic pathological event, caused by sudden reduction of cerebral blood flow, is currently associated to a high unmet need due to the lack of effective neuroprotective therapies [8] able to protect cerebral tissues, avoiding irreversible neurological impairments. Neuronal damage results from an abnormal overload of Ca^{2+} into the post-synaptic neurons, through the overactivation, by glutamate, of the ionotropic NMDA receptors [9]. The crucial role of glycine as *co*-agonist of glutamate, in the activation of the ion channel associated to the NMDA receptor, has been well documented [10,11].

In the last decade the strychnine-insensitive glycine binding site has been hypothesised to be a key target for the identification of suitable antagonists [12–19] as potential neuroprotective agents. Following this working hypothesis, some years ago we became interested in the glycine antagonists field. In particular, the exploration of indole-2-carboxylates led to the identification of the indole-2-carboxylate (**1**) (GV150526), shown in Fig. 1, as a selective glycine antagonist endowed with an outstanding neuroprotective activity in animal models of cerebral ischaemia. [20,21]. To further explore the SAR of the C-3 side chain belonging to this indole template, several classes of modified indole-2-carboxylates derivatives were prepared [22–25] and two examples of particular relevance to this paper are reported in Fig. 1 (**2** and **3**). In the present study, the synthesis and the biological and pharmacological characterisation of some compounds of the general structure **4** [Fig. 1: $X = (CH_2)_n$, $n = 1, 2$; $Y = N, O$] were performed in order to evaluate the effect of the introduction of different heteroatoms within cyclised and conformationally restricted structures.

2. Chemistry

Compounds **2** and **3** were previously synthesised following the general sequential aldol-type condensa-

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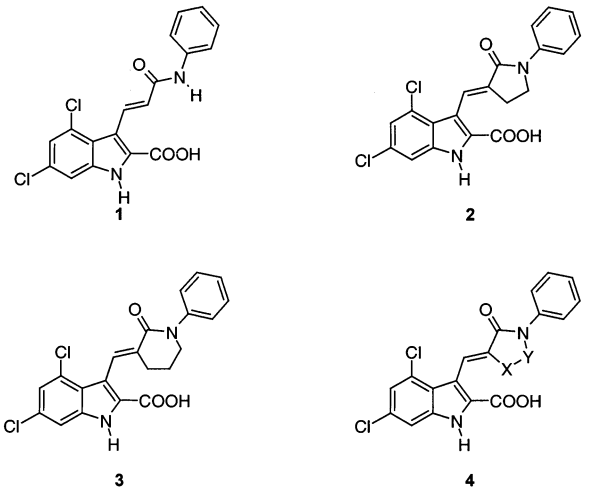


Fig. 1. GV150526 and conformationally restricted indole-2-carboxylate derivatives. X = $(CH_2)_n$, $n = 1, 2$; Y = O, NH, N–CH_3, N–Boc.

tion–lactonisation–elimination reaction as reported in Ref. [26].

All the new indole-2-carboxylate derivatives were characterised in terms of in vitro affinity at the glycine-binding site, assessed by displacement of [^{3}H]-glycine according to Kishimoto [27].

To better define the structure–activity relationship (SAR) of the conformationally restricted analogues of compound **1**, and to test the effect of the introduction on these cyclic scaffolds of different heteroatoms as previously attempted [20,21], a selected number of analogues of derivatives **2** and **3** were prepared [Fig. 1: X = $(CH_2)_n$, $n = 1, 2$; Y = O, NH, N–CH_3, N–Boc].

To synthesise this second series of compounds a modified version of the previously described [26] aldol-type condensation–lactonisation–elimination reaction was set up. This time, the main problem to be tackled was related to the potential chemical instability of these new compounds in the strong acid and/or basic conditions necessary to remove the N–SEM and the ethyl ester protecting groups, respectively. In this event, the N–Boc derivative of intermediate **5** was prepared. Actually, the N–Boc protecting group was easier to remove using milder conditions with respect to the N–SEM one. However, when **6** was submitted to the previous aldol-type condensation reaction, a different reactivity with respect to the analogous SEM-derivative was observed.

As an example (Scheme 1), when aldehyde derivative **6** was treated with the Li enolate of pyrazolidinone **14a**, prepared in three steps from phenylhydrazine **11** (Scheme 2), at −50 °C in THF and then at room temperature, a mixture of free carboxyl derivative **7**, the corresponding ethyl ester **8** and the chemically stable *anti*-lactone **9** (ratio 4.6:2.7:1) was isolated [28]. Notably, the presence of compound **9** confirmed the condensation–lactonisation–elimination mechanism previously proposed [26]. Moreover, it is worth underlining that all isolated products were devoid of the Boc protecting group on the indole nitrogen, suggesting a more complex reaction mechanism with respect to the previous case [26].

As far as the regioselectivity of the reaction is concerned, the presence of the *anti*-lactone **9** in the reaction mixture suggests that this compound, probably for steric reasons, cannot reach the necessary antiperiplanar conformational arrangement to give the elimination reaction. The corresponding *syn*-analogue instead, should evolve rapidly affording the desired *E*-olefin derivative **7**.

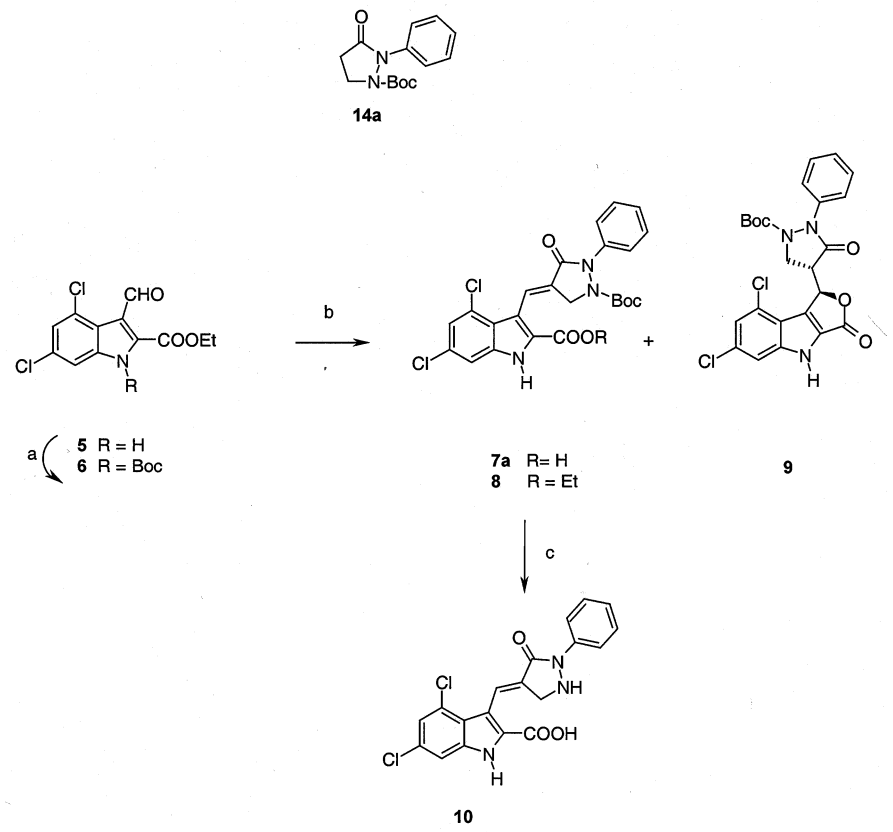
Due the presence of ethyl ester **8** in the reaction mixture, at the moment, it is difficult to explain the different mechanisms controlling this condensation reaction. However, the liability of the Boc protecting group on the indole nitrogen in basic medium (release of 1 eq. LiOEt after lactonisation) should play an essential role in the formation of **8**. Further studies are currently going on to clarify these particular aspects and will be published in due time. To obtain the target compound **10**, the free carboxyl derivative **7** was crystallised and submitted to the following deprotection reaction of the N–Boc group to give **10** in high yield. After the optimisation of the reaction conditions, the same synthetic route was successfully applied to the preparation of the different compounds shown in Table 1 (entries **15**–**17**) (Scheme 3) [29].

3. Results and discussion

3.1. In vitro affinity studies

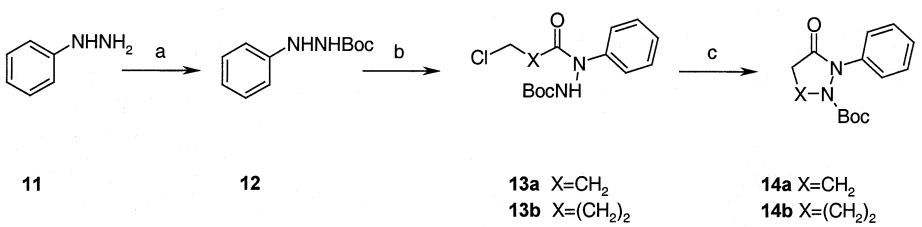
All the prepared derivatives were characterised in terms of in vitro affinity to the glycine-binding site.

The K_i values were measured from at least six-point inhibition curves and they are the geometric means of at least three independent experiments. The standard error of the mean was less than 0.05 for all the derivatives tested. All the derivatives showed complete selectivity (> 1000 fold) for other ionotropic receptors, namely NMDA, AMPA and KA. From the results achieved by this limited series of compounds, shown in Table 1, the reduction of both the lipophilic character (**10** versus **2**) and the steric bulk seem to maximise the affinity at the receptor in the five-membered ring series (**10** versus **15** and **7a**, respectively). In the six-membered ring series (**16** versus **3**) the reduction in lipophilicity seems to have poor effect, possibly because of an already exceeded steric hindrance within the receptor pocket (excluded volumes). In particular, the *N*-phenyl pyrazolidinone derivative **10** was identified as the most



a. Boc_2O, AcOEt, DMAP, reflux, 92%; b. **14a**, LiHMDS, THF, -20°C then r.t., 27% (compound **7a**); c. TFA, CH_2Cl_2, 90%.

Scheme 1. Synthesis of the pyrazolidinone derivatives.



a. Boc_2O, THF, r.t., 15 h, quantitative; b. 3-Cl propionyl choride or 4-Br butyryl chloride DMF, K_2CO_3, r.t., 9 h, 75% and 70%, respectively.

Scheme 2. Synthesis of the *N*-phenyl pyrazolidinone intermediates.

potent compound of this series of indole-2-carboxylate ($pK_i = 8.0$ versus 8.5 for compound **1**).

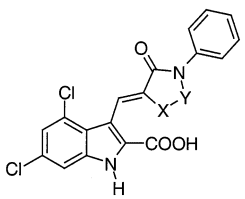
3.2. In vivo characterisation in animal model of stroke

In view of the promising in vitro activity to the glycine-binding site associated to the NMDA receptor, compound **10** was tested in vivo in the NMDA-induced convulsion model in mice [30], a surrogate model of stroke, and the research complied with national legislation, with the company policy on the Care and Use of Animals and with the related codes of practice.

This indole derivative was found to be as effective as compound **1** in blocking convulsions induced by prior i.c.v. administration (1 min) of the exogenous agonist *N*-methyl-D-aspartate ($ED_{50} = 0.1$ and 0.06 mg/kg, i.v., for compound **10** and **1**, respectively). In particular, animals were observed for the occurrence of generalised seizures during the first 30 min after the treatment with NMDA and were considered protected if convulsions did not occur within this period. In view of this result, compound **10** evaluated in the middle cerebral artery occlusion model (MCAo) [31] in rats, exhibiting a high neuroprotective activity when administered i.v. both

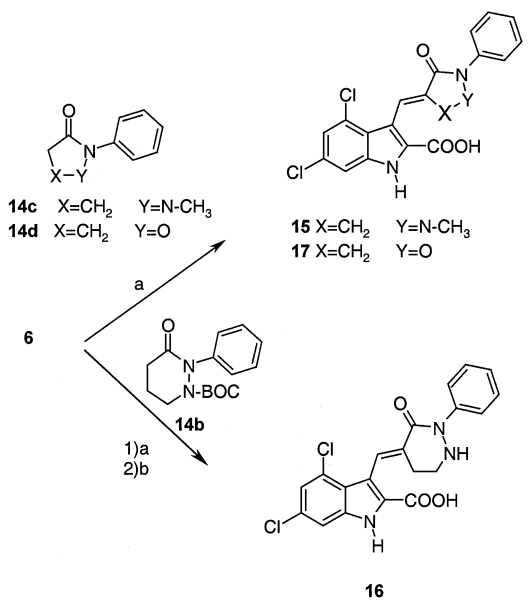
pre- and post-ischaemia. In particular, in the latter protocol, this novel indole derivative, as in the case of compound **1**, was able to stop the progression of the brain damage [20] when given, as a single 3 mg/kg i.v. bolus, up to 6 h after the occlusion of the middle cerebral artery (MCA).

Table 1
In vitro affinity to the glycine-binding site



Compound	X	Y	pKi[a]
1	-	-	8.5
2	CH_2	CH_2	7.5
3	$(CH_2)_2$	CH_2	7.3
7a	CH_2	N-Boc	6.0
10	CH_2	NH	8.0
15	CH_2	N-CH_3	7.5
16	$(CH_2)_2$	NH	7.1
17	CH_2	O	7.1

Displacement of [^{3}H]-glycine. K_i values were measured from at least six-point inhibition curves and they are the geometric means of at least three independent experiments. The standard error of the mean was less than 0.05 for all compounds.



a. **14b-d**, LiHMDS, THF, -20°C then r.t.; b. TFA, CH_2Cl_2, 90%.

Scheme 3. Synthesis of conformationally restrained indole derivatives.

4. Conclusions

The further exploration of indole-2-carboxylate allowed to identify a novel series of glycine antagonists exhibiting high in vitro affinity to the glycine-binding site and outstanding neuroprotective profile in the animal model of cerebral ischemia. In particular, the pyrazolidinone derivative compound **10**, the most potent glycine antagonist belonging to this selected series of indole-2-carboxylates, in view of the observed activity in the MCAo model in rats can be considered as one of the most in vivo potent glycine antagonists identified to date.

5. Experimental

5.1. Chemistry

Nuclear magnetic resonance spectra were recorded with a Varian VXR5000S spectrometer (300 MHz); chemical shifts are expressed as δ units (part per million) downfield from TMS. Infrared spectra were recorded in a Bruker IFS48 spectrometer as Nujol emulsion. The FAB-mass spectra were measured in a Fisons VG-4 instrument. Elemental analysis (C, H, N) were performed in a CHNS-O EA-1108 Elemental Analyser and were within $\pm 0.4\%$ of the theoretical values. Melting points are uncorrected and were determined with a capillary melting point apparatus. TLC was performed in Merck silica gel 60, F_{254} plates. Flash column chromatography was carried out in silica gel (230–400 mesh, G60 Merck). All reagents were of commercial quality from freshly opened containers. Compounds **2** and **3** have been prepared as previously reported [24].

5.2. Ethyl 1-tert-butoxycarbonyl-3-formyl-4,6-dichloroindole-2-carboxylate (6)

To a stirred suspension of the ester **5** (2.2 g, 7.7 mmol) in CH_2Cl_2 (70 ml) was added 4-dimethylamino pyridine (0.01 g, 0.08 mmol) and a solution of di-*tert*-butyl dicarbonate (2 g, 9.16 mmol) in CH_2Cl_2 (20 ml) and the reaction mixture was stirred for 1 h. The solvent was evaporated and the crude residue was triturated in Et_2O/petroleum (10:10 ml) to yield compound **6** as a white solid (2.7 g, 91%): m.p. 142–144 °C. IR (Nujol): ν_{max} 1768, 1742, 1692 cm^{-1}. MS; *m/e*: 386 ($M+1$). ^{1}H NMR ($CDCl_3$): δ 10.77 (s, 1H), 8.24 (d, 1H), 7.42 (d, 1H), 4.50 (q, 2H), 1.65 (s, 9H), 1.43 (t, 3H). *Anal.* ($C_{17}H_{17}Cl_2NO_5$): C, H, N.

5.3. General procedure A for the preparation of compounds **7a**, **7b**, **15** and **17**

To a stirred solution of the intermediates **14a–d** (1.2 eq.) in THF was added a 1 M solution of lithium bis(trimethylsilyl)amide in THF (1.4 eq.) at −50 °C, then the solution was stirred for 30 min at −20 °C. After cooling at −50 °C, a solution of the aldehyde **6** (1.0 eq.) in THF was added dropwise, then the reaction mixture was slowly warmed at room temperature (r.t.) and stirred for 2 h. The solution was diluted with AcOEt and washed with a 0.1 N solution of HCl and water, then the organic layer was separated, dried over Na_2SO_4, and evaporated in vacuo. The obtained compounds were purified by crystallisation or by flash column chromatography.

5.3.1. (E)-3-(1-tert-Butoxycarbonyl-3-oxo-2-phenyl-pyrazolidin-4-ylidenemethyl)-4,6-dichloro-1H-indole-2-carboxylic acid (**7a**)

Compound **7a** was prepared from **6** (0.46 g, 1.75 mmol) and **14a** according to general procedure A. The crude compound was crystallised from AcOEt/hexane to yield compound **7a** as a pale-yellow solid (0.26 g, 27%): m.p. >240 °C. IR (Nujol): ν_{max} 3400–3200, 1715, 1688, 1659 cm^{-1}. MS; *m/e*: 502 (*M* + 1). ^1H NMR (DMSO-d_6): δ 12.6 (bs, 1H), 7.81 (t, 1H), 7.58 (d, 2H), 7.48 (d, 1H), 7.41 (t, 2H), 7.30 (d, 2H), 7.17 (t, 1H), 4.53 (d, 2H), 1.20 (s, 9H). *Anal.* ($C_{24}H_{21}Cl_2N_3O_5$): C, H, N.

The liquor mother was evaporated and purified by flash chromatography using cyclohexane/EtOAc as elution solvent to yield compounds **8** and **9**.

8: Yellowish solid. M.p. = 190 °C. IR (Nujol): ν_{max} 3321, 1728, 1709, 1674 cm^{-1}. MS; *m/e*: 530 (*M* + 1). ^1H NMR ($CDCl_3$): δ 9.27 (bs, 1H), 7.96 (t, 1H), 7.68 (d, 2H), 7.38 (t, 2H), 7.35 (d, 1H), 7.20 (d, 1H), 7.17 (m, 1H), 6.59 (d, 2H), 3.93 (s, 3H), 1.34 (q, 2H), 1.32 (t, 3H), 1.24 (s, 9H). *Anal.* ($C_{26}H_{25}Cl_2N_3O_5$): C, H, N.

9: White solid. M.p. = 220 °C. IR (Nujol): ν_{max} 3279, 1792, 1728, 1680 cm^{-1}. MS; *m/e*: 502 (*M* + 1). ^1H NMR (DMSO-d_6): δ 13.00 (bs, 1H), 7.59 (d, 1H), 7.52 (dd, 2H), 7.44 (d, 1H), 7.40 (t, 2H), 7.17 (tt, 1H), 6.27 (d, 1H), 3.95 (ddd, 1H), 3.91 (dd, 1H), 3.48 (dd, 1H), 1.19 (s, 9H). *Anal.* ($C_{24}H_{21}Cl_2N_3O_5$): C, H, N.

5.3.2. (E)-3-(1-tert-Butoxycarbonyl-3-oxo-2-phenyl-tetrahydro-pyridazin-4-ylidenemethyl)-4,6-dichloro-1H-indole-2-carboxylic acid (**7b**)

Compound **7b** was prepared from **6** (0.43 g, 1.1 mmol) and **14b** according to general procedure A. The crude compound was crystallised from AcOEt/hexane at 0 °C to yield compound **7b** as a pale-yellow solid (0.2 g, 35%): m.p. >240 °C. IR (Nujol): ν_{max} 1717, 1672, 1657 cm^{-1}. MS; *m/e*: 516 (*M* + 1). ^1H NMR (DMSO-d_6, 60 °C): δ 13.5 (bs, 1H), 12.8 (bs, 1H), 8.0 (t, 1H), 7.60 (dd, 2H), 7.46 (d, 1H), 7.37 (t, 2H), 7.19 (d, 1H), 7.19 (tt, 1H), 3.84 (bs, 2H), 2.56 (bs, 2H), 1.28 (s, 9H). *Anal.* ($C_{25}H_{23}Cl_2N_3O_5$): C, H, N.

5.3.3. (E)-4,6-Dichloro-3-(1-methyl-3-oxo-2-phenyl-pyrazolidin-4-ylidenemethyl)-1H-indole-2-carboxylic acid (**15**)

Compound **15** was prepared from **6** (0.37 g, 0.96 mmol) and **14c** according to general procedure A. The crude compound was purified by trituration in Et_2O to obtain compound **15** as a pale-yellow solid (0.06 g, 15%): m.p. >220 °C. IR (Nujol): ν_{max} 3244, 1676, 1653 cm^{-1}. MS; *m/e*: 416 (*M* + 1). ^1H NMR (DMSO-d_6): δ 13.75 (bs, 1H), 12.48 (s, 1H), 7.84 (d, 2H), 7.83 (s, 1H), 7.45 (d, 1H), 7.42 (t, 2H), 7.27 (d, 1H), 7.16 (t, 1H), 4.08 (bs, 1H), 3.63 (bs, 1H), 3.31 (s, 3H). *Anal.* ($C_{20}H_{15}Cl_2N_3O_3$): C, H, N.

5.3.4. (E)-4,6-Dichloro-3-(3-oxo-2-phenyl-isoxazolidin-4-ylidenemethyl)-1H-indole-2-carboxylic acid (**17**)

Compound **17** was prepared from **6** (0.95 g, 2.5 mmol) and **14d** according to general procedure A. The crude residue was purified by flash chromatography using CH_2Cl_2/MeOH (9:1) as elution solvent to yield compound **17** as a pale-yellow solid (0.16 g, 16%): m.p. >220 °C. IR (Nujol): ν_{max} 3304, 1672, 1645 cm^{-1}. MS; *m/e*: 403 (*M* + 1). ^1H NMR (DMSO-d_6): δ 13.89 (bs, 1H), 12.59 (s, 1H), 7.85 (t, 1H), 7.74 (dd, 2H), 7.47 (d, 1H), 7.45 (t, 2H), 7.30 (d, 1H), 7.20 (t, 1H), 5.07 (d, 2H). *Anal.* ($C_{19}H_{12}Cl_2N_2O_4$): C, H, N.

5.4. General procedure B for the preparation of compounds **14a** and **14b**

To a stirred mixture of 1-*tert*-butoxycarbonyl-2-phenylhydrazine (**12**) (1 eq.) and K_2CO_3 (1.7 eq.) in DMF was added dropwise the appropriate acyloyl chloride (1.4 eq.) and the reaction mixture was stirred at reflux for 9 h and at r.t. overnight. The mixture was diluted with Et_2O and washed with water; then the organic layer was separated, dried over Na_2SO_4, and evaporated in vacuo. The crude residue was purified by flash chromatography using cyclohexane/EtOAc (8:2) as elution solvent. Notably, when the previous reaction was run in two steps isolating intermediates **13a** and **13b**, compounds **14a** and **14b** were obtained in higher yields.

5.4.1. 3-Oxo-2-phenyl-pyrazolidine-1-carboxylic acid tert-butyl ester (**14a**)

Compound **14a** was prepared from **12** (5.6 g, 27 mmol) and 3-chloropropionyl chloride according to general procedure B and obtained as a white solid (5.4 g, 75%): m.p. = 150 °C. IR (Nujol): ν_{max} 1773, 1692 cm^{-1}. MS; *m/e*: 263 (*M* + 1). ^1H NMR (DMSO-d_6): δ 7.45 (m, 2H), 7.35 (m, 2H), 7.12 (m, 1H), 4.07 (t, 2H), 2.74 (t, 2H), 1.26 (s, 9H). *Anal.* ($C_{14}H_{18}N_2O_3$): C, H, N.

*5.4.2. 3-Oxo-2-phenyl-tetrahydro-pyridazine-1-carboxylic acid tert-butyl ester (**14b**)*

Compound **14b** was prepared from **12** (1 g, 4.8 mmol) and 4-bromobutyryl chloride according to general procedure B and obtained as a white solid (0.951 g, 70%): m.p. = 69–71 °C. IR (Nujol): ν_{max} 1771, 1690 cm^{-1}. MS; *m/e*: 277 ($M+1$). ^1H NMR (DMSO-d_6): δ 7.55 (d, 2H), 7.38 (t, 2H), 7.19 (t, 1H), 4.5–3.0 (broad, 2H), 2.5–1.0 (broad, 13H). *Anal.* ($C_{15}H_{20}N_2O_3$): C, H, N.

*5.4.3. 1-Methyl-2-phenyl-3-pyrazolidinone (**14c**)*

A solution of compound **14a** (4.6 g, 17.5 mmol) in trifluoroacetic acid/CH_2Cl_2 was stirred for 1 h, then the solvent was evaporated and the crude residue was dissolved in AcOEt and washed with a saturated solution of $NaHCO_3$ and water. The organic layer was separated, dried over Na_2SO_4, and evaporated in vacuo to obtain 2-phenyl-3-pyrazolidinone (2.6 g, 92%). The latter was dissolved in DMF (35 ml), cooled at −20 °C, then methyl trifluoromethanesulfonate (2.54 ml, 23.2 mmol) was added dropwise. The reaction mixture was slowly warmed at r.t. and stirred for 3 h. The solution was diluted with Et_2O and washed with a saturated solution of NH_4Cl and water, then the organic layer was separated, dried over Na_2SO_4, and evaporated in vacuo. The crude residue was purified by flash chromatography using AcOEt/cyclohexane (1:1) as elution solvent to yield compound **14c** as a white foam (0.75 g, 25%): IR (Nujol): ν_{max} 1697 cm^{-1}. MS; *m/e*: 177 ($M+1$). ^1H NMR ($CDCl_3$): δ 7.78 (d, 2H), 7.37 (t, 2H), 7.13 (tt, 1H), 3.8–3.6 (bm, 1H), 3.3–3.0 (bm, 2H), 2.7–2.4 (bm, 1H), 2.65 (s, 3H). *Anal.* ($C_{10}H_{12}N_2O$): C, H, N.

*5.4.4. 2-Phenyl-3-isoxazolidinone (**14d**)*

To a suspension of 5% Rh/C (0.13 g) in THF (23 ml) was added nitrobenzene (5 g, 40.6 mmol). The mixture was cooled at 0 °C and hydrazine monohydrate (2 g, 40.6 mmol) was added portionwise, maintaining the temperature below 25 °C throughout the addition. After that the reaction mixture was stirred at 25 °C for 2 h, then filtered and concentrated in vacuo. The residue was dissolved in DMF (30 ml) and cooled at −5 °C, then K_2CO_3 (5.6 g) and 3-chloropropionyl chloride (3.8 ml, 39.6 mmol) were added. The reaction mixture was stirred at 25 °C for 1 h, then diluted with Et_2O and washed with water. The organic layer was separated, dried over Na_2SO_4, and evaporated in vacuo. The crude residue was purified by crystallisation from AcOEt and cyclohexane to yield *N*-(3-chloropropionyl)-*N*-phenylhydrohylamine (4 g, 46%). The latter was dissolved in acetone (150 ml), then K_2CO_3 (2.5 g) was added. The reaction mixture was stirred for 6 h, then concentrated in vacuo. The crude residue was purified by flash chromatography using AcOEt/cyclohexane (8:2) as elution solvent to yield compound **14d** as an oil (2.5 g, 82%): IR (Nujol): ν_{max} 1697 cm^{-1}. MS; *m/e*: 164 ($M+1$). ^1H NMR ($CDCl_3$): δ 7.69 (m, 2H), 7.37 (m, 2H), 7.14 (tt, 1H), 4.52 (t, 2H), 3.00 (t, 2H). *Anal.* ($C_9H_9NO_2$): C, H, N.

*5.5. General procedure C for the preparation of compounds **10** and **16***

A solution of compounds **7a** and **7b** (1 eq.) in trifluoroacetic acid/CH_2Cl_2 was stirred for 1 h, then the solvent was evaporated and the crude residue was purified by trituration in Et_2O.

*5.5.1. 4,6-Dichloro-3-(5-oxo-1-phenyl-pyrazolidin-4-ylidenemethyl)-1H-indole-2-carboxylic acid (**10**)*

Compound **10** was prepared from **7a** (0.255 g, 0.51 mmol) according to general procedure C and obtained as a solid (0.18 g, 87%): m.p. >250 °C. IR (Nujol): ν_{max} 3250–3150, 1717, 1684 cm^{-1}. MS; *m/e*: 402 ($M+1$). ^1H NMR (DMSO-d_6): δ 13.70 (bs, 1H), 12.49 (bs, 1H), 7.90 (d, 2H), 7.70 (t, 1H), 7.46 (d, 1H), 7.39 (t, 2H), 7.27 (d, 1H), 7.12 (t, 1H), 6.45 (bs, 1H), 3.83 (bs, 2H). *Anal.* ($C_{19}H_{13}Cl_2N_3O_3$): C, H, N.

*5.5.2. 4,6-Dichloro-3-(3-oxo-2-phenyl-tetrahydro-pyridazin-4-ylidenemethyl)-1H-indole-2-carboxylic acid (**16**)*

Compound **16** was prepared from **14b** (0.12 g, 0.24 mmol) according to general procedure C and obtained as a solid (0.05 g, 50%): m.p. >250 °C. IR (Nujol): ν_{max} 3277, 1672, 1612 cm^{-1}. MS; *m/e*: 416 ($M+1$). ^1H NMR (DMSO-d_6): δ 13.50 (bs, 1H), 12.36 (s, 1H), 8.0 (t, 1H), 7.63 (d, 2H), 7.43 (d, 1H), 7.33 (t, 2H), 7.22 (d, 1H), 7.12 (t, 1H), 6.02 (t, 1H), 3.35 (m, 2H), 3.10 (m, 2H). *Anal.* ($C_{20}H_{15}Cl_2N_3O_3$): C, H, N.

5.6. Biology

5.6.1. Binding assay

NCEs were dissolved at a 5 mM concentration in 100% DMSO and tested at seven different concentrations in duplicate in the [^{3}H]-glycine displacement experiments and, in the other binding assays, at five concentrations (from 10 nM to 100 μM). A reference compound was always included as internal control.

Affinity to the glycine-binding site was measured by inhibition of the binding of [^{3}H]-glycine to crude synaptic membranes prepared from adult rat cerebral cortex, as described by Kishimoto et al. [27]. Incubation (20 min at 4 °C) was carried out in 50 mM Tris/citrate (pH 7.10) using 20 nM [^{3}H]-glycine. Data for displacement experiments, performed to determinate the inhibition constants (K_i) of the displacer ligands, were analysed using the nonlinear curve-fitting software LIGAND [32]. The K_i values were measured from at least six-point

inhibition curves and are geometric means of at least three different experiments.

5.6.2. Anticonvulsant activity

NCEs were evaluated in vivo by assessing their anticonvulsant effect [30] when convulsions were induced in male CD-1 mice (18–29 g) by i.c.v. injection of NMDA (1 nmol/mouse), 1 min after i.v. administration of the NCE. Animals were observed for the occurrence of generalised seizures during the first 30 min after the treatment with NMDA and were considered protected if convulsions did not occur within this period. The percentage of animals showing anticonvulsant activity in each treatment group was recorded and ED_{50} values were estimated along with their 95% confidence limits.

5.6.3. Neuroprotective activity

5.6.3.1. MCAo (distal MCA occlusion). The experimental procedure was performed according to Tamura et al. [31], with minor modifications. Male Sprague–Dawley rats (280–350 g) were anaesthetised with chloral hydrate (400 mg/kg, i.p.). The animals were maintained normothermic by means of a heating pad and placed under an operating microscope. A skin incision was made, the temporalis muscle was reflected and a craniectomy was performed by drilling at the junction between the medial wall and the roof of the infero-temporal fossa. The dura was opened and the MCA was permanently occluded by electrocoagulation proximal to the frontal branch. The muscles and the skin were reported to the original position and the skin incision was closed. Rats were allowed to recover for 24 h before the evaluation of the ischaemic damage.

5.6.3.2. Histology. Animals were sacrificed 24 h after MCAo, the brain removed carefully and sectioned coronally at 0.5 mm intervals by using a motorised vibroslice. The brain slices were immersed in a solution of 2% triphenyltetrazolium chloride (TTC) at 37 °C for 20 min and then stored in 4% neutral buffered formalin. The TTC staining clearly distinguishes between the normal tissue (stained in red) and the ischaemic area (white, not stained).

The area of cerebral damage in each coronal section was assessed by using an imagine analyser (Imaging Research Inc., Canada). The infarct volumes were calculated by using the trapezoidal rule method.

5.6.3.3. Drugs and statistical analysis. The compounds were dissolved in DMSO and then diluted to a final 3% DMSO concentration with distilled water. Solutions were prepared fresh at the day of the experiment.

Values are expressed as mean ± SEM. Statistical differences between groups were analysed using the Student's *t*-test for unpaired data using the GBSTAT 5.3 program (Dynamic Microsystem).

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