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Aryl azoles with neuroprotective activity—Parallel synthesis and attempts at target identification

Giuseppe Cocconcelli,[a,*] Enrica Diodato,[a,*] Andrea Caricasole,[a] Giovanni Gaviraghi,[a] Eva Genesio,[a] Chiara Ghiron,[a] Letizia Magnoni,[a] Elena Pecchioli,[a] Pier Vincenzo Plazzi[b] and Georg C. Terstappen[a]

[a]*Siena Biotech S.p.A., via Fiorentina 1, 53100 Siena, Italy*
[b]*Dipartimento Farmaceutico, Università degli Studi di Parma, V.le G.P. Usberti, 27/A, I-43100 Parma, Italy*

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Abstract—A parallel synthesis of aryl azoles with neuroprotective activity is described. All compounds obtained were evaluated in an in vitro assay using a NMDA toxicity paradigm showing a neuroprotective activity between 15% and 40%. The potential biological target of the active compounds was investigated by extensive literature searches based around similar scaffolds with reported neuroprotective activity. The most interesting molecules active in the NMDA toxicity assay (**3a** and **2g**) showed moderate but significant activity in the inhibition of the Site 2 Sodium Channel binding assay at 10 μM. To confirm our hypothesis compounds **3a**, **c**, **f** and **2g** were tested in the Veratridine assay which is one of the excitotoxicity assays of revelance to NaV channels. The compounds tested showed an activity between 40% and 70%. The identification of neuroprotective small molecules and the identification of NaV channels as the potential site of action were the most important goals of this work.

1. Introduction

Neurodegenerative disorders are characterized by a progressive dysfunction and death of neurons from specific regions of the brain. These disorders include debilitating diseases as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). Although clinical and neuropathological aspects of these syndromes are distinct, their unifying feature is that each one has a characteristic pattern of neuronal degeneration in anatomically defined regions.[1]

Neuroprotection may be defined as the interference with the cellular and functional outcomes of neurotoxic insults, with the aim to preserve neuronal survival and function.[2] The result of a neuroprotective therapy may be the salvage, recovery or regeneration of the nervous system, its cells, structure and functions. Presently pharmacological therapy of neurodegenerative disorders is mostly limited to symptomatic treatments. In Parkinson's disease for example, where the neurochemical deficit produced by the disease is comparatively well-defined, symptomatic treatment is in general relatively successful.[3,4] Carbidopa/levodopa, bromocriptine (a dopamine receptor agonist), entacapone (a caethcol-*O*-methyl-transferase inhibitor), selegiline (a MAO-B inhibitor) and amantidine are examples of commonly used medications for the treatment of PD. Drugs approved by US Food and Drug Administration for the treatment of AD are galantamine, rivastigmine, donepezil, tacrine (cholinesterase inhibitors) and memantine (an NMDA antagonist). The pharmacological treatment of Huntington's disease is currently limited to the amelioration of movement and psychiatric abnormalities associated with the illness and includes antipsychotic agents and benzodiazepines. Likewise, current therapy for ALS is symptomatic and includes GABA-B agonists and benzodiazepines. These symptomatic treatments do not alter the course of these disorders. The lack of disease-modifying therapies for most neurodegenerative disorders commands significant efforts towards the development of novel neuroprotective strategies. These efforts have resulted in the identification of general mechanisms such as excitotoxicity (determined by an

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* Corresponding authors. Tel.: +39 0577 381416; fax: +39 0577 381410 (G.C.); e-mail: gcocconcelli@sienabiotech.it

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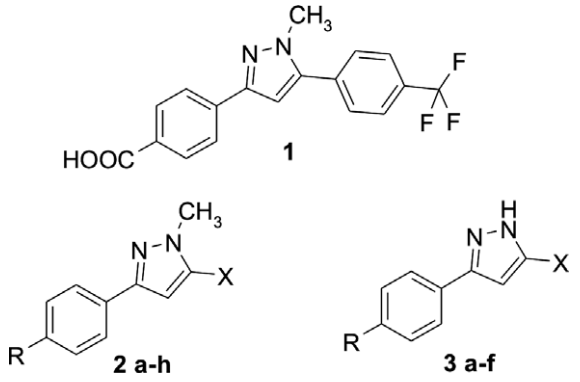


Figure 1. Erythropoietin sensitizer **1** and compounds **2a**–**h**; **3a**–**f**.

excess of glutamate in the brain[5]), aberrant neuronal energy metabolism[6] and the production of reactive compounds, such as peroxides and radicals,[7] as contributors to the cell death observed in various neurodegenerative diseases. In addition to generic toxicity responses which contribute to many neurodegenerative conditions, disease-specific mechanisms have been identified through the study of familial cases, resulting in the definition of key proteins associated with individual pathologies such as the amyloid precursor protein (APP) and the secretases (β- and γ-secretase) in AD, Parkin in PD, Huntingtin in HD and Superoxide Dismutase 1 (SOD1) in ALS. Many of these proteins represent targets currently exploited for pharmacological intervention in drug discovery efforts, aimed at defining disease-modification approaches. In addition, the mechanisms underlying the neuroprotective properties of specific proteins (such as erythropoietin,[8] NGF and others[9]) are being studied to identify tractable targets upon which neuroprotective pharmacological strategies could be developed.

As part of a research project directed towards the development of small neuroprotective molecules, we were initially interested in compounds which could act as erythropoietin mimetics as these might also display neuroprotective properties.[8] Compound **1** has been reported to improve the erythropoietic process by sensitizing red blood cells to the effect of erythropoietin and possesses a chemically tractable chemical scaffold.[8] We therefore decided to consider **1** as starting point for our work and test it in in vitro neuroprotection assays. As the molecule did indeed show some level of neuroprotective activity, we explored the chemical class further by synthesizing a small array of compounds (Fig. 1) and investigating their neuroprotective properties. Finally, we proceeded to attempt deconvoluting the cellular target of this chemical class. As a result, classical erythropoietin receptor-mediated responses were excluded and NaV channels were identified as the likely targets for the neuroprotective action of these compounds.

2. Results and discussion

2.1. Chemistry

Numerous methods for the synthesis of 1,3,5-substituted pyrazoles are known.[10] One frequently reported method is the reaction of 1,3-dicarbonyl compounds with hydrazine hydrate. However, when substituted hydrazines and unsymmetrical ketones are used, the reaction often results in a mixture of regioisomeric pyrazoles.[11]

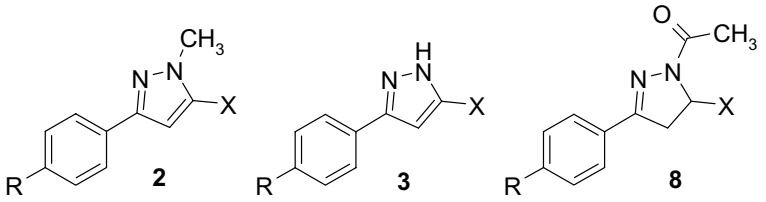
The 3,5-diarylpyrazoles (**2a**–**h** and **3a**–**f**) described in this paper (see Table 1) were obtained following the synthetic routes represented in Scheme 1. Since the desired pyrazoles are substituted in position 1 and the aryl groups in position 3 and 5 of the heterocyclic ring are different (**2a**–**h**), the development of a regioselective synthesis was necessary. In order to obtain compounds **2a**–**h**, we therefore decided to explore the reaction of substituted hydrazines with α,β-unsaturated ketones; this reaction has been reported to lead to the regioselective formation of 4,5-dihydro-1H-pyrazoles that can then be oxidized to the corresponding pyrazoles.[12,13]

Compounds **6a**, **c**, **f** are commercially available. Compounds **6b**, **d**, **e**, **g** were synthesized under basic conditions, according to reported literature methods.[14] Compound **6h** was successfully synthesized under microwave irradiation (μW) with acidic catalysis after the observation that 1*H*-indole-3-carbaldehyde did not react under basic conditions, possibly due to a tautomeric equilibrium between the indole NH and the carbonyl.[15]

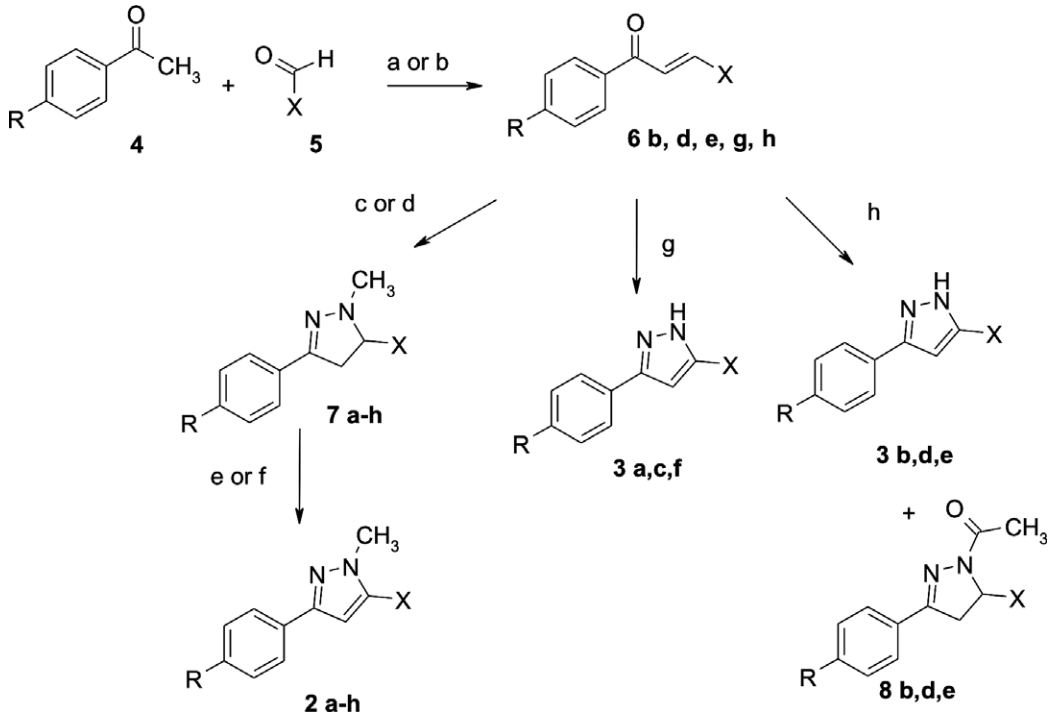
Although products **2a**–**h** could be obtained directly from compounds **6a**–**h**,[12] the intermediate 4,5-dihydro-1*H*-pyrazoles **7a**–**h** were also isolated and characterized in order to test them in our biological assay. However, these intermediates proved to be air-sensitive especially in solution (spontaneous slow oxidative aromatization was observed), and their biological evaluation was not possible. Compounds **7a**–**d**, **7h** were synthesized from the respective ketones under basic conditions, while compounds **7e**–**g** could only be obtained under neutral conditions, as they showed a high degree of instability when submitted to our standard procedure (see Scheme 1 and Table 1).

3,5-Diaryl-4,5-dihydro-1*H*-pyrazoles **7a**–**f** were then oxidized under Pd-catalysis in acetic acid according to the procedure described by Nakamichi et al.[16] This oxidative aromatization procedure in acidic medium led however in our hands to a high degree of decomposition for the molecules bearing an indole moiety. To circumvent this problem we resorted to a different method for 4,5-dihydro-1*H*-pyrazoles **7g**, **h** which were finally successfully oxidized using MnO_2 in toluene. The regioselective mechanism of this synthetic route was confirmed by ^1H NMR–NOESY (see Supplementary material). Since we noted that compounds **7a**–**h** underwent a spontaneous oxidative aromatization, we planned to obtain the 3,5-diarylpyrazoles **3a**–**f** directly from compounds **6a**–**h** by treatment with hydrazine. However, using literature reported conditions for this kind of reaction (ethanol at room temperature),[17] only low yield of the desired products was obtained. These were only deemed acceptable for commercially available starting α,β-unsaturated ketones **6a**, **c**, **f**. Investigating what parameters would affect the reaction profile, we tried to use acetic acid as the solvent at refluxing temperature. Although we

Table 1. Chemical structure of final synthesized compounds **2a**–**h**, **3a**–**f**, **8b**, **d**, **e**



R	OCH_3	H	Cl	H	NO_2	H	H	H
X	*	* OMe	*	* Cl	*	* NO_2	* N H	* HN
	a	**b**	**c**	**d**	**e**	**f**	**g**	**h**



Scheme 1. Reagents and conditions: (a) NaOH, EtOH (**6b**, **d**, **e**, **g**); (b) M_W, 65 W, 65 °C, 4 × 10 min (**6h**); (c) methylhydrazine sulfate, KOH, EtOH (**7a**–**d**, **h**); (d) methylhydrazine, EtOH (**7e**–**g**); (e) 10% Pd/C, CH_3COOH (**2a**–**f**); (f) MnO_2, toluene (**2g**, **h**); (g) hydrazine hydrate, EtOH (**3a**, **c**, **f**); (h) hydrazine hydrate, CH_3COOH (**3b**, **d**, **e**; **8b**, **d**, **e**).

observed an increase in the yield of the desired pyrazoles, this was accompanied by the formation of side products identified as the *N*-acetyl-4,5-dihydro-1*H*-pyrazoles **8b**, **d**, **e**. As we did not observe further oxidation to the corresponding *N*-acetylpyrazoles, we supposed compounds **8b**, **d**, **e** to be more stable than the corresponding *N*-methyl and *N*-H 4,5-dihydro-1*H*-pyrazoles. The *N*-acetyl-4,5-dihydro-1*H*-pyrazoles proved indeed to be more stable and compounds **8b**, **d**, **e** could be isolated and tested in our biological assays. The structures of these *N*-acetyl-4,5-dihydro-1*H*-pyrazoles were confirmed by ^{1}H NMR–NOESY (see Supplementary material). Some of the final compounds are described in the literature.[31–37]

2.2. In vitro pharmacology

The neuroprotective properties of compounds **1**, **2a**–**h**, **3a**–**f**, **8b**, **8d**, and **8e** were first evaluated at the single dose of 10 μM (co-treatment, see Sections 4.2.2 in 4.2) in the NMDA toxicity paradigm in mixed rat cortical neurons. The results for each compound are presented in Table 2

Table 2. Results of neuroprotective effect of all compounds (10 μM, co-treatment) tested in NMDA toxicity assay

N	Mean	SEM	R	E
Co-treatment (10 μM)				
1	61.1*	5.29	4	3
2a	70.4	7.01	4	2
2b	78.3	6.49	4	3
2c	87.3	5.38	4	3
2d	65.6	5.67	4	3
2e	86.3	5.67	4	3
2f	85.5	4.79	4	3
2g	67.1*	3.26	8	2
2h	76.7*	5.05	4	3
3a	67.2*	3.28	8	4
3b	76.0*	3.34	8	4
3c	66.1*	3.41	8	4
3d	96.4	3.47	8	4
3e	89.0	3.91	8	4
3f	75.6*	3.21	8	4
8b	81.0*	3.42	8	4
8d	86.4	3.72	8	3
8e	85.5*	3.28	8	4
9	80.3*	3.05	8	2

Insult: NMDA (60 μM); positive control: MK801 (10 μM). Normalized data (expressed as percentage of LDH released by NMDA) are displayed as means and SEM of at least two independent experiments with at least four technical replicates for each sample. R, replicates; E, experiments.

* $p < 0.05$ Tukey's honestly significant difference criterion post ANOVA test.

and show the percentage of cell damage due to the toxic insult (NMDA) in presence of the tested compounds. The neuroprotective effect is therefore deduced as difference between the maximum insult (100%) and the cell damage in presence of test compound. All compounds tested demonstrated neuroprotective activity (15–40%), with compounds **3a**, **c**, **f**; **2g**, **d** and **1** demonstrating the strongest neuroprotective effects.

More detailed experiments were then carried out in order to better profile the neuroprotective activity of these compounds. In the NMDA toxicity assay in mixed rat cortical cultures compounds **3a** and **2g** were evaluated at the doses of 1 μM and 10 μM in both co-treatment and pre-treatment paradigms. The results are shown in Table 3. Compounds **3a** and **2g** displayed significant protection from toxicity induced by NMDA at both 1 μM and 10 μM, when tested in the co-treatment paradigm. In addition, when tested at three concentrations (0.1 μM, 1 μM and 10 μM), compounds **3a** and **2g** showed activity only at the higher concentrations (i.e., were inactive at 0.1 μM; data not shown), suggestive of a concentration-dependent neuroprotective activity. However, when tested in the pre-treatment paradigm, no neuroprotective activity was evident. This may be due to a number of reasons, for example, the compounds may elicit a transient cellular response whose neuroprotective effect is efficaceous only when delivered simultaneously with the neurotoxic insult. For instance, this effect may be produced as the result of desensitization of a receptor recognizing the small molecules.

Table 3. Results of neuroprotective effects of compounds **2g** and **3a** (1 μM and 10 μM co- and pre-treatment) tested in NMDA toxicity assay

N	1 μM		10 μM		R	E
	Mean	SEM	Mean	SEM		
Pre-treatment						
2g	90.2	2.03	86.0	2.03	8	3
3a	91.1	2.65	95.0	2.60	8	3
Co-treatment						
2g	80.3*	2.65	72.0*	2.65	8	3
3a	81.7*	2.32	77.5*	2.22	8	3

Insult: NMDA (60 μM); positive control: MK801 (10 μM). Normalized data (expressed as percentage of LDH released by NMDA) are displayed as means and SEM of three independent experiments with eight technical replicates for each sample. R, replicates; E, experiments.

* $p < 0.05$ Tukey's honestly significant difference criterion post ANOVA test.

Intrigued by the levels of neuroprotection shown by the molecules synthesized and by the observation that erythropoietin, in contrast to the behaviour of our compounds, can act as neuroprotectant agent in pre-treatment assays,[18] we reasoned that the observed neuroprotection could possibly be related to activity on targets other than those associated with classical erythropoietin signalling. Furthermore, the structural differences between our compounds and starting molecule **1** prompted us to make a further attempt at the definition of putative targets for our molecules through literature searches based around generic azole scaffolds with reported neuroprotectant activity. Structurally related acetyl-4,5 dihydro-1H-pyrrazoles were recently reported[19] to display neuroprotectant activity, however our attention was focused on structural analogies with Sodium Channel Site 2 ligands and AMPA receptor antagonists (i.e., compound **9**—Fig. 2), suggesting these proteins as possible targets for our compounds.[20]

We therefore decided to test **3a** and **2g**, the best performing amongst the synthesized compounds, in binding assays against the above-mentioned receptors.[21–23] We found evidence supporting our hypothesis, with **3a** and **2g** showing moderate activity in the inhibition in the Site 2 Sodium Channel binding assay at 10 μM as shown in Table 4. Negligible activity was detected on the AMPA and Kainate receptors. All compounds synthesized were then tested on Site 2 Sodium Channel binding assay (Table 4).

Example 17 from WO99/54314 (compound **9**) prepared according to the patent experimental description was also tested, and its activity as neuroprotectant was confirmed in our NMDA (co-treatment) toxicity assay as

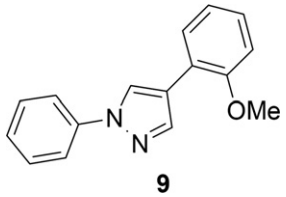


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Figure 2. Example 17 from WO99/54314.

Table 4. Percent inhibition of Veratridine (V) specific binding in the Na^+ channel (site 2) assay as measured by displacement of 10 nM [^{3}H[batrachotoxinin by synthesized compounds **1**, **2a**–**h**, **3a**–**f**, **8b**, **d**, **e** and **9**

N	% Inhibition[a]	N	% Inhibition[a]
V	100%	**3a**	30
1	Inactive	**3b**	27
2a	8	**3c**	16
2b	12	**3d**	21
2c	13	**3e**	14
2d	15	**3f**	15
2e	8	**8b**	16
2f	4	**8d**	27
2g	41	**8e**	17
2h	14	**9**	Inactive

[a] Concentration of positive control (Veratridine): 300 μM: concentration of test compounds: 10 μM.

shown in Table 2. Surprisingly, compound **9** did not show activity in the Site 2 Sodium Channel binding assay (Table 4). Although these data are supported by the inactivity on the Veratridine toxicity assay described after, perhaps indicating that the compounds from our study are more potent against Site 2 Sodium Channel, the binding data in Table 4 should be evaluated in a qualitative way and more experiments will be necessary to allow for structure–activity-relationship analysis. We next sought evidence for the expression of NaV genes in the primary neuronal cultures employed in the neuroprotection assays. To this end, levels of mRNA of NaV genes known to be expressed in CNS neurons were evaluated by real time PCR. At least four NaV α subunits (Ia, IIa, IIIa and VIIIa) are known to be expressed in CNS neurons in the rat.[24] In rat primary cortical cultures, Scn1a, 2a, 3a and 8a are all expressed in both pure cultured neurons and in mixed cortical cultures of neurons and glia, with higher expression levels of Scn2a relatively to other subunits (Fig. 3). The expression profile in pure versus mixed cortical cultures suggests a predominantly neuronal expression of the analyzed NaV subunits, consistent with published data.[24] One of the excitotoxicity assays of relevance to NaV channels is the Veratridine assay. Analogues of compound **1**, showing significant neuroprotection against NMDA, were tested in the Veratridine toxicity assay.[25] Compounds **3a**, **c**, **f** and **2g** were tested in both pre- and co-treatment at the doses of 1 μM and 10 μM in both pure neuronal and mixed cortical cultures. While no protective effects were evident in the pre-treatment paradigm (data not shown), the dose of 10 μM was neuroprotective (**2g**: 40%, **3a**: 50%; **3c**, **f**: 70%) respectively, against Veratridine-induced neuronal toxicity in the co-treatment paradigm for all tested compounds in mixed rat cortical neurons (Table 5). At the lower dose (1 μM), only compounds **3a**, **c** and **f** demonstrated a low but significant (ca. 20%) neuroprotective activity, while no activity was observed at the concentration of 0.1 μM (data not shown). These data are suggestive of a concentration-dependent neuroprotective activity of the compounds. In pure neuronal cultures, all tested compounds displayed significant neuroprotective activity (ca. 40–50%) at the dose of 10 μM. Significant but limited (20–30%) neuroprotective effects were observed at 1 μM for compounds **3a** and **3c**, while compounds **3f** and **2g** were inactive. In agreement to the binding data on Side 2 Sodium Channel, compound **1** showed no significant activity in the Veratridine paradigm; this compound could be acting as neuroprotectant agent exploiting another mechanism. Thus, all compounds tested demonstrated neuroprotective activity against excitotoxicity induced by NMDA (15–40%) and in the Veratridine assay (40–70%). As the observed toxicity (measured as LDH release) induced by NMDA and Veratridine in the mixed cultures is comparable (data not shown), these data are consistent with the compounds acting as NaV blockers, as their neuroprotective activity increases in a more

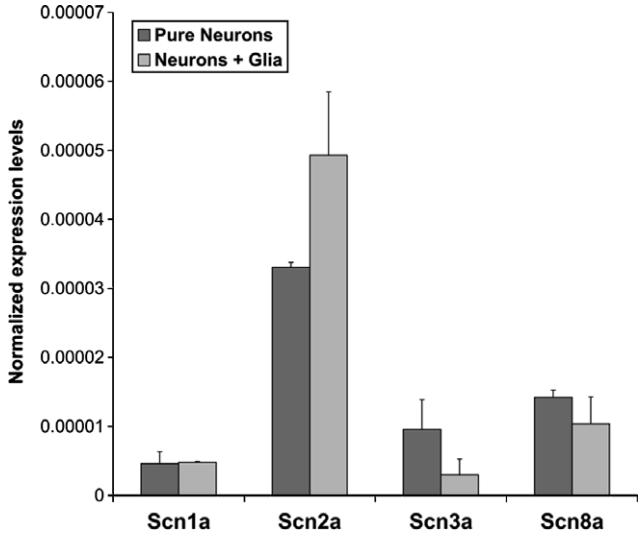

Figure 3. Messenger RNA expression levels of Scn1a, 2a, 3a and 8a in pure and mixed rat cortical cultures. Data are normalized relatively to β-actin levels, and are expressed as SEM ± SD. A representative experiment is shown.

Table 5. Neuroprotective effects of compounds **2g**, **3a**, **3c** and **3f** in Veratridine (60 μM) toxicity assay

N	1 μM		10 μM		R	E
	Mean	SEM	Mean	SEM		
Mixed rat cortical neurons						
3a	80.9*	5.58	49.2*	6.37	8	3
3c	74.1*	4.82	24.2*	5.85	8	3
3f	77.6*	4.93	26.6*	3.94	8	3
2g	87.9	3.10	63.2*	2.90	8	3
Pure rat cortical neurons						
3a	70.4*	5.81	64.9*	2.87	8	3
3c	75.0*	3.25	56.5*	6.10	8	3
3f	80.9	9.17	51.2*	9.45	8	3
2g	82.7	8.12	56.8*	7.41	8	3

Compounds were tested at the doses of 1 μM and 10 μM in co-treatment paradigm using mixed rat cortical neurons and pure rat cortical neurons. Insult: Veratridine. Normalized data (expressed as percentage of LDH or XTT released by Veratridine) are displayed as means and SEM of three independent experiments with eight technical replicates for each sample. R, replicates; E, experiments.

* $p < 0.05$ Tukey's honestly significant difference criterion post ANOVA test.

direct assay of NaV activation-mediated neurotoxicity. In particular, analogues **3a**, **c**, **f** and **2g** showed the most significant neuroprotective effects in our toxicity assay. These data, together with the results demonstrating moderate binding of our class of compounds to the NaV channel, point to the inhibition of NaV channel as an important component of the neuroprotective properties of these compounds, consistent with the known neuroprotective properties of published inhibitors of voltage-gated sodium channels.[26,27]

3. Conclusion

In summary, we have identified novel neuroprotective small molecules active against excitotoxic insults, and have identified the inhibition of NaV channels as a likely site of action of their neuroprotective activity.

4. Experimental

4.1. Chemistry

Melting points are not corrected and were determined with a Gallenkamp melting point apparatus. Purity of final compounds **2a**–**f**, **2h**, **3a**–**c**, **3f** and **8b**, **d** was determined by ThermoQuest (Italia) FlashEA 1112 Elemental Analyzer, for C, H, and N. The percentages found were within ±0.4% of the theoretical values. Characterization of final compounds **2g**, **3e** and **8e** was determined by HR-MS using a Thermo LTQ Orbitrap Mass Spectrometer. ^{1}H NMR spectra were recorded on a Bruker Avance 300 Spectrometer (300 MHz); chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent. ^{1}H NMR spectra are reported in the following order: multiplicity, approximate coupling constants (J value) in Hertz (Hz) and number of protons. Reactions were monitored by TLC, on Kieselgel 60 F 254 (DC-Alufolien, Merck). Final compounds and intermediates were purified by chromatography on preparative Gilson MPLC, using a SiO_2 column (LiChroprep, Si 60, 25-40 μm, Merck). Microwave reactions (MW) were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC). Abbreviations for solvents are the following: EtOAc: ethyl acetate, DMSO: dimethylsulfoxide.

4.1.1. Preparation of 1,3-diarylpropenones (6a–h)

4.1.1.1. General synthesis of compounds 6b, d, e, g. To a solution of 0.10 mol of ketone **4** and 0.10 mol of aldehyde **5**, in a minimum amount of ethanol, 5 mL NaOH (40% w/v) was added dropwise. The mixture was stirred for 2 h, then the residue was filtered and washed with cold EtOH, H_2O and cold EtOH again.

4.1.1.2. 3-(3-Methoxyphenyl)-1-phenylpropenone (6b). The product was purified by chromatography using CH_2Cl_2 as eluant. Yield: 67%; mp 56–57 °C (lit. 59–61 °C[28]). ^{1}H NMR (DMSO-d_6) δ 3.82 (s, 3H), δ 7.03 (d, 1H), δ 7.34–7.75 (m, 7H) δ 7.95 (d, J = 15.7 Hz, 1H) δ 8.16 (d, 2H).

4.1.1.3. 3-(3-Chlorophenyl)-1-phenylpropenone (6d). The product was used without further purification in the next reaction. Yield: 89%; mp 74–75 °C (lit. 73–74 °C[28]). ^{1}H NMR ($CDCl_3$) δ 7.32–7.40 (m, 2H) δ 7.48–7.63 (m, 6H) δ 7.73 (d, J = 15.6 Hz, 1H) δ 8.01 (d, 2H).

4.1.1.4. 1-(4-Nitrophenyl)-3-phenylpropenone (6e). The product was purified by crystallization from ethanol. Yield: 82%; mp 115–116 °C (lit. 146–148 °C[29]). ^{1}H NMR (DMSO-d_6) δ 7.47–7.49 (m, 3H) δ 7.77–7.98 (m, 4H) δ 8.33–8.40 (m, 4H).

4.1.1.5. 3-(1*H*-Indol-5-yl)-1-phenylpropenone (6g). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant. Yield: 80%; mp 168–170 °C. ^{1}H NMR (DMSO-d_6) δ 6.52 (br s, 1H) δ 7.42 (t, 1H) δ 7.46 (d, 1H) δ 7.57 (t, 2H) δ 7.63–7.71 (m, 2H) δ 7.82 (d, J = 15.6 Hz, 1H) δ 7.89 (d, J = 15.0 Hz, 1H) δ 8.06 (s, 1H) δ 8.15 (d, 2H) δ 11.36 (s, 1H).

4.1.1.6. Synthesis of 3-(1*H*-indol-3-yl)-1-phenylpropenone (6h). A mixture of 0.15 mol of ketone **5**, 0.10 mol of aldehyde **6** and 10 g Al_2O_3 [activated, acid (Brockman I)] was irradiated in the microwave reactor (65 W, 65 °C, 4 × 10 min). The mixture was then extracted with CH_3OH and the solvent was evaporated under reduced pressure. The product was purified by chromatography using CH_2Cl_2/CH_3OH (10:1) as eluant. Yield: 76%; mp 168–169 °C (lit. 166–167 °C[30]). ^{1}H NMR (DMSO-d_6) δ 7.20–7.27 (m, 2H) δ 7.48–7.67 (m, 5H) δ 8.03–8.12 (m, 5H) δ 11.91 (s, 1H).

4.1.2. Preparation of 3,5-diaryl-1-methyl-4,5-dihydro-1H-pyrazoles (7a–h)

4.1.2.1. General synthesis of compounds 7a–d, h. A solution of 10.0 mmol of 1,3-diarylpropenones **6a**–**d**, **h**, 37.5 mmol (5.4 g) of methylhydrazine sulfate and 94.5 mmol (5.3 g) of KOH in 100 mL of EtOH was refluxed for 3 h under a nitrogen atmosphere. The solvent was then evaporated under reduced pressure and the residue was diluted with H_2O and extracted with Et_2O. The organic phases were combined, dried over Na_2SO_4 and evaporated under reduced pressure.

4.1.2.2. 3-(4-Methoxyphenyl)-1-methyl-5-phenyl-4,5-dihydro-1*H*-pyrazole (7a). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining an air-sensitive solid. Yield: 69%. ^{1}H NMR ($CDCl_3$) δ 2.81 (s, 3H) δ 2.92–3.16 (m, 1H) δ 3.43–3.60 (m, 1H) δ 3.83 (s, 3H) δ 4.03–4.29 (m, 1H) δ 6.90 (d, J = 8.7 Hz, 2H) δ 7.32–7.48 (m, 5H) δ 7.61 (d, J = 8.7 Hz, 2H).

4.1.2.3. 5-(3-Methoxyphenyl)-1-methyl-3-phenyl-4,5-dihydro-1*H*-pyrazole (7b). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining an oil. Yield: 61%. ^{1}H NMR ($CDCl_3$) δ 2.84 (s, 3H) δ 3.01–3.11 (m, 1H) δ 3.52 (dd, 1H) δ 3.82 (s, 3H) δ 4.10–4.32 (m, 1H) δ 6.88 (d, 1H) δ 7.00–7.04 (m, 2H) δ 7.29–7.41 (m, 4H) δ 7.67 (d, 2H).

4.1.2.4. 3-(4-Chlorophenyl)-1-methyl-5-phenyl-4,5-dihydro-1*H*-pyrazole (7c). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining an oil. Yield: 69%. ^{1}H NMR ($CDCl_3$) δ 2.86 (s, 3H) δ 3.00 (dd, J = 16.1, 14.4 Hz, 1H) δ 3.47 (dd, J = 16.1, 10.0 Hz, 1H) δ 4.18 (dd, J = 14.3, 10.1 Hz, 1H) δ 7.33–7.45 (m, 5H) δ 7.49 (d, 2H) δ 7.60 (d, 2H).

4.1.2.5. 5-(3-Chlorophenyl)-1-methyl-3-phenyl-4,5-dihydro-1*H*-pyrazole (7d). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining an oil. Yield: 67%. ^{1}H NMR ($CDCl_3$) δ 2.86 (s, 3H) δ 3.02 (dd, J = 16.1, 14.1 Hz, 1H) δ 3.54 (dd, J = 16.2, 10.0 Hz, 1H) δ 4.17 (dd, J = 13.9, 10.5 Hz, 1H) δ 7.32–7.43 (m, 6H) δ 7.51 (s, 1H) δ 7.67 (dd, 2H).

4.1.2.6. 3-(2-Methyl-5-phenyl-3,4-dihydro-2*H*-pyrazol-3-yl)-1*H*-indole (7h). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining an oil. Yield: 50%. ^{1}H NMR ($CDCl_3$) δ 2.90 (s, 3H) δ 3.25 (dd, J = 13.5, 16.2 Hz, 1H) δ 3.53 (dd, J = 10.2, 16.2 Hz, 1H) δ 4.52–4.58 (m, 1H) δ 7.10–7.23 (m, 2H) δ 7.30–7.43 (m, 5H) δ 7.70 (d, 3H) δ 8.24 (s, 1H).

4.1.2.7. General synthesis of compounds 7e–g. A solution of 10.0 mmol of the 1,3-diarylpropenones **6e**–**g** and 37.5 mmol (1.73 g) of methylhydrazine in 100 mL of ethanol was refluxed for 3 h under nitrogen. The solvent was then evaporated under reduced pressure.

4.1.2.8. 1-Methyl-3-(4-nitrophenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazole (7e). The product was purified by crystallization from ethanol. Yield: 56%; mp 115–116 °C. ^{1}H NMR (DMSO-d_6) δ 2.79 (s, 3H) δ 2.97 (dd, J = 14.4, 16.5 Hz, 1H) δ 3.63 (dd, J = 10.5, 16.5 Hz, 1H) δ 4.35 (dd, J = 10.5, 14.1 Hz, 1H) δ 7.31–7.48 (m, 5H) δ 7.83 (d, J = 8.7 Hz, 2H) δ 8.23 (d, J = 8.9 Hz, 2H).

4.1.2.9. 1-Methyl-5-(3-nitrophenyl)-3-phenyl-4,5-dihydro-1*H*-pyrazole (7f). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining an oil. Yield: 73%. ^{1}H NMR ($CDCl_3$) δ 2.88 (s, 3H) δ 3.02 (dd, J = 16.1, 14.1 Hz, 1H) δ 3.61 (dd, J = 16.2, 10.2 Hz, 1H) δ 4.29 (dd, J = 14.0, 10.2 Hz, 1H) δ 7.40 (d, 3H) δ 7.60 (t, J = 7.9 Hz, 1H) δ 7.67 (dd, J = 8.0, 1.9 Hz, 2H) δ 7.88 (d, J = 7.8 Hz, 1H) δ 8.22 (d, J = 8.18 Hz, 1H) 8.37 (s, 1H).

4.1.2.10. 5-(2-Methyl-5-phenyl-3,4-dihydro-2*H*-pyrazol-3-yl)-1*H*-indole (7g). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining a air-sensitive solid. Yield: 50%. ^{1}H NMR (DMSO-d_6) δ 2.70 (s, 3H) δ 2.89–299 (m, 1H) δ 3.53 (dd, 1H) δ 4.18 (dd, 1H) δ 6.41 (br s, 1H) δ 7.21 (dd, 1H) δ 7.34–7.41 (m, 5H) δ 7.60–7.66 (m, 3H) δ 11.11 (s, 1H).

4.1.3. Preparation of 3,5-diaryl-1-methylpyrazoles

4.1.3.1. General synthesis of compounds 2a–f. A solution of 10.0 mmol of 3,5-diaryl-1-methyl-4,5-dihydro-1*H*-pyrazole (**7a**–**f**) and 0.5 g 10% Pd/C in 45 mL of CH_3COOH was heated under stirring for 6 h. The mixture was then filtered on Hyflo Super Cel® medium, carefully basified with an aqueous solution of NaOH and extracted with EtOAc. The organic phases were collected and evaporated under reduced pressure.

4.1.3.2. 3-(4-Methoxyphenyl)-1-methyl-5-phenyl-1*H*-pyrazole (2a). The product was purified by chromatography using CH_2Cl_2/EtOAc (10:1) as eluant. Yield: 35%; mp 90–91 °C (lit. 92–93 °C[31]). ^{1}H NMR (DMSO-d_6) δ 3.78 (s, 3H) δ 3.87 (s, 3H) δ 6.79 (s, 1H) δ 6.79 (d, J = 9.0 Hz, 2H) δ 7.43–7.60 (m, 5H) δ 7.75 (d, J = 8.7 Hz, 2H). MS (ES$^+$) 265 [M+1]$^+$. Anal. Calcd for $C_{17}H_{16}N_2O$ (264.32). C, 77.24; H, 6.10; N, 10.60. Found: C, 77.19; H, 6.04; N, 10.30.

4.1.3.3. 5-(3-Methoxyphenyl)-1-methyl-3-phenyl-1*H*-pyrazole (2b). The product was purified by chromatography using CH_2Cl_2/EtOAc (10:1) as eluant obtaining an oil. Yield: 52%. ^{1}H NMR (DMSO-d_6) δ 3.83 (s, 3H) δ 3.90 (s, 3H) δ 6.90 (s, 1H) δ 7.03 (d, 1H) δ 7.12–7.17 (m, 2H) δ 7.30 (t, 1H) δ 7.38–7.46, (m, 3H) δ 7.83, (d, 2H). MS (ES$^+$) 265 [M+1]$^+$. Anal. Calcd for $C_{17}H_{16}N_2O$ (264.32). C, 77.24; H, 6.10; N, 10.60. Found: C, 76.97; H, 6.01; N, 10.55.

4.1.3.4. 3-(4-Chlorophenyl)-1-methyl-5-phenyl-1*H*-pyrazole (2c). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant. Yield: 56%; mp 127–129 °C (lit. 127–128 °C[31]). ^{1}H NMR (DMSO-d_6) δ 3.89 (s, 3H) δ 6.92 (s, 1H) δ 7.45–7.60 (m, 7H) δ 7.85 (d, 2H). MS (ES$^+$) 269 [M+1]$^+$. Anal. Calcd for $C_{16}H_{13}ClN_2$ (268.74). C, 71.51; H, 4.87; N, 10.42. Found: C, 71.34; H, 4.76; N, 10.27.

4.1.3.5. 5-(3-Chlorophenyl)-1-methyl-3-phenyl-1*H*-pyrazole (2d). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant obtaining an oil. Yield: 55%. ^{1}H NMR (DMSO-d_6) δ 3.91 (s, 3H) δ 6.98 (s, 1H) δ 7.32 (d, 1H) δ 7.39–7.44 (m, 2H) δ 7.53–7.59 (m, 3H) δ 7.69 (s, 1H) δ 7.83 (d, 2H). MS (ES$^+$) 269 [M+1]$^+$. Anal. Calcd for $C_{16}H_{13}ClN_2$ (268.74). C, 71.51; H, 4.87; N, 10.42. Found: C, 71.80; H, 4.98; N, 9.98.

4.1.3.6. 1-Methyl-3-(4-nitrophenyl)-5-phenyl-1*H*-pyrazole (2e). The product was purified by chromatography using CH_2Cl_2/EtOAc (40:1) as eluant. Yield: 43%; mp 138–139 °C (lit. 141–142 °C[31]). ^{1}H NMR (DMSO-d_6) δ 3.94 (s, 3H) δ 7.12 (s, 1H) δ 7.46–7.63 (m, 5H) δ 8.10 (d, J = 8.1 Hz, 2H) δ 8.28 (d, J = 8.7 Hz, 2H). MS (ES$^+$) 280 [M+1]$^+$. Anal. Calcd for $C_{16}H_{13}N_3O_2$ (279.29). C, 68.80; H, 4.69; N, 15.05. Found: C, 68.63; H, 4.59; N, 15.03.

4.1.3.7. 1-Methyl-5-(3-nitrophenyl)-3-phenyl-1*H*-pyrazole (2f). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant. Yield: 33%; mp 125–127 °C (lit. 98–95 °C[32]). ^{1}H NMR (DMSO-d_6) δ 3.96 (s, 3H) δ 7.10 (s, 1H) δ 7.32 (t, 1H) δ 7.43 (t, 2H) δ 7.80–7.87 (m, 3H) δ 8.09 (d, 1H) δ 8.31 (d, 1H) δ 8.40 (s, 1H). MS (ES$^+$) 280 [M+1]$^+$. Anal. Calcd for $C_{16}H_{13}N_3O_2$ (279.29). C, 68.80; H, 4.69; N, 15.05. Found: C, 68.54; H, 4.68; N, 14.80.

4.1.3.8. General synthesis of compounds 2g, h. A solution of 1.0 mmol of 3,5-diaryl-1-methyl-4,5-dihydro-1*H*-pyrazole (**7g**, **h**) and 13.0 mmol (1.13 g) of MnO_2 in 45 mL of toluene was refluxed for 2 h. The mixture was then filtered on Hyflo Super Cel® medium and the solvent was evaporated under reduced pressure.

4.1.3.9. 5-(2-Methyl-5-phenyl-2*H*-pyrazol-3-yl)-1*H*-indole (2g). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant. Yield: 36%; mp 161–162 °C. ^{1}H NMR (DMSO-d_6) δ 3.89 (s, 3H) δ 6.52 (br s, 1H) δ 6.81 (s, 1H) δ 7.25–7.32 (m, 2H) δ 7.38–7.46 (m, 3H) δ 7.52 (d, 1H) δ 7.74 (s, 1H) δ 7.84 (d, 2H) δ 11.31 (s, 1H). HR-MS *m*/*z* 274.13363 $[M+1]^+$. Calcd mass for $C_{18}H_{16}N_3$ 273.13387 (deviation 0.9 ppm).

4.1.3.10. 3-(2-Methyl-5-phenyl-2*H*-pyrazol-3-yl)-1*H*-indole (2h). The product was purified by chromatography using CH_2Cl_2/EtOAc (10:1) as eluant and then crystallized from ethanol/water. Yield: 41%; mp 206–207 °C. ^{1}H NMR (DMSO-d_6) δ 3.93 (s, 3H) δ 6.91 (s, 1H) δ 7.10–7.23 (m, 2H) δ 7.29 (t, 1H) δ 7.41 (t, 2H) δ 7.49 (d, 1H) δ 7.69–7.73 (m, 2H) δ 7.88 (d, 2H) δ 11.58 (s, 1H). MS (ES^+) 274 $[M+1]^+$. Anal. Calcd for $C_{18}H_{15}N_3$ (273.33). C, 79.10; H, 5.53; N, 15.37. Found: C, 78.97; H, 5.36; N, 15.26.

4.1.4. Preparation of 3,5-diarylpyrazoles (3a–f) and 1-(3,5-diaryl)-4,5-dihydro-pyrazol-1-yl-ethanones (8b, d, e)

4.1.4.1. General synthesis of compounds 3a, c, f. A solution of 10.0 mmol of 1,3-diaryl-propenone (**6a**, **c**, **f**) and 20.0 mmol (1.0 g) of hydrazine hydrate in 100 mL of ethanol was stirred for 48 h at room temperature. The solvent was then evaporated under reduced pressure.

4.1.4.2. 3-(4-Methoxyphenyl)-5-phenyl-1*H*-pyrazole (3a). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant and then crystallized from CH_2Cl_2/petroleum ether. Yield: 8%; mp 158–159 °C (lit. 159–160 °C[33]). ^{1}H NMR (DMSO-d_6) δ 3.79 (s, 3H) δ 6.96–7.07 (m, 2H) δ 7.08 (s, 1H) δ 7.26–7.52 (m, 3H) δ 7.72–7.92 (m, 4H) δ 13.20 (s, 1H). MS (ES^+) 251 $[M+1]^+$. Anal. Calcd for $C_{16}H_{14}N_2O$ (250.29). C, 76.78; H, 5.64; N, 11.19. Found: C, 76.53; H, 5.50; N, 11.08.

4.1.4.3. 3-(4-Chlorophenyl)-5-phenyl-1*H*-pyrazole (3c). The product was purified by chromatography using CH_2Cl_2/EtOAc (20:1) as eluant and then crystallized from CH_2Cl_2/petroleum ether. Yield: 12%; mp 216–217 °C (lit. 216–217 °C[34]). ^{1}H NMR (DMSO-d_6) δ 7.23 (s, 1H) δ 7.31–7.57 (m, 5H) δ 7.79–7.90 (m, 4H) δ 13.44 (s, 1H). MS (ES^+) 255 $[M+1]^+$. Anal. Calcd for $C_{15}H_{11}ClN_2$ (254.71). C, 70.73; H, 4.35; N, 11.00. Found: C, 70.60; H, 4.27; N, 11.10.

4.1.4.4. 3-3-Nitrophenyl)-5-phenyl-1*H*-pyrazole (3f). The product was purified by chromatography using CH_2Cl_2/EtOAc (5:1) as eluant. Yield: 9%; mp 198–199 °C (lit. 204–205 °C[35]) ^{1}H NMR (DMSO-d_6) δ 7.36–7.52 (m, 4H) δ 7.71–7.91 (m, 3H) δ 8.16–8.33 (m, 2H) δ 8.68 (d, 1H) δ 13.65 (d, 1H). MS (ES^+) 266 $[M+1]^+$. Anal. Calcd for $C_{15}H_{11}N_3O_2$ (265.27). C, 67.92; H, 4.18; N, 15.84. Found: C, 67.71; H, 4.11; N, 15.24.

4.1.4.5. General synthesis of compounds 3b, d, e; 8b, d, e. A solution of 10.0 mmol of 1,3-diarylpropenone (**6b**, **d**, **e**) and 20.0 mmol (1.0 g) of hydrazine hydrate in 150 mL of CH_3COOH was refluxed for 6 h. The mixture was carefully basified with an aqueous solution of NaOH and extracted with EtOAc. The organic phases were collected and evaporated under reduced pressure.

4.1.4.6. 5-(3-Methoxyphenyl)-3-phenyl-1*H*-pyrazole (3b). The product was purified by chromatography using CH_2Cl_2/EtOAc (5:1) as eluant. Yield: 20%; mp 100–102 °C. ^{1}H NMR (DMSO-d_6) δ 3.82 (s, 3H) δ 6.91 (s, 1H) δ 7.22 (s, 1H) δ 7.31–7.47 (m, 6H) δ 7.80–7.89 (m, 2H) δ 13.38 (s, 1H). MS (ES^+) 251 $[M+1]^+$. Anal. Calcd for $C_{16}H_{14}N_2O$ (250.29). C, 76.78; H, 5.64; N, 11.19. Found: C, 76.49; H, 5.52; N, 11.40.

4.1.4.7. 1-[5-(3-Methoxyphenyl)-3-phenyl-4,5-dihydro-pyrazol-1-yl]-ethanone (8b). The product was purified by chromatography using CH_2Cl_2/EtOAc (5:1) as eluant. Yield: 40%; mp 112–113 °C (lit. 115–117 °C[36]). ^{1}H NMR (DMSO-d_6) δ 2.32 (s, 3H) δ 3.12 (dd, J = 4.8, 18.0 Hz, 1H) δ 3.72 (s, 3H) δ 3.82 (dd, J = 12.0, 18.0 Hz, 1H) δ 5.52 (dd, J = 5.1, 12.0 Hz, 1H) δ 6.72–6.75 (m, 2H) δ 6.80–6.84 (m, 1H) δ 7.24 (t, 1H) δ 7.44–7.46 (m, 3H) δ 7.76–7.79 (m, 2H). MS (ES^+) 295 $[M+1]^+$. Anal. Calcd for $C_{18}H_{18}N_2O_2$ (294.35). C, 73.45; H, 6.16; N, 9.52. Found: C, 73.21; H, 6.14; N, 9.40.

4.1.4.8. 5-(3-Chlorophenyl)-3-phenyl-1*H*-pyrazole (3d). The product was purified by chromatography using CH_2Cl_2/EtOAc (10:1) as eluant. Yield: 12%; mp 177–178 °C (lit. 168–169 °C[34]). ^{1}H NMR (DMSO-d_6) δ 7.30–7.51 (m, 6H) δ 7.79–7.91 (m, 4H) δ 13.47 (d, 1H). MS (ES^+) 255 $[M+1]^+$. Anal. Calcd for $C_{15}H_{11}ClN_2$ (254.71). C, 70.73; H, 4.35; N, 11.00. Found: C, 70.45; H, 4.29; N, 11.26.

4.1.4.9. 1-[5-(3-Chlorophenyl)-3-phenyl-4,5-dihydropyrazol-1-yl]-ethanone (8d). The product was purified by chromatography using CH_2Cl_2/EtOAc (10:1) as eluant. Yield: 46%; mp 124–125 °C. ^{1}H NMR (DMSO-d_6) δ 2.31 (s, 3H) δ 3.18 (dd, J = 4.5, 18.3 Hz, 1H) δ 3.85 (dd, J = 12.3, 18.0 Hz, 1H) δ 5.55 (dd, J = 5.1, 12.3 Hz, 1H) δ 7.15 (d, 1H) δ 7.26 (s, 1H) δ 7.30–7.39 (m, 2H) δ 7.46–7.48 (m, 3H) δ 7.77–7.80 (m, 2H). MS (ES^+) 299 $[M+1]^+$. Anal. Calcd for $C_{17}H_{15}ClN_2O$ (298.77). C, 68.34; H, 5.06; N, 9.38. Found: C, 67.95; H, 4.98; N, 9.25.

4.1.4.10. 3-(4-Nitrophenyl)-5-phenyl-1*H*-pyrazole (3e). The product was purified by chromatography using CH_2Cl_2/CH_3OH (5:1) as eluant. Yield: 8%; mp 270 °C dec. (lit. 278–279 °C[37]). ^{1}H NMR (DMSO-d_6) δ 7.39–7.53 (m, 4H) δ 7.81–7.90 (m, 2H) δ 8.07–8.16 (m, 2H) δ 8.30–8.38 (m, 2H) δ 13.73 (s, 1H). HR-MS *m*/*z* 266.09259 $[M+1]^+$. Calcd mass for $C_{15}H_{12}N_3O_2$ 266.09240 (deviation 0.7 ppm).

4.1.4.11. 1-[3-(4-Nitrophenyl)-5-phenyl-4,5-dihydro-pyrazol-1-yl]-ethanone (8e). The product was purified by chromatography using CH_2Cl_2/CH_3OH (5:1) as eluant. Yield: 44%; mp 194–195 °C. ^1H NMR (DMSO-d_6) δ 2.34 (s, 3H) δ 3.21 (dd, J = 5.4, 18.6 Hz, 1H) δ 3.93 (dd, J = 11.4, 17.4 Hz, 1H) δ 5.60 (dd, J = 4.8, 12.0 Hz, 1H) δ 7.19–7.36 (m, 5H) δ 8.03 (d, J = 9.6 Hz, 2H) δ 8.30 (d, J = 9.0 Hz, 2H). HR-MS m/z 310.11900 $[M+1]^+$. Calcd mass for $C_{17}H_{16}N_3O_3$ 310.11862 (deviation 1.2 ppm).

4.2. Biology

4.2.1. Primary neuronal cultures. Pure neuronal and mixed primary cortical cultures were prepared from E16 embryos Sprague–Dawley rat neocortex by mechanical dissociation[38] and were grown in Neurobasal medium supplemented with B27 (Invitrogen—pure cortical neurons), or with Neurobasal medium supplemented with 5% of FBS (Fetal Bovine Serum—Hyclone-mixed cortical cultures). Cultures were maintained in a humidified incubator with an atmosphere of 95% air/ 5% CO_2 at 37 °C and the culture medium was replaced every 3–4 days. All primary neuronal cultures were grown in 24-well plates (500 μL medium/ well) and used after 12 days in vitro.

4.2.2. In vitro neuroprotection assay. Two different in vitro models of excitotoxicity, *N*-methyl-D-aspartate and Veratridine, were implemented in mixed and pure cortical neurons, respectively. Primary neuronal cultures from rodents challenged with different neurotoxic agents (e.g., NMDA or other excitotoxins, Aβpeptide) represent well-validated and characterized in vitro models of neurodegeneration.[39–42] They were therefore employed to analyze the capacity of compounds to protect neurons from neuronal cell death induced by excitotoxicity mediated by overactivation of NMDA receptors or from activation of Sodium Channels. *N*-Methyl-D-aspartate (NMDA) (Sigma) was added to culture medium in presence or absence of 10 μM dizolcipine maleate (MK-801) (Sigma). MK801 is a non-selective NMDA channel blocker, and represents the positive control for activity in the neuroprotection assay as it can reverse the neuronal cell death induced by NMDA. Experimental compounds were added to culture medium 24 h (pretreatment) or 20 min (co-treatment) before addition of either NMDA or Veratridine. In both experimental paradigms, neuronal viability was assessed 24 h after the excitotoxic insult. Neuronal cell death was determined according to manufacturer's instructions using a Cytotoxicity Detection Kit (LDH) (Roche Applied Science, IN, USA) or a Cell Proliferation kit II (XTT) (Roche Applied Science, IN, USA) in mixed cortical cultures or pure neuronal cultures, respectively. The XTT assay measures mitochondrial activity, which is inversely proportional to cell death. The LDH assay measures the presence in the medium of an intracellular enzyme (lactate dehydrogenase) whose release outside the cell is inversely proportional to cell membrane integrity, and is therefore directly proportional to neuronal cell death. This assay is typically preferred in mixed neuronal cultures (comprising both a neuronal and a glial component).[43,44] Data have been background-subtracted (naïve cells) and expressed as percentages of LDH or XTT release by NMDA or Veratridine (respectively) for each independent experiment and have been analyzed with a blocked ANOVA model (each experiment was at least replicated twice) followed by Tukey's honestly significant difference criterion for identifying compounds significantly different from NMDA (or Veratridine) toxicity ($p < 0.05$).

4.2.3. RT-PCR. Total RNA was extracted as described previously.[45] Total RNA was subjected to DnaseI treatment (Roche, Hertforshire, UK) and 2 μg of total RNA/ sample was used for cDNA synthesis using Superscript II (Invitrogen, San Diego, CA) and an oligo(dT) primer. Each reverse transcriptase (RT) product was diluted to 100 μL with sterile, distilled water, and 1 μL of cDNA was used in the subsequent PCR amplification. Real-time PCR was performed using an I-Cycler and the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Primer sequences were designed relevant (neuronal expressed) to rat NaV coding sequences and to the rat β-actin gene. Primer sequences were as follows: Scn1a-FOR 5′-GAG GAACAGCCTGTCATGGAA-3′; Scn1a-REV 5′-CTG CACACAGCCTTCAGTGAA-3′; Scn2a1-FOR 5′-TC TTGCTGATGCCGCTGTAT-3′; Scn2a1-REV 5′-CG ACGTATGGCTTCCCTGTT-3′; Scn3a-FOR 5′-CCC GGGACCTGAGAGCTT-3′; Scn3a-REV 5′-GCAGC ACGCTTTTCGATAGC-3′; Scn8a1-FOR 5′-TGCA CCGTTCACTGTATTTGG-3′; Scn8a1-REV 5′-AGG TGTACGAAAGAATCCTACACTGA-3′; β-actin-FOR 5′-CCCTGGCTCCTAGCACCAT-3′; β-actin-REV 5′-GAGCCACCAATCCACACAGA-3′. Reaction conditions were as follows: 95 °C/5′; 35 × (95 °C/30″; 55 °C/30″). Data were analyzed with the I-Cycler Optical System software version 3.0a. Results are represented as mean normalized expression levels ± SEM.

4.2.4. In vitro pharmacology. AMPA, Kainate and Site 2 Sodium Channel binding assays were performed by Cerep-France (www.cerep.com).

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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.2007.10.090.

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