Synthesis and Pharmacological Evaluation of Phenylacetamides as Sodium-Channel Blockers†

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The synthesis and structure–activity relationships of a series of phenylacetamides related to *N*-[3-(2,6-dimethyl-1-piperidinyl)propyl]-α-phenylbenzeneacetamide (1) (PD85639) acting at the voltage-dependent Na^+ channel are described. All structural variations for this study were made in the phenylacetic acid portion of these molecules, and the compounds were synthesized by coupling the appropriately substituted phenylacetic acid derivative with 3-[1-(2,6-dimethyl)piperidinyl]-propanamine using standard methods of amide formation. Compounds were tested as inhibitors of [^{3}H]batrachtoxinin binding in rat neocortical membranes and also as inhibitors of veratridine-induced Na^+ influx in Chinese hamster ovary cells expressing type IIA Na^+ channels. Diphenylacetic acid derivatives with halogenated aromatic rings (**12-15**) were very potent in both assays, while alkoxy and alkyl substitution did not affect activity (**16** and **17**). Selected compounds were tested as potential neuroprotective agents in two cell culture assays involving inhibition of veratridine-induced and hypoxia-induced lactate dehydrogenase release. Compound **15** was equipotent with flunarizine, a reference compound in both neuroprotection assays.

Introduction

Voltage-dependent sodium channels have been proposed as the site of action for Class IA, B, and C antiarrhythmic drugs,[1] anticonvulsant drugs,[2] and local anesthetics.[3,4] While there is some degree of overlap among members of these three classes of drugs (i.e., lidocaine and phenytoin exhibit antiarrhythmic activity in addition to their function as a local anesthetic and an anticonvulsant, respectively), these drugs are assumed to act on different sites and/or different conformational states of the sodium channel.[5]

Recent advances in electrophysiological methods and molecular biology techniques and the availability of specific neurotoxins have contributed to a better understanding of voltage-sensitive sodium channels.[6] Sodium channels are large glycoproteins which exist as heterotrimers (containing a 260-kDA α subunit, a 36-kDA β_1 subunit, and a 33-kDA β_2 subunit) in neuronal cells, while in cardiac and skeletal muscle cells, they exist as dimers (containing the 260-kDA α subunit and a 38-kDA β subunit). The sodium channels in cardiac and skeletal muscle cells have different electrophysiological and pharmacological properties than those in the neurons. In some species, the sodium channels consist of a single α subunit (e.g., the eel electroplax). Many different subtypes of sodium channels have been cloned, and in the case of the rat brain, four distinct channels have been found.[7] These are designated as types I, II, IIA, and III. Type I sodium channels of neuronal cells are found primarily in cell bodies and may be a site of action for anticonvulsant drugs, while type II sodium channels are found in the axons of the neuronal cells and are presumed to be the site of action for the local anesthetics.

The sodium channel shifts between three distinct conformational states: active, resting, and inactivated. Each state is capable of conducting electrical signals and allowing sodium-ion permeability to a different extent or not at all.[5] In general, after the neuronal cell membrane is depolarized, the permeability of the membrane to sodium ions increases, with sodium ions rushing through into the cell, and shortly after that in a rate- and voltage-dependent manner, the permeability to sodium returns to its original level. Some neurotoxins bind specifically to sodium channels, causing either blocking or opening of the channel. These toxins can distinguish not only different types of channels but also different states of the channels by either inducing or preventing them from adopting a specific conformational state. The sites of their action[8-12] are defined as follows: neurotoxin receptor site 1, where water-soluble tetrodotoxin and saxitoxin act by blocking sodium permeation; neurotoxin receptor site 2, where lipid-soluble toxins like veratridine, aconitine, and batrachotoxin act causing prolonged activation; neurotoxin receptor site 3, where α-scorpion toxin and sea anemone neumatocytes bind inhibiting inactivation; neurotoxin receptor site 4, where β-scorpion toxins act by shifting activation; and finally, neurotoxin receptor site 5, where ciguatoxins act to prolong activation.

The site of action of the antiarrhythmic, anticonvulsant, and local anesthetic drugs is believed to be the intracellular side of the sodium channel. They allosterically inhibit interaction with neurotoxin receptor site 2, in a frequency- and voltage-dependent manner.[10] Selective high-affinity binding of these inhibitors to the inactivated state of the sodium channel is believed to be the property that makes them specific for blocking abnormally firing sodium channels without inhibiting normal cardiac and neuronal sodium-channel functions.[5]

The α subunit of the sodium channel is sufficient to form an active, voltage-gated ion channel.[13-19] When it is cloned and expressed in Chinese hamster ovary (CHO) cells, it behaves as a fully functional channel, having the pharmacological and electrophysiological properties of native sodium channels.[15] *N*-[3-(2,6-dimethyl-1-piperid-

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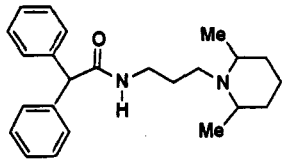
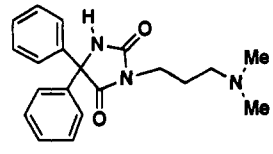
inyl)propyl]-α-phenylbenzeneacetamide (PD85639; 1) was shown to act as a local anesthetic with high selectivity for the type IIA sodium channels.[16]



1 (PD85639)

In this paper, the synthesis and the biochemical characterization of a number of derivatives of 1 are reported. Compounds 2–4, which are restricted analogs of 1, were synthesized and tested as sodium-channel blockers. In order to rapidly identify those analogs interacting with voltage-sensitive sodium channels, they were initially screened for their ability to inhibit [^{3}H]-batrachtoxinin ([^{3}H]BTX) binding to rat neocortical membranes. Further, since it was possible that these agents would preferentially inhibit sodium channels localized to axons, the compounds active in the [^{3}H]BTX binding assay were further tested for inhibition of veratridine-stimulated Na^+ flux (NaFl) into CHO cells expressing type IIA sodium channels. In addition, selected compounds were tested as potential neuroprotective agents in cell cultures using inhibition of veratridine- and hypoxia-induced lactate dehydrogenase (LDH) release as assays for measuring neuroprotective activity.



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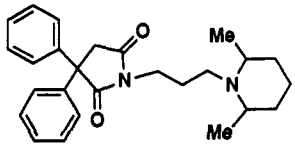

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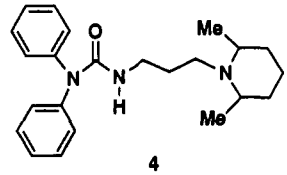

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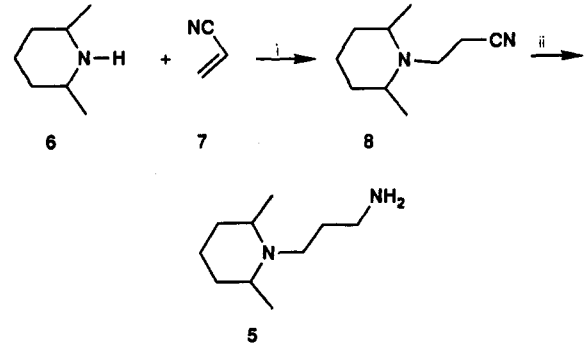
Chemistry

The structural modifications of 1 primarily involved alterations in the diphenylacetic acid portion of the molecule. (2,6-Dimethyl-1-piperidino)propanamine (5) was a key intermediate in the preparation of all derivatives of this study. It was prepared (Scheme 1) by treatment of acrylonitrile 7 with *cis*-2,6-dimethylpiperidine (6) in a Michael-type reaction to afford the addition product 8 in 79% yield. Nitrile 8 was catalytically reduced to produce the desired primary amine 5 in 85% yield.

The targeted amides (Table 1) were prepared utilizing coupling methodology that was developed for peptide synthesis (Scheme 2). For example, procedure A involved the treatment of carboxylic acids with 1,1′-carbonyldiimidazole (CDI) in dimethylformamide followed by the addition of amine 5. This method was used to prepare compounds 9, 14–16, 18, 19, and 21. Procedure B was used in the preparation of amides 13, 17, and 20 by coupling the amine and the appropriate acid with 1,3-dicyclohexylcarbodiimide (DCC) in the presence of 3-hydroxybenzotriazole in dichloromethane. Amides 4 and 10–12 were prepared by reacting the carboxylic acid chlorides (prepared by treating the carboxylic acids with thionyl chloride or with oxalyl chloride and dimethylformamide) with amine 5 (procedure C).

Most carboxylic acids were commercially available or prepared by methodology previously described in the literature. The precursor carboxylic acid for the synthesis of amide 15 was prepared as shown in Scheme 3. The glyoxylic acid 22 was converted to the acetate, and the carboxylic acid was esterified with methanolic hydrochloric acid to give 23. Deoxygenation was achieved with samarium iodide,[20] and the ester was subsequently hydrolyzed to the acid 24, which was converted to amide 15 according to procedure A.

Scheme 1[a]



[a] (i) $HCONH_2$, H_2O; (ii) H_2, Raney Ni, $MeOH/NH_3$.

Biological Testing

Inhibition of [^{3}H]BTX binding to sodium channels in rat brain preparations has been used to detect compounds with affinity for sodium channels. Previous studies have shown that local anesthetics (like 1) markedly inhibit [^{3}H]-BTX binding in neuronal membrane preparations and that this measure was a useful index for comparing potencies within a given chemical series.[21,22] In this study, analogs of 1 were tested to inhibit [^{3}H]BTX binding in rat neocortical membranes.

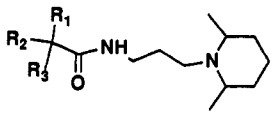
The ability of the compounds to block Na^+ influx in CHO cells expressing the α subunit of rat brain type IIA sodium channels was determined on a CNaIIA-1 cell line[17–19] as follows. After application of the test compounds to the CHO cells, the cells were treated with veratridine in the presence of α-scorpion toxin, which resulted in opening of the sodium channels. Inhibition of the [^{14}C]-guanidinium influx was used to measure sodium-channel blockade,[23,24] and the IC_{50} values were determined.

Selected compounds were tested in two assays for neuroprotection, based on their ability to inhibit lactate dehydrogenase release from neuronal cells. LDH is a cytosolic enzyme released from cells following breakdown in plasma membrane integrity. Prolonged opening of veratridine-sensitive Na^+ channels is neurotoxic in rat brain neuronal cultures, and release of LDH has been used as a biochemical marker for measuring cell death.[25–28] In the hypoxic-mediated cell-injury assay, the ability of compounds to protect against neuronal damage caused by hypoxic exposure is determined similarly by measuring LDH release.[29]

Biological Results and Discussion

Tetrodotoxin is known to block neurotoxin receptor site 1 of the Na^+ channel and has an IC_{50} of 14 nM in the NaFl

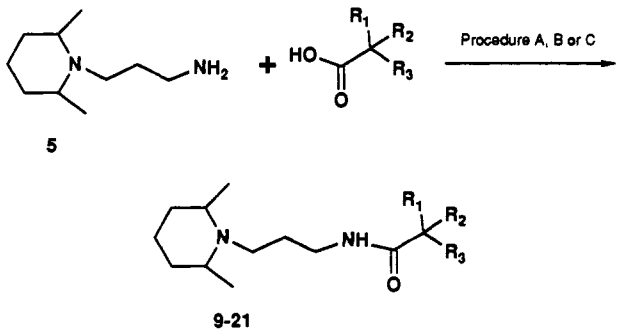
Table 1. Physical Data for the PD85639 Analogs



compd	R_1	R_2	R_3	mp, °C	formula	method
1	H	Ph	Ph	152	$C_{24}H_{32}N_2O$	A
9	CH_3	Ph	Ph	177	$C_{25}H_{34}N_2O$[a]	A
10	H	9-fluorenyl	9-fluorenyl	197	$C_{24}H_{30}N_2O$[b]	C
11	H	9-xanthenyl	9-xanthenyl	192	$C_{24}H_{30}N_2O_2$[b]	C
12	H	Ph-4-Br	Ph-4-Br	171	$C_{24}H_{30}Br_2N_2O$[b]	C
13	H	Ph-4-Cl	Ph-4-Cl	156	$C_{24}H_{30}Cl_2N_2O$	B
14	H	Ph-4-F	Ph-4-F	129	$C_{24}H_{30}F_2N_2O$	A
15	H	Ph-2-Cl	PH-2-Cl	162	$C_{24}H_{30}Cl_2N_2O$	A
16	H	Ph-4-OMe	Ph-4-OMe	118	$C_{26}H_{36}N_2O_3$	A
17	H	Ph-4-Me	Ph-4-Me	149	$C_{26}H_{36}N_2O$	B
18	H	1-naphthyl-4-OMe	1-naphthyl-4-OMe	219	$C_{34}H_{40}N_2O_3$	A
19	H	Ph	H	83	$C_{18}H_{22}N_2O$	B
20	H	1-naphthyl	H	131	$C_{22}H_{30}N_2O$	A
21	Ph	cyclopentyl	H	116	$C_{23}H_{36}N_2O$	A
22	Ph	$(CH_2)_5$	$(CH_2)_5$	94	$C_{23}H_{36}N_2O$	B

[a] Oxalate salt. [b] Hydrochloride salt.

Scheme 2[a]



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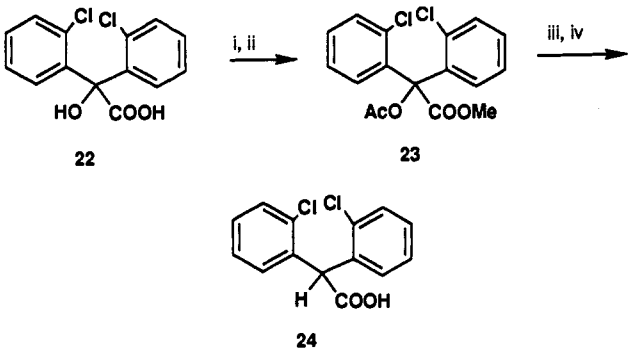
9-21

[a] Procedure A: 1-hydroxybenzotriazole, DCC, CH_2Cl_2, 0 °C. Procedure B: CDI, DMF, 45 °C to room temperature. Procedure C: oxalyl chloride/DMF.

Scheme 3[a]



22 23 24

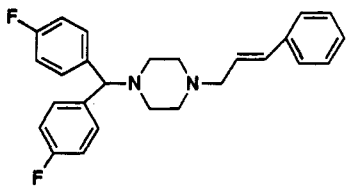
[a] (i) Ac_2O, Et_3N, DMAP; (ii) Me_2SO_4, K_2CO_3; (iii) SmI_2, MeOH, HMPA; (iv) NaOH/EtOH.

assay (Table 2). Flunarizine and lidoflazine are characterized principally as Ca^{2+} channel antagonists, but they also block Na^+ channels. They inhibit [^{3}H]BTX binding (K_i = 53 nM for flunarizine and 77 nM for lidoflazine), while in the NaFl assay, flunarizine (IC_{50} = 420 nM) was more potent than lidoflazine (IC_{50} = 2.4 μM). Compound 1 is also active (K_i = 260 nM) in the [^{3}H]BTX binding assay and demonstrates good activity in the NaFl assay (IC_{50} = 2.7 μM). For this series of compounds, activity in the [^{3}H]BTX binding assay and activity in NaFl are highly correlated (correlation coefficient r = 0.841), as can be seen in Figure 1. The hydantoin derivative 2,[30] which is structurally similar to the anticonvulsant drug phenytoin, had low affinity for the Na^+ channel in both assays

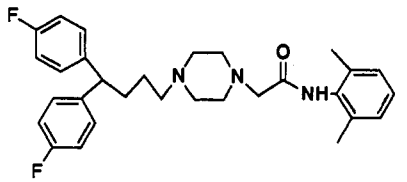
Table 2. Summary of Results for *in Vitro* Biological Assays[a]

			LDH release assays IC_{50}, μM	
compd	[^{3}H]BTX K_i, μM	NaFl IC_{50}, μM	veratridine-induced	hypoxia-induced[b]
tetrodotoxin	c	0.014		
flunarizine	0.053	0.42	2	7.3
lidoflazine	0.077	2.4		
1 (PD85639)	0.26	2.7	5	89
2	21	72		
3	1.0	3.6		
4	15	106	30 (25%)	>100
9	2.0	32		
10	2.3	29	30 (37%)	51
11	0.41	13		
12	0.064	5.7		
13	0.11	4.5		
14	0.13	5.8		
15	0.073	3.5	6	4.8
16	1.0	3.1		
17	0.48	7.5		
18	0.24	13		4.4
19	21	>100	30 (8%)	>100
20	6.9	>100		
21	0.19	12		
22	0.38	4.3	12	61

[a] $n \geq 2$ unless indicated. Individual values were within ±15% of each other. [b] n = 1. [c] Tetrodotoxin is routinely used in this assay to block its binding site on the sodium channel.



Flunarizine



Lidoflazine

([^{3}H]BTX K_i = 21 μM; NaFl IC_{50} = 72 μM). Its succinimide analog 3, which is also structurally similar to

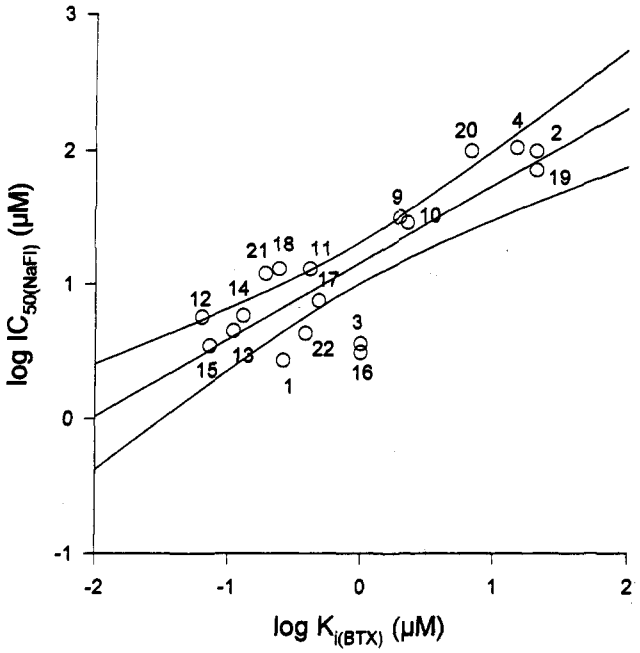


Figure 1. Correlation between inhibition of [^{3}H]BTX binding (log K_i) and inhibition of veratridine-induced Na^+ influx (log IC_{50}). r = 0.841 (p < 0.001). The results for tetrodotoxin, flunarizine, and lidoflazine are not included. The outer lines represent the confidence limits (±95%).

1, had moderate activity in the [^{3}H]BTX binding assay (K_i = 1.0 μM) and was almost equipotent with 1 in the NaFl assay (IC_{50} = 3.6 μM). When the methine carbon atom of the diphenyl system of 1 was replaced with a nitrogen atom to give urea 4, activity in both assays was reduced dramatically. Analog 9, where the hydrogen of the α carbon of the amide was replaced with a methyl group, had considerably lower affinity for the Na^+ channel in both the [^{3}H]BTX binding assay (K_i = 2.0 μM) and the NaFl assay (IC_{50} = 32 μM). Incorporation of the diphenyl system into the rigid tricyclic analogs 10 and 11 retained good activity in the [^{3}H]BTX binding assay (K_i = 2.3 and 0.41 μM, respectively) but had a more pronounced effect on the NaFl assay where activity was reduced (IC_{50} = 29 and 13 μM, respectively). The introduction of halogen substituents onto the phenyl rings of the diphenyl system (12–15) resulted in compounds that had very high activity in the [^{3}H]BTX binding assay, with compounds 12 (K_i = 0.064 μM) and 15 (K_i = 0.073 μM) having an activity equal to flunarizine. The activity of the halogenated derivatives in the NaFl assay was not affected, and 12–15 were approximately equipotent with 1. Alkyl and alkoxy substituents on the phenyl rings gave compounds 16 and 17 which were less active in the [^{3}H]BTX binding assay (K_i = 1 and 0.48 μM, respectively), but the activity of these derivatives in the NaFl assay was not greatly changed compared to that of 1. The bulkier dinaphthalene derivative 18 had a K_i = 0.24 μM in the [^{3}H]BTX binding assay but had lower activity for the Na^+ channel in the NaFl assay (IC_{50} = 13 μM). The monoarylacetic acid analogs 19 and 20 showed poor activity in both assays, demonstrating the need for a second phenyl group. While the α-cyclopentyl derivative 21 was equipotent with 1 in the [^{3}H]BTX binding assay (K_i = 0.19 μM), it retained only moderate activity in the NaFl assay (IC_{50} = 12.2 μM). The spiro analog 22 had very good activity in the [^{3}H]-BTX binding assay (K_i = 0.38 μM) as well as good activity in NaFl (IC_{50} = 4.3 μM).

Selected compounds were further tested on cultured fetal rat neocortical neurons to determine the ability of sodium-channel blockers to function as neuroprotective agents. When veratridine was used as the potential stimulus, good agreement was noted between inhibition of NaFl and neuroprotective activity, as seen in Table 2. Compounds 1, 15, and 22 and flunarizine all showed good activity in NaFl, while derivatives 4, 10, and 19 either were inactive or demonstrated only moderate activity. In the veratridine-induced LDH release assay, compounds 1 (IC_{50} = 4.6 μM), 15 (IC_{50} = 6 μM), and 22 (IC_{50} = 12 μM) had activities comparable with that of flunarizine (IC_{50} = 2 μM), while compounds 4, 10, and 19 were considerably less active at 30 μM. In the hypoxic-induced LDH release assay, several compounds including flunarizine, 15, and 18 showed good activity (IC_{50} < 10 μM). These results are consistent with the results for flunarizine and compound 15 in NaFl (IC_{50} = 0.42 and 3.5 μM, respectively). The results of the hypoxic-induced LDH release assay for the inactive compounds 4 and 19 (IC_{50} > 100 μM) and the weakly active 10 (IC_{50} = 51 μM) are also in agreement with the results from the NaFl assay for the same compounds (IC_{50}s for 4, 10, and 19 in NaFl were 106, 29, and >100 μM, respectively). Compound 1 (IC_{50} = 89 μM) and compound 22 (IC_{50} = 61 μM) were only weak inhibitors of hypoxia-induced LDH release. These results for compounds 1 and 22 were surprising since they are inconsistent with the good activity the compounds demonstrated in NaFl (IC_{50} = 2.7 and 4.3 μM, respectively). While many explanations are possible, one rationale for these anomalous data may lie with the kinetics of these compounds. Compound 1 has been extensively studied[16] and was found to have a slow association rate with sodium channels (half time = 1.2 ± 0.46 min).[16b] When a veratridine stimulus is used, a sodium channel is held open for an extended period. This extended channel opening may not be mimicked by a hypoxic stimulus, and this difference could be important for the weak neuroprotective activity against hypoxia which was observed in our experiments.

In summary, a number of derivatives of 1 were synthesized and tested for inhibitory activity in the Na^+ channel, with many of them clearly demonstrating greater potency than the parent compound in the [^{3}H]BTX binding assay. Changes of substituents on the aromatic rings do not seem to affect the affinity of the compounds for the Na^+ channel, as long as the diphenyl system itself remains part of the molecule. The amide functionality (or succinimide in 3) seems in improve Na^+-channel blockade, while a change to either hydantoin 2 or urea 4 resulted in a considerable loss of activity. Several of the compounds were examined for neuroprotection in cultured fetal rat neocortical neurons. In regard to the veratridine-induced LDH release, the compounds tested showed a good correlation between activity in this assay and activity in NaFl. Finally, eight compounds were studied in a cell culture assay subjected to hypoxia for an 8-h period. Several of these compounds were found to be neuroprotective in this paradigm, suggesting that sodium-channel blockers may be therapeutically useful agents in ischemic stroke.

Experimental Section

Chemistry. All melting points were determined on a MELT-TEMP II capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were determined in KBr on a Mattson Cygnus 100 or a Nicolet MX1 instrument. Proton magnetic

resonance (NMR) spectra were recorded on either a Varian XL-300 or a Bruker AM250 spectrometer; shifts are reported in δ units relative to internal tetramethylsilane. All mass spectra were obtained on a Finnigan 4500 GCMS or a VG analytical 7070 E/F spectrometer. Elemental analyses were performed on a CEC Model 240 elemental analyzer, and all compounds prepared had analytical results = 0.4% of the theoretical values. Medium-pressure liquid chromatography utilized E. Merck silica gel, 230–400 mesh.

3-[1-(2,6-Dimethyl)piperidinyl]propanenitrile (8). A mixture of formamide (90 g, 2 mol) and 2,6-dimethylpiperidine (226 g, 2 mol) was added over a period of 30 min to a flask containing acrylonitrile (212 g, 4 mol) and H_2O (8 mL, 0.44 mol). The aqueous solution was extracted with Et_2O (3 × 500 mL), and the organic layer was washed with H_2O and a saturated aqueous solution of NaCl. The ether layer was dried (Na_2SO_4) and filtered, and the volatiles were removed. The yellow oil residue was distilled (115–119 °C, 1.5–2 mmHg) to afford 8 as a colorless liquid (261 g, 79% yield). Anal. ($C_{10}H_{18}N_2$) C,H,N.

3-[1-(2,6-Dimethyl)piperidinyl]propanamine (5). Compound 8 (261 g, 1.591 mol) was dissolved in NH_3/MeOH (1000 mL) and reduced with Raney nickel (40 g) at 15.5 psig H_2. When the theoretical amount of H_2 was consumed, the catalyst was filtered off and the solvents were removed in a rotary evaporator to give a light brown liquid, which was purified by distillation (101–102 °C, 2 mmHg) to afford 226 g of pure 5 (85% yield). Anal. ($C_{10}H_{22}N_2$) C,H,N.

Procedure A. The carboxylic acid (10 mmol) and 1,1′-carbonyldiimidazole (15 mmol) were stirred together in 30 mL of dry DMF at 40–45 °C until gas evolution had ceased (about 20 min). A solution of amine 5 (30 mmol) in 20 mL of dry DMF was added at room temperature to the above reaction flask, and the mixture was stirred at room temperature. The reaction was monitored with TLC (SiO_2; MeOH/CH_2Cl_2 1:12). After 30 min, the reaction mixture was poured into an excess of 0.5 N aqueous Na_2CO_3 solution and the white solid that precipitated was collected by filtration, washed with H_2O, and then dried under reduced pressure to give the product.

Procedure B. The carboxylic acid (10 mmol) and 1-hydroxybenzotriazole hydrate (10 mmol) were dissolved in CH_2Cl_2 at 0 °C, and then, 1,3-dicyclohexylcarbodiimide (10 mmol) was added with stirring at 0 °C. A suspension was formed, which was treated with amine 5 (10 mmol), and the reaction mixture was stirred at room temperature overnight. The white solid was filtered off and washed with CH_2Cl_2, the filtrates and washings were combined and washed with H_2O, and the volatiles were evaporated to afford an off-white solid, which was dissolved in EtOAc and filtered; the filtrate was extracted with a 10% aqueous citric acid solution. The aqueous layer was isolated, washed with Et_2O, made alkaline with concentrated NH_4OH, and then extracted with Et_2O. The organic layer was washed with H_2O and saturated aqueous NaCl and dried (Na_2SO_4). Evaporation of the solvents afforded the product, which was purified by column chromatography.

Procedure C. The carboxylic acid (10 mmol) and oxalyl chloride (10 mmol) were mixed in a flask, and a few drops of DMF were added, which initiated an evolution of gas that lasts for a few minutes. After the reaction had subsided, the volatiles were evaporated, the residue was taken up to 25 mL of CH_2Cl_2, placed in an ice–water bath, and treated with amine 5 (50 mmol). The mixture was left stirring at room temperature overnight. The reaction was washed with a 10% aqueous Na_2CO_3 solution, H_2O, and a saturated aqueous NaCl solution and dried (Na_2SO_4). Evaporation of the solvents gave the product as a free base, which was further purified by chromatography.

1-[3-(2,6-Dimethyl-1-piperidinyl)propyl]-3,3-diphenyl-2,5-pyrrolidinedione (3). A mixture of 3,3-diphenyl-2,5-pyrrolidinedione (2.51 g, 10 mmol) and amine 5 (5.1 g, 30 mmol) was heated in an oil bath at 1500 °C for 3 h. The mixture was chromatographed (MeOH/CH_2Cl_2 5:95), and the volatiles were evaporated to afford 2.63 g of an oil, which solidified on standing under reduced pressure for 7 days (65.1% yield); mp 55 °C. Anal. ($C_{26}H_{32}N_2O_2$) C,H,N. H: found, 7.26; calcd, 7.97.

***N′*-[3-(2,6-Dimethyl-1-piperidinyl)propyl]-*N*,*N*-diphenylurea (4).** Diphenylcarbamyl chloride (4.64 g, 20 mmol) was dissolved in 50 mL of CH_2Cl_2 containing Et_3N (5 g, 50 mmol) and amine 5 (1.7 g, 10 mmol). The mixture was stirred at room temperature for 18 h, and then, it was extracted with a 2 N aqueous HCl solution; the aqueous phase was isolated, washed with EtOAc, made alkaline (10% aqueous NaOH solution), and extracted with EtOAc. The organic phase was washed with H_2O and saturated aqueous NaCl and dried (Na_2SO_4). Removal of the volatiles under reduced pressure afforded an off-white solid (2.74 g, 75% yield) which was analytically pure; mp 70 °C. Anal. ($C_{23}H_{31}N_3O$) C,H,N.

α-(Acetyloxy)-2-chloro-α-(2-chlorophenyl)benzeneacetic Acid, Methyl Ester (23). A solution of α-(hydroxy)-2-chloro-α-(2-chlorophenyl)benzeneacetic acid (22) (5 g, 17 mmol) in 30 mL of THF was treated with 1.71 g of Ac_2O (17 mmol), 2.34 mL of Et_3N (17 mmoL), and 5 mg of DMAP. The solution was stirred at room temperature under N_2 overnight. A mixture of 200 mL of EtOAc/Et_2O (1:1) was added, and the solution was extracted with 200 mL of Et_2O. The ethereal layer was washed with water, saturated aqueous NaCl and dried over anhydrous Na_2SO_4. The solvent was removed, and the residue was dried under reduced pressure to give 4.8 g of crude product which was dissolved in 25 mL of CH_3COCH_3. Solid K_2CO_3 (3.14 g, 14 mmoL) and 1.7 g of $(CH_3O)_2SO_2$ (14 mmoL) were added to the reaction solution, and the mixture was stirred at room temperature for 45 min. The volatiles were removed, and the residue was partioned between EtOAc and H_2O. The organic layer was washed with saturated aqueous NaCl and dried over Na_2SO_4. The solvents were removed, and the residue was further dried under reduced pressure to a final weight of 4.15 g of analytically pure product (83% yield), mp 127 °C. Anal. ($C_{17}H_{14}Cl_2O_4$) C,H,Cl.

2-Chloro-α-(2-Chlorophenyl)benzeneacetic Acid (24). The ester 23 (4 g, 11 mmoL) was dissolved in THF and mixed with 0.435 mL of MeOH, 5.6 mL of HMPA, and 11.5 g (28.25 mmoL) of freshly prepared SmI_2. The mixture was stirred at room temperature for 1 h, and then, 250 mL of a saturated aqueous Na_2CO_3 solution was added followed by the addition of 500 mL of Et_2O. The organic layer was isolated, washed with H_2O and saturated aqueous NaCl, and dried over anhydrous Na_2SO_4. Removal of the volatiles gave 3.1 g of the crude methyl ester, which was taken up to 25 mL of EtOH (95%) and added to a 100-mL round-bottomed flask containing a solution of 5 g of NaOH in 20 mL of EtOH (95%). The mixture was stirred at room temperature for 2 h, most of the solvents were removed, and the residue was treated with a 2 N aqueous HCl solution until strongly acidic. It was then extracted with Et_2O (2 × 50 mL), and the organic layer was washed with H_2O and saturated aqueous NaCl and dried over anhydrous Na_2SO_4. The solvents were removed under reduced pressure, and the residue was crystallized from 95% aqueous EtOH to afford 1.68 g of analytically pure acid (53% yield), mp 169–172 °C. Anal. ($C_{14}H_{10}Cl_2O_2$) C,H,Cl.

Biological Methods. [^{3}H]Batrachotoxinin Binding Assay. Male rats (Sprague–Dawley, 200–220 g) were killed by decapitation; neocortices were isolated by blunt dissection, cleaned of white matter, pooled, homogenized in ice-cold 50 mM HEPES–NaOH buffer (pH 7.4 at 22 °C) with a tissue homogenizer (position 6, 30 s; Polytron, PTA 20 S), and centrifged at 1000*g* for 10 min at 5 °C. The resultant supernatant was centrifuged at 4800*g* for 10 at 5 °C. This step was repeated once more after resuspending the pellet in fresh buffer. The final pellet was suspended in fresh buffer and homogenized with a glass/Teflon homogenizer (800 rpm, 8 strokes; Potter Elvehjem). The resultant suspension was frozen rapidly and stored in liquid nitrogen until it was used. In the [^{3}H]batrachotoxinin binding assay, incubation mixtures (250 μL) consisted of 100 μL of 50 mM HEPES–NaOH buffer (pH 7.4), 50 μL of membrane suspension (~25 μg of protein), 25 μL of tetrodotoxin (final concentration 1 μM), 25 μL of scorpion venom (final concentration 5 μg/mL), 25 μL of either assay buffer (for total binding) or a solution containing an interacting substance or aconitine (final concentration 100 μM for nonspecific binding), and 25 μL of [^{3}H]batrachotoxinin (final concentration 5 nM; 2.04 TBq/mmol). Incubations were terminated after 30 min at 37 °C by rapid filtration through presoaked (0.1% PEI) glass-fiber filters (GFC). The filters were immediately rinsed with three 4-mL volumes of ice-cold buffer. Radioactivity of the filters was measured by a liquid scintillation

counter at the efficiency of 51%. Specific binding of [^{3}H]-batrachotoxinin was defined as the difference between total and nonspecific binding. The results of competition experiments were analyzed by nonlinear curve-fitting algorithms,[31] and they are expressed as K_is according to the Cheng–Prusoff equation.[32]

Inhibition of Veratridine-Induced Na^+ Influx. These experiments were performed using the cell line CNaIIA-1, derived from a Chinese hamster ovary cell line (CHO-K1; American Type Cultures) which was transfected with the vector ZEM2580 containing a cDNA encoding the rat brain IIA Na^+ channel.[17-19] The rat IIA sequence used contained the natural leucine at position 860, conferring normal voltage-dependent properties. CNaIIA-1 cells were cultured in 12 well plates at 37 °C and 5% CO_2 in RPMI medium 1640 (GIBCO) containing 10% fetal calf serum, 2 mL of geneticin solution (5 mg/mL), and penicillin/streptomycin (final concentration 20 units/mL penicillin G sodium and 20 μg/mL streptomycin sulfate). Incubation experiments were run on a 500-μL scale by first adding 425 μL of freshly oxygenated KRH (Krebs–Ringer HEPES) buffer (pH 7.4), 25 μL of drug solution in DMSO/H_2O 1:9 (final concentrations ranged from 0.01 to 100 μM), and 25 μL of a 0.6 mM veratridine solution (final concentration 30 μM) containing 0.124 μL of α-scorpion venom solution (prepared by dissolving 5 mg of α-scorpion venom in a mixture of 200 μL of DMSO, 100 μL of H_2O, and 100 μL of a 2.9 N aqueous HCl solution) followed by an 11-min incubation at 37 °C in a 5% CO_2 incubator, and finally, 25 μL of [^{14}C]guanidine containing KRH buffer solution was added (final concentration 250 μM "cold" guanidine and 0.005 μCi of [^{14}C]guanidine). The KRH buffer used contained KCl (final concentration 4.75 mM), $CaCl_2$ (1.25 mM), KH_2PO_4 (1.20 mM), $MgSO_4$ (1.18 mM), HEPES (22 mM), dextrose (11 mM), and choline hydrochloride (130 mM). The pH of the buffer was brought to 7.4 with a 3 M TRIS solution. The buffer was refrigerated and made fresh every week. The 0.6 mM veratridine solution was prepared by dissolving veratridine in equal volumes of 0.1 N HCl and 0.1 N NaOH and a few drops of 1 N HCl followed by the addition of an α-scorpion venom solution (3.1 μg/mL). The drug solutions were prepared by dissolving the appropriate amount of either the salt or the free base of the drug to a 10% solution of DMSO in H_2O so that the final concentration of the drugs ranged from 0.01 to 100 μM. The control solution contained equal volumes of 0.1 N HCl and 0.1 N NaOH. The experiments were terminated 25 min after the first addition (KRH buffer) by washing the wells containing the cell cultures with PBS buffer (2 × 2 mL) and then adding a 3% solution of Triton X-100 (0.5 mL), and 30 min later, the well contents were transferred to a scintillation vial containing 10 mL of Ready Gel scintillation solution, with the amount of radioactivity remaining determined using a scintillation counter.

Veratridine-Induced LDH Release Assay. Neuronal cultures were prepared from the cerebral cortices of Charles River rat embryos as described elsewhere;[25,26] 17- or 18-day old cultures were exposed to a veratridine solution of the following composition (mM): NaCl (120), KCl (5.4), $MgCl_2$ (0.8), $CaCl_2$ (1.8), HEPES pH 7.4 at 25 °C (20), glucose (15), and veratridine (100 μM). After 10 min, the exposure solution was thoroughly replaced with culture medium (lacking serum) by triple exchange and the plates were returned to the incubator for LDH measurements the following day. Control experiments showed little or no cell injury produced by this protocol if veratridine was omitted. The drugs tested were added to the veratridine exposure medium, and incubated with the culture along with veratridine for 10 min, and the exposure medium was then replaced with culture medium (lacking serum) by triple exchange. Overall neuronal injury was quantitatively assessed by measurement of LDH released into the culture medium 1 day after veratridine exposure, as it has been previously described.[27,28] By using a minor modification of this method, we were able to perform a kinetic enzyme assay for the purpose of LDH measurement; 25-μL medium samples from each well were placed into a clean 96-well microtiter place and mixed with 225 μL of 0.1 M phosphate buffer (pH 7.5 at 25 °C) containing 30 μg of NADH. Following a 10-min incubation at 37 °C, the reaction was initiated by the addition of 30 μL of 2.4 mM sodium pyruvate to each well. The absorbance of each well at 340 nM, an index of NADH concentration, was measured repetitively with a Multiskan MCC/340 plate reader with an interval time of 5 s (sufficient to complete a single reading of 96 samples) for a total of 2 min. LDH activity (conventional units/mL) was then calculated from the absorbance readings by applying a correction factor for temperature and light path. Accuracy of the assay was verified by periodic checks with a standard LDH enzyme solution. A small amount of LDH was present in the media of cultures carried through the exposure protocol but not exposed to veratridine. No significant LDH efflux occurred when cultures of cortical glia were similarly exposed to veratridine.

Hypoxic-Mediated Cell Injury. Cerebrocortical cultures were derived from Sprague–Dawley fetal rat brain on the 18th day of gestation. Dissected cortical hemispheres were trypsinized (2.5 g/L trypsin in Mg^{2+}- and Ca^{2+}-free HBSS) for 15 min, washed thrice, triturated into a single-cell suspension, and then diluted to a concentration of 1 brain/20 mL in growth medium containing 42% Delbeco's Modified Eagle's (DME) medium, 42% Hams F12 Nutrient (HamF12), 10% horse serum, and 6% fetal calf serum pH 7.4. A 100-μL aliquot of the cell suspension was dispensed into individual wells of 96-well tissue culture plates precoated with poly(L-lysine) to aid in cell adherence. Plates were then stored in a humidified, 2.5% CO_2, 37 °C incubator. Mitotic division of non-neuronal components of the cell culture was halted after 4 days of growth by adding 100 μL of 30 μg/mL 5-fluoro-2′-deoxyuridine and 70 μg/mL uridine in 45% DME/45% HamF12/10% horse serum. Subsequent feedings (half volume replacements) using 45% DMF/45% HamF12/10% horse serum were performed at 2–3-day intervals thereafter. Cells were tested 17 days postisolation. Prior to hypoxic exposure, the growth medium in the culture plates was aspirated out and quickly replaced with 50 μL of test medium (HBSS containing no Mg^{2+}, 1.8 mM Ca^{2+}, and 1 g/L D-glucose pH 7.4) with or without test agents. Plates were placed in a humidified incubator set to 1% O_2, 8% CO_2, and 91% N_2 and incubated for 8.5 h at 37 °C. Immediately following the initial hypoxic exposure, the wells received an additional 50 μL (100 μL total volume) of test medium containing the appropriate test agent concentrations, 1.6 mM Mg^{2+}, and 7 g/L D-glucose. Plates were then stored under a humidified, normoxic atmosphere (20% O_2, 8% CO_2, and 72% N_2) at 37 °C for an additional 15.5 h. Final Mg^{2+} and D-glucose concentrations during the reoxygenation period were 0.8 mM and 4 g/L, respectively. LDH release determinations were performed 24 h after experiment initiation using a kinetic assay which is described in the previous protocol for Veratridine-Induced LDH Release Assay. Absorbance readings were determined at 5-s intervals for a period of 1 min.

References

(1) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th ed.; Gilman, A. G.; Rall, T. W.; Nies, A. S.; Taylor, P., Eds.; Pergamon Press: New York, 1991; p 847.
(2) (a) See ref 1, p 440. (b) See ref 1, p 447.
(3) See ref 1, p 312.
(4) Triggle, D. J.; Langs, D. A. Ligand gated and voltage-gated ion channels. *Annu. Rep. Med. Chem.* **1990**, *25*, 225–234.
(5) Catterall, W. A. Common modes of drug action on Na^+ channels: local anesthetics, antiarrhythmics and anticonvulsants. *Trends Pharmacol. Sci.* **1987**, *8*, 57–65.
(6) Hille, B. *Ionic Channels of Excitable Membranes*, 2nd ed.; Sinauer Associates, Inc.: Sunderland, MA, 1992.
(7) Catterall, W. A. Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev.* **1992**, *72* (Suppl. 4), S15–48.
(8) Catterall, W. A. Structure and function of voltage-sensitive ion channels. *Science* **1988**, *242*, 50–61.
(9) Strichartz, Z.; Rando, T.; Wang, G. K. An intergrated view of the molecular toxicology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* **1987**, *10*, 237–267.
(10) Catterall, W. A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 15–43.
(11) Lombet, A.; Bidard, J.-N.; Lazdunski, M. Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na^+ channel. *FEBS Lett.* **1987**, *219*, 355–359.
(12) Agnew, W. S. Voltage-regulated sodium channel molecules. *Annu. Rev. Physiol.* **1984**, *46*, 517–530.
(13) Auld, V. J.; Goldin, A. L.; Krafte, D. S.; Marshall, J.; Dunn, J. M.; Catterall, W. A.; Lester, H. A.; Davidson, N.; Dunn, R. J. A rat brain Na^+ channel *alpha* subunit with novel gating properties. *Neuron* **1988**, *1*, 449–461.

(14) Suzuki, H.; Beckh, S.; Kubo, H.; Yahagi, N.; Ishida, H.; Kayano, T.; Noda, M.; Numa, S. Functional Expression of cloned cDNA encoding sodium channel III. *FEBS Lett.* **1988**, *228*, 195–200.
(15) Raysdale, D. S.; Scheuer, T.; Catterall, W. A. Frequency and voltage-dependent inhibition of type IIA Na^+ channels, expressed in a mammalian cell line, by local anesthetic, antiarrhythmic, and anticonvulsant drugs. *Mol. Pharmacol.* **1991**, *40*, 756–765.
(16) (a) Ragsdale, D. S.; Numann, R.; Catterall, W. A.; Scheuer, T. Inhibition of Na^+ channels by the novel blocker PD85639. *Mol. Pharmacol.* **1993**, *43*, 949–954. (b) Thomsen, W.; Hays, S. J.; Hicks, J. L.; Schwarz, R. D.; Catterall, W. A. Specific binding of the novel Na^+ channel blocker PD85639 to the α subunit of rat brain Na^+ channels. *Mol. Pharmacol.* **1993**, *43*, 955–964.
(17) Scheuer, T.; Auld, V. J.; Boyd, S.; Offord, J.; Dunn, R.; Catterall, W. A. Functional properties of rat brain sodium channels expressed in a somatic cell line. *Science* **1990**, *247*, 854–858.
(18) West, J. W.; Scheuer, T.; Maechler, L.; Catterall, W. A. Efficient expression of type IIA Na^+ channel α subunit in a somatic cell line. *Neuron* **1992**, *5*, 59–70.
(19) Auld, V. J.; Goldin, A. L.; Krafte, D. S.; Catterall, W. A.; Lester, H. A.; Davidson, N.; Dunn, R. J. A neutral amino acid change in segment IIS4 dramatically alters the gating properties of the voltage-dependent sodium channel. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 323–327.
(20) Kusuda, K.; Inanaga, J.; Yamaguchi, M. A highly efficient deoxygenation of α-oxygenated ester via SmI_2-induced electron transfer process. *Tetrahedron Lett.* **1989**, *30*, 2945–2948.
(21) Pauwels, P. J.; Leysen, J. E.; Laduron, P. M. [^{3}H]-Batrachotoxinin A 20-α-benzoate binding to sodium channels in rat brain: characterization and pharamacological significance. *Eur. J. Pharmacol.* **1986**, *124*, 291–298.
(22) Catterall, W. A.; Morrow, C. S.; Daly, J. W.; Brown, G. B. Binding of batrachotoxinin A 20-α-benzoate to a receptor site associated with sodium channels in synaptic nerve ending particles. *J. Biol. Chem.* **1981**, *256*, 8922–8927.
(23) Reith, M. E. A. [^{14}C]Guanidinium ion influx into Na^+ channel preparations from mouse cerebral cortex *Eur. J. Pharmacol.-Mol. Pharmacol. Sect.* **1990**, *188*, 33–41.
(24) Hille, B. The permeability of the sodium channel to organic cations in myellenated nerve *J. Gen. Physiol.* **1971**, *58*, 599–619.
(25) Koh, J. Y.; Choi, D. W. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay *J. Neurosci. Methods* **1987**, *20*, 83–90.
(26) Klingman, J. G.; Hartley, D. M.; Choi, D. W. Automated determination of excitatory amino acid neurotoxicity in cortical culture *J. Neurosci. Methods* **1989**, *31*, 47–51.
(27) Pauwels, P. J.; Van Assouw, H. P.; Leysen, J. E.; Janssen, P. A. J. Ca^{++}-Mediated neuronal death in rat brain neuronal cultures by veratridine: protection by flunarizine *Mol. Pharmacol.* **1989**, *36*, 525–531.
(28) Pauwels, P. J.; Van Assouw, H. P.; Leysen, J. E. Attenuation of neurotoxicity following anoxia or glutamate receptor activation in EGF- and hippocampal extract treated neuronal cultures *Cell. Signal* **1989**, *1*, 45–54.
(29) Madden, K. P.; Clark, W. M.; Marcoux, F. W.; Probert, A. W.; Jr.; Weber, M. L.; Rivier, J.; Zivin, J. A. Treatment with conotoxin, an 'N-type' calcium channel blocker, in neuronal hypoxic-ischemic injury *Brain Res.* **1990**, *537*, 256–262.
(30) (a) Compound **2** was first reported in 1958 by Chit, W.; Selleri, R. Chemical structure and local anesthetic action. Tertiary alkyleneamine derivatives of diphenylhydantoin. *Farmaco, Ed. Sci.* **1958**, *13*, 261–285. It is a white solid, mp 112 °C. (b) Chit, W.; Superbi, C.; Chiarini, P. Hydantoin and Thiohydantoin derivatives with myolytic and local anesthetic activity. *Farmaco, Ed. Sci.* **1960**, *15*, 809–820.
(31) Rodbard, D.; Cole, B. R.; Munson, P. J. Development of a friendly, self-teaching, interactive statistical package for analysis of clinical research data. The BRIGHT STA-PACK. *J. Med. Syst.* **1984**, *8*, 205–212.
(32) Cheng, Y.-C.; Prusoff, W. H. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent reduction (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.