A Structure−Activity Relationship Study of Novel Phenylacetamides Which Are Sodium Channel Blockers

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A structure−activity relationship study of a series of novel Na^+ channel blockers, structurally related to *N*-[3-(2,6-dimethyl-1-piperidinyl)propyl]-α-phenylbenzeneacetamide (**1**, PD85639) is described. The diphenylacetic acid portion of the molecule was left unchanged throughout the study, while structural features in the amine portion and the amide alkyl linkage of the molecule were modified. The compounds were tested for inhibition of veratridine-stimulated Na^+ influx in CHO cells expressing type IIA Na^+ channels. Several derivatives show a trend toward more potent Na^+ channel blockade activity with increasing lipophilicity of the amine portion of the molecule. The presence of a phenyl ring near the amine increases inhibitory potency. A three-carbon spacer between the amide and amine is optimal, and a secondary amide linkage is preferred.

Introduction

Voltage-dependent sodium channels have been implicated as the site of action for many types of drugs including antiarrhythmics, anticonvulsants, and local anesthetics.[1] Additionally, sodium channel blockers have demonstrated neuroprotection in experimental models of ischemic stroke.[2,3] Sodium channel blocking agents are believed to act by interfering with the rapid influx of Na^+ ions which is responsible for the generation of action potentials in excitable cells.[4] Sodium channels in neuronal cells are large trimeric glycoproteins (containing a 260 kDa α subunit, a 36 kDa β_1 subunit, and a 33 kDa β_2 subunit) having different electrophysiological and pharmacological properties from sodium channels in cardiac and skeletal muscle (which contain two subunits). Different subtypes of sodium channels have been cloned (I, II, IIA, and III from rat brain) with the type I found primarily in neuronal cell bodies and type II in the axons of the neuronal cells.[5] In some species (e.g. eel electroplax) the sodium channel consists of a single α subunit. When the α subunit alone (type IIA) was cloned and expressed in Chinese hamster ovary (CHO) cells, fully functional sodium channels were obtained.[6,7]

The sodium channel can shift between three distinct conformational states: active, resting, and inactivated. 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 resting level.[1] The availability of specific neurotoxins has contributed to a better understanding of voltage-sensitive sodium channels. 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 site 5, where ciguatoxins act to prolong activation.

The site of action of the class III 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 allows them to selectively block abnormally firing sodium channels without inhibiting normal cardiac and neuronal sodium channel functions.[1]

N-[3-(2,6-Dimethyl-1-piperidinyl)propyl]-α-phenylbenzeneacetamide (PD85639, **1**) has been shown to act as a local anesthetic with high selectivity for the type II sodium channels.[13,14] As part of a previous structure−activity relationship (SAR) study on derivatives of **1**, we reported that these compounds inhibited [^{3}H]batrachotoxin binding in rat neocortical membranes and Na^+ influx in CHO cells expressing the α subunit of type IIA sodium channels.[15] Some derivatives also inhibited veratridine- and hypoxia-induced lactate dehydrogenase (LDH) release from cell cultures, which has been used as a measure of neuroprotection.[16−18] These data suggest that they may function as neuroprotecting agents and potentially be useful for the treatment of ischemic stroke. The previous SAR study of derivatives of **1** involved changes in the diphenylacetyl portion of the molecule.[15] In the present study, structural modifications were explored in the linkage (part B) and amine portion (part C) of compound **1**, while the diphenylacetic acid moiety (part A) was held unchanged throughout the series. The synthesis and sodium channel blockade activity of these derivatives are described.

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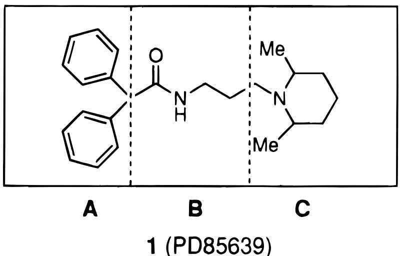

A B C

1 (PD85639)

Chemistry

To study the effect of the spacer and amine portions of **1**, a variety of targets were designed and synthesized. The diamines that were not commercially available were prepared as shown in Scheme 1. The starting amines (**2A–I**) were treated with acrylonitrile in methanol to afford the nitriles in good yield, and the nitriles were subsequently converted to the diamines (**3A–I**) by Raney nickel catalyzed hydrogenation. The products (**3A–I**) from this procedure are shown in Table 1.

The targeted compounds (**7–28**) were prepared as shown in Schemes 2 and 3. The diphenylacetic acid **4** was treated with *endo-N*-hydroxy-5-norborene-2,3-dicarbodiimide **5**[19] in the presence of 1,3-dicyclohexylcarbodiamide (DCC) to form the activated ester **6** as shown in Scheme 2. The ester **6** was mixed with 3-aminopropionitrile to afford the amide which was then reduced via catalytic hydrogenation to produce the primary amine **7**. The remainder of the amide targets were synthesized either by coupling of the activated ester derivative of diphenylacetic acid **6** with the appropriate

Scheme 1

R_1–N(H)–R_2 **2A-I** → (1. Acrylonitrile; 2. H_2, Raney Nickel) → R_1–N(R_2)–$CH_2CH_2CH_2$–NH_2 **3A-I**

diamine in dichloromethane (Scheme 3, procedure A) or by reacting diphenylacetic acid, 1,1′-carbonyldiimidazole (CDI) and the diamine in dimethylformamide (Scheme 3, procedure B).

Biological Testing

Inhibition of [^{3}H]batrachotoxin ([^{3}H]BTX) binding in rat neocortical membranes has been used as a rapid screening method for identifying compounds that block voltage-sensitive sodium channels.[20,21] The existence of a good correlation ($r = 0.841$) between inhibition of [^{3}H]-BTX binding and inhibition of veratridine-stimulated Na^+ influx (NaFl) in CHO cells expressing the α subunit of rat brain type IIA sodium channels has been previously demonstrated.[15] In this report the ability of the synthesized compounds to block Na^+ influx (NaFl) was used exclusively to determine their interaction with the sodium channel. After application of test compounds to the CHO cells for 5 min, the cells were treated with a veratridine solution containing α-scorpion toxin, which results in opening of the sodium channels. A [^{14}C]-guanidinium solution was then added to the cells, the inhibition of the [^{14}C]guanidinium influx was used to measure sodium channel blockade,[22] and the IC_{50} values were determined by linear regression analysis (see Table 2).

Table 1. Structure and Physical Constants of Compounds Prepared by Scheme 1

Compound Number	Starting Amine (2)	Product (3)	mp(°C)	Formula
A			138[a]	$C_9H_{20}N_2O \cdot 2HCl \cdot 1.15\ H_2O$
B			154[a]	$C_{11}H_{18}N_2 \cdot 2HCl \cdot 0.6\ H_2O$
C			227[a]	$C_{14}H_{23}N_3O \cdot 3HCl \cdot 0.5\ H_2O$
D			a	$C_{14}H_{23}N_3 \cdot 3HCl \cdot 1.0\ H_2O$
E			b	$C_{15}H_{22}N_4O \cdot 1.5\ H_2O$
F			161[a]	$C_{14}H_{22}N_2O \cdot 2HCl \cdot 1.2\ H_2O$
G			b,c	$C_{15}H_{24}N_2$
H			c	$C_{14}H_{22}N_2O$
I			d	$C_{15}H_{24}N_2 \cdot 2HCl \cdot 0.2\ H_2O$

[a] hydrochloride salt; [b] free base; [c] used without purification; [d] 2-methylacryolnitrile was used

Scheme 2

4 + HON 5 —DCC→ 6 + NC~~NH2 —1. 1,4-dioxane/THF 2. H_2, Raney Nickel→ 7

Scheme 3

R_1, H, R_2–N–( )$_n$–N–R_3 —Procedure A (6) / Procedure B (4, CDI)→ 8-28

Results and Discussion

The local anesthetic **1** inhibits Na^+ influx in CHO cells expressing the α subunit of type IIA sodium channels with an IC_{50} of 2.7 μM. The vasodilator flunarizine, which is characterized principally as a Ca^{2+} channel blocker, is also a very potent Na^+ blocker (IC_{50} = 0.42 μM) as is the antipsychotic drug, pimozide (IC_{50} = 0.34 μM). Sodium channel blockade was determined experimentally for these reference compounds and can be viewed in Table 2. All of these compounds contain a diphenylmethyl moiety, an amine, and a lipophilic tail.

Flunarizine Pimozide

The primary amine **7** (IC_{50} = 40 μM), the dimethylamino analog **8** (IC_{50} = 20 μM), the diethylamino derivative **9** (IC_{50} = 4.4 μM), and the methylbenzylamine derivative **10** (IC_{50} = 1.2 μM) show a trend toward more potent Na^+ channel blockade activity with the increasing lipophilicity of the amine portion of the molecule. While the piperidine, 2,5-dimethylpyrrolidine, and the morpholine derivatives **11**, **12**, and **13** demonstrated good inhibition of Na^+ influx (IC_{50} = 5.3, 4.6, and 6.5 μM, respectively), only the slightly more lipophilic 2,6-dimethylmorpholine derivative **14** was equipotent with the parent compound **1** (IC_{50} = 2.7 μM).

An enormous difference in activity was found between the various piperazine derivatives. The 4-methylpiperazine analog **15** was essentially inactive (IC_{50} > 200 μM) while the 4-(2-methoxy)phenyl analog **16** has activity (IC_{50} = 1.2 μM) on the same order as the parent **1** and the benzylpiperazine analog **17** (IC_{50} = 0.7 μM) has activity approaching that of flunarizine or pimozide. This large discrepancy in activity of these compounds may be a function of the basicity of the piperazine nitrogen atoms. The most basic center in **15** is the N-methyl nitrogen atom whereas the electron-withdrawing effects of the aryl and benzyl groups make the corresponding nitrogen atoms of **16** and **17** less basic.[23] As a result, the protonation of compound **15** would occur on the methylated nitrogen atom while the positive charge would reside on the alternative nitrogen of the other derivatives. This change in the position of protonation would certainly affect alignment of the compound within the channel. The phenyl rings may also play a critical role in anchoring inhibitors **16** and **17** into the Na^+ channel. This is further supported by the good activity observed for piperidine analogs such as compounds **18**–**22** which contain a phenyl moiety in this region of the molecule. These derivatives have IC_{50}s < 2.2 μM and **20** (IC_{50} = 0.6 μM) was the most potent of any of the new analogs synthesized. Compound **22** differs only from **18** in the methyl group that it has in the spacer. The two compounds are essentially equipotent (IC_{50}'s 1.5 and 1.1 μM, respectively). Derivative **23** with the benzimidazolone substitution in the 4-position of the piperidine ring mimics the heterocyclic

portion of pimozide. This compound (IC_{50} = 0.72 μM) like pimozide demonstrates potent sodium channel inhibition.

A number of derivatives of **1** were targeted that explored the linkage between the diphenylacetyl and amine functionalities. If the propyl spacer of **11** (IC_{50} = 5.3 μM) is shortened to two carbons (analog **24**), or lengthened to four carbons (analog **25**), the compounds decrease their ability to block Na^+ channel activity (IC_{50}s = 7.2 and 10.0 μM). This suggests that the three carbon spacer is optimal. When the spacer is part of a conformationally restricted ring such as **26** or **27** (IC_{50}s = 32 and 95 μM), which may function to reorient the tertiary amine at the Na^+ channel binding site, inhibitory activity is decreased. A secondary amide linkage would seem optimal since methyl alkylation of the amide **8** (IC_{50} = 20 μM) to afford the tertiary amide **28** resulted in a complete loss of activity (compound **28**; IC_{50} > 200 μM).

Most clinically useful local anesthetics consist of both hydrophilic (usually a tertiary amine) and hydrophobic (usually an aromatic ring) domains that are linked by an amide or ester bond and separated by an alkyl chain.[24,25] This simplified description of local anesthetic structure−activity relations (SAR) is appropriate for derivatives of **1.** While the amine moiety is hydrophilic in its protonated state, additional lipophilicity in the amine portion of the molecule greatly enhances sodium channel inhibition for this group of compounds. Ragsdale and co-workers[26] have identified phenylalanine1764 and tyrosine1774 as determinants of the local anesthetic binding site of the voltage-dependent Na^+ channel. Substitution of either of these residues with alanine destabilizes drug binding by reducing the hydrophobicity and aromaticity at these positions in the channel. Hydrophobic moieties at either end of the local anesthetic molecule that could interact with these residues is clearly beneficial for Na^+ blockade activity. This suggests that an appropriately positioned phenyl ring in the amine portion of these analogs may be beneficial as a result of interactions with other aromatic moieties in the channel. In conclusion, several compounds more active than **1** in blocking the veratridine-stimulated Na^+ influx have been identified. On the basis of previous functional data,[15] these derivatives should also be neuroprotectant in cell-based and animal models of neuronal injury.

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) were recorded either on 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 they were within 0.4% of the theoretical values. Medium-pressure liquid chromatography utilized E. Merck silica gel, 230−400 mesh. All reactions were run under N_2, unless indicated otherwise. All analytical (C, H, N) and spectroscopic (^{1}H NMR, IR, MS) data were in agreement with the proposed structures.

Synthesis of Amines (3A−I, Scheme 1). The appropriate starting amine (**2A−I**) and an equimolar amount of acrylonitrile were added to dry MeOH at 0 °C, and the mixture was stirred at room temperature for 24 h. The volatiles were removed under reduced pressure, and the residue was purified either by distillation or chromatography. The purified product was dissolved in MeOH (saturated with NH_3 to reduce the formation of secondary and tertiary amines) and was hydrogenated over Raney Ni. The catalyst was filtered off, and the residue was purified by either chromatography or distillation.

1,7-Dimethyl-3,5-dioxo-4-azatricyclo[5.2.1.0^{2,6}]dec-8-en-4-yldiphenylacetic Acid Ester (6) (Scheme 2).[19] Diphenylacetic acid **3** (84.8 g, 0.4 mol) and *endo-N*-hydroxy-5-norborene-2,3-dicarboximide (**2**; 80.55 g, 0.45 mol) were dissolved in THF/1,4-dioxane (1:1) (1.2 L) cooled to 0 °C in an ice water bath and treated with 1,3-dicyclohexylcarbodiimide (92.7 g, 0.45 mol). The mixture was stirred for 16 h at room temperature, the white solid precipitate was filtered off, and the filtrates were washed with a 5% $NaHCO_3$, water, and saturated NaCl and dried (Na_2SO_4). The volatiles were evaporated under reduced pressure, and the residue was recrystallized from CH_2Cl_2 to afford 125.8 g of analytically pure product (84.3% yield). mp 188−190 °C. Anal. ($C_{23}H_{19}N_1O_4$) C, H, N.

***N*-(3-Aminopropyl)-2,2-diphenylacetamide (7) (Scheme 2).** The activated ester **6** (7.46 g, 20 mmol), 3-aminopropionitrile fumarate (3.68 g, 20 mmol), and triethylamine (10 mL, 72 mmol) were dissolved in CH_2Cl_2 (100 mL) and stirred together for 16 h, and the reaction was worked up as in procedure A to give 4.65 g of an analytically pure white solid: mp 123 °C. Anal. ($C_{17}H_{16}N_2O$) C, H, N. This product was dissolved in CH_3OH saturated with NH_3 (100 mL) and hydrogenated over 1.5 g of Raney Nickel catalyst. After the catalyst was filtered off, the volatiles were removed under reduced pressure to afford the product, which was taken up in acetone (10 mL), and the oxalate salt was formed by treatment with oxalic acid (720 mg) in acetone (10 mL). The solid which precipitated was recrystallized from CH_3OH/Et_2O to afford pure product **7** (2.55 g, 35.5% overall yield): mp 217−220 °C; IR (KBr) 1644 cm^{-1}; NMR (DMSO-d_6) δ 1.62−1.65 (m, 2H), 2.66−2.70 (m, 2H), 3.07−3.11 (m, 2H), 4.89 (s, 1H), 7.15 (m, 12H). Anal. $C_{17}H_{20}N_2O \cdot C_2H_2O_4$ (C, H, N).

Example of Procedure A (Scheme 3). *N*-[3-(4-Benzylpiperidin-1-yl)propyl]-2,2-diphenylacetamide (20). Activated ester **6** (3.1 g, 10 mmol) and amine **3G** (2.3 g, 10 mmol) were dissolved in CH_2Cl_2 (25 mL) and stirred at 23 °C for 18 h. The reaction mixture was washed with 5% aqueous $NaHCO_3$ (20 mL), H_2O (20 mL), and saturated NaCl (20 mL), and the organic phase was dried over Na_2SO_4. The volatiles were removed under reduced pressure, and the crude residue (5.2 g) was recrystallized from hot 95% EtOH. The off-white solid was collected by filtration (2.18 g, 51% yield): mp 95−97 °C; IR (KBr) 1639 cm^{-1}; NMR ($CDCl_3$) δ 1.00−1.10 (m, 2H), 1.40−1.63 (m, 5H), 1.71−1.77 (t, 2H), 2.27−2.31 (t, 2H), 2.47 (d, 2H), 2.76 (d, 2H), 3.1−3.5 (m, 2H), 4.77 (s, 1H), 7.06−7.31 (m, 16H). Anal. $C_{29}H_{34}N_2O$ (C, H, N).

Example of Procedure B (Scheme 3). 2,2-Diphenyl-*N*-(3-piperidin-1-ylpropyl)acetamide (11). The diphenylacetic acid (2.17 g, 10 mmol) and 1,1′-carbonyldiimidazole (2.43 g, 15 mmol) were stirred together in dry DMF (20 mL) at 23 °C until gas evolution had ceased (about 20 min). A solution of the 1-piperidinepropanamine (30 mmol) in dry DMF (20 mL) was added and the mixture was stirred at 23 °C under a nitrogen atmosphere. After 30 min the reaction mixture was poured into an excess of 0.5 N Na_2CO_3 solution, and the white solid that precipitated was collected by filtration, washed with water, and dried under reduced pressure to afford the product. The product recrystallized from $EtOH/H_2O$ to afford the amine **11** as a fine cotton-like white solid (2.2 g, 65% yield): TLC (SiO_2; $MeOH/CH_2Cl_2$, 1:12); mp 101−103 °C; IR (KBr) 1640 cm^{-1}; NMR ($CDCl_3$) δ 1.35−1.46 (m, 6H), 1.6 (m, 2H), 2.25−2.34 (m, 6H), 3.35 (m, 2H), 4.8 (s, 1H), 7.2−7.3 (m, 11H). Anal. $C_{22}H_{28}N_2O$ (C, H, N).

Biological Methods. Inhibition of Veratridine-Induced Na^+ Influx. These experiments were performed using the cell line CNaIIA-1, derived from a Chinese hamster ovary (CHO) cell line (CHO-K1; American Type Cultures) which were transfected with the vector ZEM2580 containing a cDNA encoding the rat brain IIA Na^+ channel.[5] The rat IIA sequence

Table 2. Physical Constants and Biological Activity of Compounds **7**–**28**

Compound	X	Method of synthesis	m.p. (°C)	Formula[d]	IC_{50} μM(NaFl)[e]
1					2.7 ± 0.08
Flunarizine					0.49 ± 0.08
Pimozide					0.34 ± 0.06
7	HN NH$_2$	A	217[a]	$C_{17}H_{20}N_2O \cdot C_2H_2O_4$	40 (26-40)
8	HN N(Me)$_2$	A	65[b]	$C_{19}H_{24}N_2O \cdot 0.04\ H_2O$	20 (16-24)
9	HN N(Et)$_2$	A	90[b]	$C_{21}H_{28}N_2O \cdot 0.05\ H_2O$	4.4 (3.8-5.0)
10	HN Me N	A	177[a]	$C_{25}H_{28}N_2O \cdot C_2H_2O_4$	1.2 (0.9-1.4)
11	HN N	B	102[b]	$C_{22}H_{28}N_2O$	5.3 (5.2-5.4)
12	HN N Me Me	B	98[b]	$C_{23}H_{30}N_2O$	4.6 (4.3-4.9)
13	HN N O	A	174[b]	$C_{21}H_{26}N_2O_2$	6.5 (6.3-6.6)
14	HN N O Me Me	A	159[b]	$C_{23}H_{30}N_2O_2 \cdot C_2H_2O_4 \cdot 0.10\ H_2O$	2.7 (1.9-3.6)
15	HN N N–Me	A	112[b]	$C_{22}H_{29}N_3O$	>200
16	HN N N MeO	A	172[a]	$C_{28}H_{33}N_3O \cdot C_2H_2O_4$	1.2 (1.1-1.3)
17	HN N N	A	240[a]	$C_{28}H_{33}N_3O \cdot C_2H_2O_4$	0.7 (0.6-0.8)
18	HN N	B	134[b]	$C_{28}H_{32}N_2O \cdot 0.11\ H_2O$	1.1 (1.0-1.1)
19	HN N OH	A	211[a]	$C_{28}H_{32}N_2O_2 \cdot C_2H_2O_4 \cdot 0.03\ H_2O$	2.2 (1.8-2.6)
20	HN N	A	96[a]	$C_{29}H_{34}N_2O$	0.60 (0.55-0.65)
21	HN N O	A	b	$C_{28}H_{32}N_2O_2$	1.4 (1.37-1.50)
22	HN Me N	A	175[a]	$C_{29}H_{34}N_2O \cdot C_2H_2O_4$	1.5 (0.8-2.2)

Table 2 (Continued)

Compound	X	Method of synthesis	m.p. (°C)	Formula[d]	IC_{50} μM(NaFl)[e]
23		A	220[c]	$C_{29}H_{32}N_4O_2 \cdot 0.55\ H_2O$	0.72 (0.70-0.75)
24		A	81[b]	$C_{21}H_{26}N_2O \cdot 0.15\ H_2O$	7.2 (5.5-8.9)
25		B	88[b]	$C_{23}H_{30}N_2O$	10 (8.8-11.2)
26		A	92[b]	$C_{21}H_{26}N_2O$	32 (24-39)
27		A	154[b]	$C_{21}H_{24}N_2O \cdot 0.23\ H_2O$	95 (90-100)
28		A	204[c]	$C_{24}H_{30}N_2O \cdot HCl$	>200

[a]: oxalate salt; [b]: free amine; [c]: hydrochloride salt; [d]: Elemental analyses were within ±0.4% of the theoretical values; [e]: NaFl measures the inhibition of veratridine-stimulated Na^+ influx in CHO cells expressing the α subunit of rat brain type IIA sodium channels. Reference compounds were tested three or more times and IC_{50} values are expressed as the mean ± SEM. Targeted compounds were tested two times and the IC_{50} values are reported as the average of the two numbers. The range of IC_{50}s is given in parentheses.

used contains 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 medium1640 (GIBCO) containing 10% fetal calf serum, 2 mL of geneticin solution (5 mg/1 mL), and penicillin/streptomycin (final concentration 20 units/mL of penicillin G sodium and 20 μg/mL streptomycin sulfate). Incubation experiments were run in a final volume of 500 μL. Freshly oxygenated KRH buffer (425 μL; pH = 7.4), drug solution in DMSO/H_2O:1/9 (25 μL), and veratridine/α-scorpion venom solution (25 μL of a 0.6 mM; final concentration 30 μM) were incubated for 11 min at 37 °C in a 5% CO_2 incubator at which time 25 μL of [^{14}C]guanidine containing KRH buffer solution was added (final concentration 250 μM of "cold" guanidine and 0.005 μCi of [^{14}C]guanidine). 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) 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 cocktail with the amount of radioactivity remaining determined using a scintillation counting techniques. The IC_{50} values were determined by linear regression analysis. The KRH (Krebs-Ringer HEPES) 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 (22mM), 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 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 in a 10% aqueous solution of DMSO so that the final concentration of drugs ranged from 0.01 to 100 μM. The control solution contained equal volumes of 0.1 N HCl and 0.1 N NaOH.

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