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Effect of polycyclic cage amines on the transmembrane potential of neuronal cells

Erika Grobler,[a] Anne Grobler,[b] Cornelis J. Van der Schyf[a,c] and Sarel F. Malan[a,*]

[a]*Department of Pharmaceutical Chemistry, North-West University, Potchefstroom, South Africa*
[b]*Department of Pharmaceutics, North-West University, Potchefstroom, South Africa*
[c]*Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, School of Pharmacy, Amarillo, TX 79106, USA*

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Abstract—A series of pentacycloundecylamine derivatives were synthesized and their influence on the transmembrane potential of human SH-SY5Y neuroblastoma cells was evaluated using laser scanning confocal microscopy in combination with the potentiometric dye tetramethylrhodamine methyl ester. Results indicate that these derivatives influence the profile of KCl-induced membrane depolarization and cause an overall reduction in cell membrane depolarization.

1. Introduction

Calcium (Ca^{2+}) plays a key role in the pathology underlying neurodegeneration although it is not the sole mechanism mediating neuronal cell death. Disregulation of Ca^{2+} influx through voltage operated Ca^{2+} channels (VOCC) as well as *N*-methyl-D-aspartate (NMDA) receptor operated channels is a major contributor to intracellular accumulation of excessive Ca^{2+}.[1–3] Although the NMDA receptor complex can be modulated by many endogenous compounds, the activation state of both aforementioned channels is regulated through changes in membrane potential.[4]

Neuronal Ca^{2+} concentrations are maintained through a multifaceted process consisting of Ca^{2+} influx and efflux, intracellular Ca^{2+} storage, and an intracellular Ca^{2+} buffering system. Calcium influx is gated by voltage operated calcium channels as well as by glutamate-controlled NMDA receptor operated channels. Efflux is controlled through calcium/sodium exchanger pumps as well as energy-dependent calcium-ATPase pumps. Despite existing homeostatic mechanisms, pathological elevations in intracellular Ca^{2+} do occur and lead to the inappropriate activation of normally dormant (or low level) calcium-dependent processes, which, in turn, result in metabolic disturbances and eventual neurodegeneration.[4,5]

Biological activity attributed to a class of polycyclic amine derivatives, the pentacycloundecylamines (Fig. 1), suggests possible neuroprotective abilities for these compounds through modulation of voltage activated sodium, potassium and Ca^{2+} channels, as well as interaction with NMDA receptor operated channels.

Studies evaluating the biological activity of these compounds indicate actions that include amongst others, neuroprotective activity, selectivity, and high affinity for the sigma-binding site.[6,7] The pentacycloundecylamine derivatives show structural similarities to known NMDA antagonists, and peripheral L-type calcium channel activity has also been described.[6,8,9] Recently, it was found that 6-benzylamino-3-hydroxyhexacyclo[6.5.0.0^{3,7}.0^{4,2}.0^{5,10}.0^{9,13}]tridecane inhibits L-type Ca^{2+} channels and sodium channels, as well as the fast

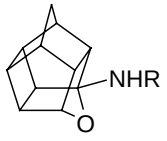

Figure 1. Generalized structure of polycyclic cage amines.

Keywords: Pentacycloundecylamines; Transmembrane potential; Laser scanning confocal microscopy; SH-SY5Y cells.
* Corresponding author. Tel.: +27 18 299 2266; fax: +27 18 299 4243; e-mail: fchsfm@puk.ac.za

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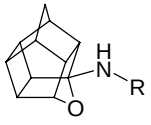
component of delayed rectifier potassium channels.[10] Although little is known about the mechanisms of CNS activity of the compounds, it is postulated that these derivatives could yet be of therapeutic value in the treatment of neurodegenerative disorders including Parkinson's and Alzheimer's diseases.[11]

Confocal microscopic imaging offers extreme high-quality images and can be used independently, or in combination with classical fluorescence techniques that are useful in studies to assess neuroprotective properties of novel drug molecules. Laser scanning confocal microscopy depends heavily on fluorescence as an imaging mode, which results in a high degree of sensitivity and enables the specific targeting of cellular components and dynamic processes in living cells. Such fluorescent probes are designed to localize in specific regions of the cell (i.e., mitochondria, endoplasmic reticulum, nucleus or membranes) or can be utilized to monitor dynamic cellular processes, for example, membrane potential, changes in pH, and concentrations of inorganic ions.[12]

In this study, the potentiometric probe tetramethyl-rhodamine methyl ester (TMRM)—used effectively in earlier studies in brain,[13] and in neurotoxicity studies in particular[14]—was employed in conjunction with laser scanning confocal microscopy to evaluate the influence of pentacycloundecylamine derivatives on the transmembrane potential in an SH-SY5Y neuroblastoma cell line.

A series of derivatives was carefully compiled to facilitate evaluation of a variety of chemical features (Table 1). The influence of an unsaturated system, cyclic and aliphatic substituents, hydrogen bonding capability, and side-chain length, volume, and flexibility were assessed. The structure of compounds of the type depicted in Table 1 have been studied extensively both in the crystal form[15] and in solution.[15,16] In solution, these cyclized aminol-type compounds exist tenaciously in the oxa-, rather than the aza-form, as depicted in the general structure seen in Table 1.[15,16] Compounds selected are all chiral and were tested as the racemate in this study. Earlier work by our group[17] showed that activities of the enantiomers of one of our selected compounds, NGP1-01 (**1**, Table 2), resolved at enantiomeric purity

Table 1. Compounds selected for synthesis and evaluation



Compound	R
1 (NGP1-01)	CH_2Ph
2	$CH_2C_6H_{11}$ (cyclohexylmethyl)
3	C_7H_{15}
4	CH_2Ph-3-OMe (3-methoxybenzyl)
5	$CH_2C_5H_4N$ (4-methylpyridine)
6	CH_3

Table 2. Average % fluorescence (±SEM) after KCl challenge

Compound	% fluorescence		
	$t = 0$ s	$t = 420$ s	$t = 870$ s
Control ($n = 4$)	100	48.44 ± 7.32	27.68 ± 3.54
1 ($n = 5$)	98.27	67.56 ± 3.45	48.97 ± 7.76
2 ($n = 4$)	98.16	56.74 ± 7.22	38.89 ± 10.38
3 ($n = 3$)	99.74	76.22 ± 8.81	37.40 ± 10.82
4 ($n = 6$)	100	58.38 ± 7.26	30.39 ± 9.69
5 ($n = 5$)	97.55	67.87 ± 5.17	42.81 ± 5.72
6 ($n = 6$)	100	73.12 ± 9.95	44.93 ± 5.42
Nifedipine ($n = 3$)	100	51.43 ± 6.46	23.50 ± 2.55

(e.e.) in excess of 96% (for both the + and the − enantiomers), did not differ from the racemate when tested in a calcium channel blocker assay.[17] Although it cannot be excluded that mechanisms by which these compounds influence membrane depolarization may be chirally dependent, we elected not to study that aspect in the current study. NGP1-01 (**1**) was selected as the lead compound in this investigation as it has thus far been the compound most extensively studied within this class.[11]

2. Results and discussion

Our data demonstrate clear differences between control experiments and the effects elicited by the respective test compounds (Table 2). Fluorescence measurements (Fig. 2) in experiments conducted in the absence of test compounds in response to KCl stimuli showed a decrease to 48.44% after 420 s with a total decline of 72.32% to a final value of 27.68% over an 870 s period. The aforementioned experiments adequately assess the response of cells to KCl stimuli without the application of additional external modulation and therefore served as control values.

At 870 s **2**, **3**, and **4** provided the least protection against KCl-induced depolarization with fluorescence decreasing to 38.89%, 37.40%, and 30.39%, respectively. Compound **3**, however, provided initial protection at $t = 420$ s with a decrease of only 23.52% compared to decreases of 51.56% in control experiments, and 41.42% and 41.62% for compounds **2** and **4**, respectively, at 420 s. It is not yet known what the in vivo neuroprotective implication of membrane depolarization protection at these levels may be.

The best overall protection against KCl-induced membrane depolarization was provided by compounds **1**, **5**, and **6** with fluorescence decreases of 30.71%, 29.68%, and 26.88% ($t = 420$ s) and final decreases (after 870 s) of 49.30%, 54.74%, and 55.07%, respectively (Figs. 3 and 4). Nifedipine failed to inhibit KCl-induced depolarization in these experiments.

3. Conclusion

Results indicate that 8-benzylamino-8,11-oxapentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**1**) and its derivatives influence the profile of KCl-induced membrane depolar-

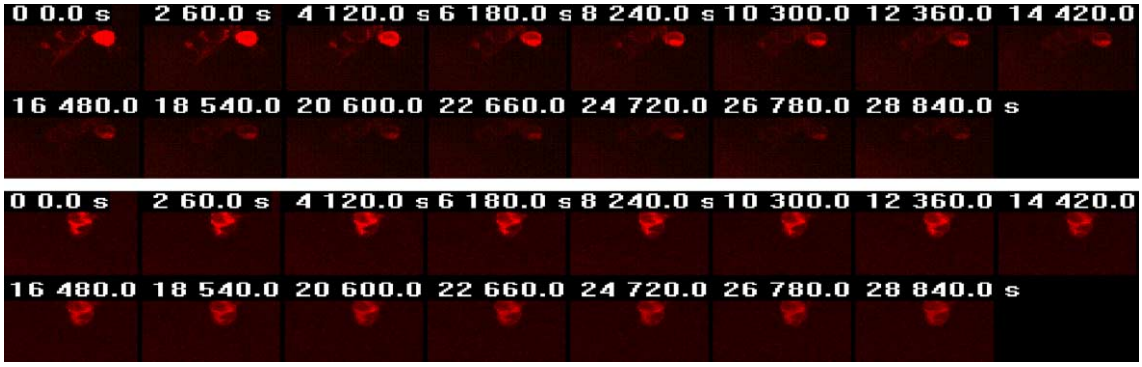


Figure 2. Confocal time series from which fluorescence values were determined. Top slide represents KCl control and bottom slide KCl in the presence of **1**.

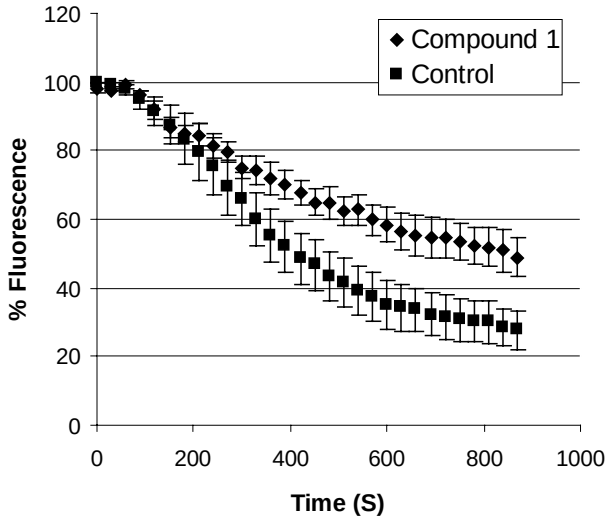


Figure 3. Fluorescence values (average ± SEM) of cells incubated with compound **1** ($n = 5$), plotted with average values of KCl-control experiments ($n = 6$).

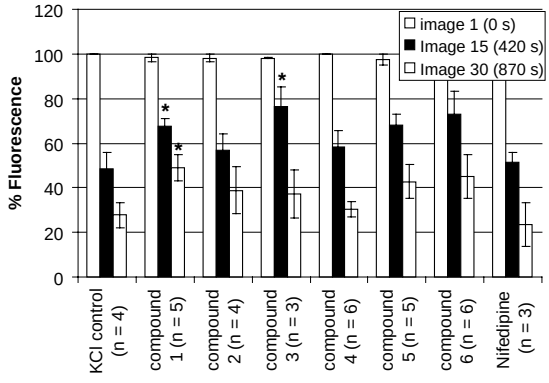


Figure 4. Average fluorescence (±SEM) values for respective experiments at images 0, 15, and 30 (*statistically significant differences from control values; $p \leqslant 0.05$). Three treatments provided statistically significant protection against KCl-induced depolarization: compound **1** at 420 and 870 s and compound **3** at 420 s (see text).

ization. The test compounds caused an overall reduction in membrane depolarization with compounds **1**, **5**, and **6** causing the most prevalent reductions in depolarization. Compound **3** caused initial stabilization of membrane potential but failed to provide statistically significant protection over the 870 s period (Fig. 4). It is difficult to speculate on the significance of this finding. Compound **1** showed the best overall inhibition of KCl-induced membrane depolarization with a significant 21.29% difference ($p \leqslant 0.05$) in fluorescence after 870 s in comparison to control values (Figs. 3 and 4). Compared to KCl control, compounds **5** and **6** exhibited an overall difference of 15.13% and 17.25% in final fluorescence while compounds **2**, **3**, and **4** showed less inhibition.

These results indicate that cyclic unsaturated substituents on the pentacycloundecane template are important for the activity of these compounds. The introduction of hydrogen bonding moieties (**5**) and a decrease in molecular volume (**6**) could also be structural changes that influence activity.

4. Experimental

4.1. General procedures

Reagents were obtained from D.H. Chemicals (UK), Sigma–Aldrich (UK), Fluka (Switzerland), and Molecular Probes (The Netherlands), and reaction and elution solvents were purchased from commercial sources. Melting points were measured with a Gallenkamp melting point apparatus and IR spectra were recorded on a Nicolet 470 FT-IR spectrophotometer. Mass spectra and HR-MS were recorded using a VG 7070E mass spectrometer. NMR spectra were acquired on a Varian Gemini 300 with ^{1}H spectra recorded at a frequency of 300.075 MHz and ^{13}C spectra at 75.462 MHz. Chemical shifts are reported in parts per million (ppm) relative to the internal standard, tetramethylsilane (TMS). The following abbreviations indicate multiplicities of the respective signals: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet and m, multiplet.

4.2. Synthesis

Pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane–8,11-dione was obtained by photocyclization of the Diels–Alder adduct

resulting from the reaction between *p*-benzoquinone and cyclopentadiene.[18] The resultant diketone (5 g, 0.029 mol) was dissolved in tetrahydrofuran (THF, 50 ml) and cooled to ca ±5 °C in ice. An equimolar quantity of the desired amine was slowly added under stirring. The colorless precipitate that formed after about 10 min was filtered and washed with cold THF to render the hydroxylamine. This product was dehydrated in dry benzene under Dean–Stark conditions for 1 h or until no more water collected in the container. Evaporation yielded the Schiff base as a yellowish oil. Reduction of this imine was done with sodium borohydride ($NaHB_4$) in dry methanol (30 ml) and dry THF (150 ml) for 24 h at room temperature. The solvent was removed under reduced pressure and water (100 ml) was added. The mixture was extracted with dichloromethane (4× 50 ml) and the combined organic fractions were washed with water before drying over magnesium sulfate. Evaporation yielded the desired products.

4.2.1. 8-Benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane (1). The yellow oil obtained was purified with column chromatography (1:1:1 petroleum–ether–dichloromethane–ethyl acetate) to produce a colorless wax. Recrystallization from anhydrous ethanol resulted in the formation of pure white crystals (yield: 3.00 g, 0.03 mol, 39.02%).

$C_{18}H_{19}NO$; mp 78 °C; HR-MS: calcd 265.147, exptl 265.146; IR (KBr) ν_{max}: 3308, 2970, 1352, 1009 cm^{-1}; MS (EI, 70 eV) *m/z*: 265 (M^+), 237, 186, 131, 91, 65, 28; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 7.42–7.09 (m, 5H, H-16, 17, 18, 19, 20), 4.74 (t, 1H, J = 5.33 Hz, H-11), 4.11 (ABq, 2H, J_{AB} = 13.46 Hz, H-14a, 14b), 2.98–2.47 (3× m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 2.27 (br s, 1H, NH), 1.95:1.62 (ABq, 2H, J = 10.11 Hz, H-4a, b); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 140.85 (s, C-15), 128.40 (d, C-16, 20), 127.84 (d, C-17, 19), 126.78 (d, C-18), 109.60 (s, C-8), 82.48 (d, C-11), 55.27 (d, C-7/9), 54.78 (d, C-7/9), 47.80 (t, C-14), 44.90 (d, 1C), 44.86 (d, 1C), 44.57 (d, 1C), 43.25 (t, C-4), 43.14 (d, 1C), 42.00 (d, 1C), 41.54 (d, 1C).

4.2.2. 8-Cyclohexylmethylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (2). The yellow oil obtained was purified with column chromatography (1:9 ethanol–ethyl acetate) to produce a yellowish oily residue (yield: 2.07 g, 0.0077 mol, 24.69%).

$C_{18}H_{25}NO$; HR-MS calcd 271.193, exptl 273.193; IR (KBr) ν_{max}: 3324, 2914, 1449, 1357, 1014; MS (EI, 70 eV) *m/z*: 271 (M^+), 188, 176, 159, 131, 91, 56, 28; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 4.67 (t, 1H, J = 5.22 Hz, H-11), 2.51–2.89 (3× m, 9H, H-1, 2, 3, 5, 6, 7/9, 10, 14a, 14,b), 2.39 (m, 1H, H-7/9), 1.93 (br s, 1H, H-13), 1.89:1.53 (ABq, 2H, J = 10.3 Hz, H-4a, 4b), 1.78–0.97 (4× m, 11H, H-15, 16, 17, 18, 19, 20); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 109.77 (s, C-8), 82.34 (d, C-11), 55.04 (d, C-7/9), 54.73 (d, C-7/9), 50.24 (t C-14), 44.84 (d, 2C), 44.48 (d, 1C), 43.23 (d, 1C), 41.88 (d, 1C), 41.51 (d, 1C), 38.29 (d, C-15), 26.64 (t, C-16, 20), 25.99 (t, C-17, 19).

4.2.3. 8-Heptylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (3). The dark yellow oil obtained was purified with column chromatography (1:1:8 ethyl acetate–dichloromethane–petroleum ether) to produce a yellowish oil (yield: 2.39 g, 0.0087 mol, 29.121%).

$C_{18}H_{27}NO$; HR-MS: calcd 273.413, exptl 273.412; IR (KBr) ν_{max}: 3308, 2924, 1465, 1357, 1019; MS (EI, 70 eV) *m/z*: 273 (M^+), 188, 131, 91, 43, 29; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 4.69 (t, 1H, J = 10.43 Hz, H-11), 2.60–2.87 (3× m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 2.41 (m, 1H, H-13), 2.00:1.56 (ABq, 2H, J = 10.53 Hz, H-4a, 4b), 1.35 (3× m, 12H, H-14a, b, 15, 16, 17, 18, 19), 0.92 (t, 3H, J = 7.0 Hz, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 109.73 (s, C-8), 82.36 (d, C-11), 55.21 (d, C-7/9), 54.75 (d, C-7/9), 44.81 (t, C-14), 44.66 (d, 1C), 44.48 (d, 1C) 43.75 (d, 1C) 43.24 (t, C-4) 43.04 (d, 1C), 41.71 (d, 1C), 41.51 (d, 1C), 31.77 (t, C-15), 31.74 (t, C-16), 31.03 (t, C-17), 29.10 (t, C-18), 27.22 (t, C-19).

4.2.4. 8-(3-Methoxybenzylamino)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (4). The dark yellow oil obtained was purified with column chromatography (1:3 ethyl acetate–petroleum–ether) to produce a yellowish oil (yield: 2.83 g, 0.0096 mol, 32.98%).

$C_{19}H_{21}NO_2$; HR-MS: calcd 295.157, exptl 295.158; IR (KBr) ν_{max}: 3324, 2965, 1603,1490, 1250; MS (EI, 70 eV) *m/z*: 295 (M^+), 136, 121, 91, 77, 28; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 7.23 (t, 1H, J = 5.23 Hz, H-19), 6.97 (m, 2H, H-16, 20), 6.81 (m, 1H, H-18), 4.78 (t, 1H, J = 5.25 Hz, H-11), 4.07 (ABq, 2H, J = 13.73 Hz, H-14a, 14b), 3.85 (s, 3H, OCH_3), 2.44–2.99 (3× m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 2.26 (br s, 1H, NH), 1.96:1.61 (ABq, 2H, J = 10.34, H-4a, 4b); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 159.71 (s, C-17), 142.58 (s, C-15), 129.28 (d, C-19), 120.28 (d, C-20), 113.29 (d, C-16), 112.32 (d, C-18), 109.57 (s, C-8), 82.47 (d, C-11), 55.72 (d, C-7/9), 55.28 (q, CH_3), 54.75 (d, C-7/9), 47.71 (t, C-14), 44.87 (d, 1C), 44.84 (d, 1C), 44.55 (d, 1C), 43.23 (t, C-4), 43.14 (d, 1C), 42.00 (d, 1C), 41.53 (d, 1C).

4.2.5. 8-[(1-Pyridinyl)methylamino]-8,11oxapentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (*5*). A yellow oil with signs of crystallization was obtained. This product was purified with column chromatography (9:1 ethyl acetate–ethanol) to produce white crystals (yield: 3.424 g, 0.0129 mol, 42.85%).

$C_{17}H_{18}N_2O$; mp 111.3 °C; HR-MS: calcd 266.142, exptl 266.142; IR (KBr) ν_{max}: 3293, 2970, 1598, 1367, 1004, 845; MS (EI, 70 eV) *m/z*: 266 (M^+), 187, 174, 131, 92, 65, 39, 28; ^1H NMR (300 MHz, $CDCl_3$) δ_H (spectrum 19): 8.63 (d, 2H, J = 4.42 Hz, H-17, 19), 7.38 (d, 2H, J = 4.67 Hz, H-16, 20), 4.73 (t, 1H, J = 5.22 Hz, H-11), 4.18 (ABq, 2H, J = 15.39 Hz, H-14a, 14b), 2.59–2.97 (3× m, 9H, H-1, 2, 3, 5, 6, 7, 9, 10, 13), 2.03:1.67 (ABq, 2H, J = 10.44 Hz, H-4a, 4b); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 150.29 (s, C-15), 149.64 (d, C-17, 19), 122.46 (d, C-16, 20), 109.39 (s, C-8), 82.48 (d, C-11), 55.32 (d, C-7/9),

54.70 (d, C-7/9), 46.51 (t, C-14), 44.86 (d, 1C), 44.79 (d, 1C), 44.52 (d, 1C), 43.21 (t, C-4), 43.10 (d, 1C), 41.91 (d, 1C), 41.48 (d, 1C).

4.2.6. 8-Methylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (6). The yellow oil obtained was purified with column chromatography (9:1 ethyl acetate–ethanol) to produce a yellowish oil (yield: 1.60 g, 0.0084 mol, 28.13%).

$C_{12}H_{15}NO$; HR-MS: calcd 189.254, exptl 189.254; IR (KBr) ν_{max}: 3324, 2965, 1367, 999, 855; MS (EI, 70 eV) *m/z*: 189 (M^+), 131, 91, 28; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 4.69 (t, 1H, J = 5.30 Hz, H-11), 2.40–2.89 (4× m, 11H, H-1, 2, 3, 5, 6, 7, 9, 10, CH_3), 2.06 (s, 1H, NH), 2.62:2.01 (ABq, 2H, J = 10.44 Hz, H-4a, 4b); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 109.84 (s, C-8), 82.45 (d, C-11), 55.05 (d, C-7/9), 54.79 (d, C-7/9), 44.78 (d, 1C), 44.76 (d, 1C), 44.53 (d, 1C), 43.25 (t, C-4), 42.96 (d, 1C), 41.87 (d, 1C), 41.52 (d, 1C), 30.01 (q, CH_3).

4.3. Membrane potential determinations

4.3.1. Cell cultures. Human SH-SY5Y neuroblastoma cells [obtained from the American Type Culture Collection (ATCC), Catalog No. CRL-2266] were used in this study. Cells were maintained in DMEM containing 10% fetal calf serum; 100 units penicillin/ml; 100 μg streptomycin/ml and 0.25 μg fungizone/ml and incubated at 37 °C in a 5% CO_2 and 95% O_2 humidified atmosphere. Cells duplicated approximately once every 48 h and, when 95% confluent, were detached from the flask bottom by means of trypsination (incubation with trypsin/versine for approximately 10 min) and seeded in new flasks at a density of no less than 1/6 confluency.

The self-adherent cells were seeded onto 25 mm diameter sterile glass coverslips at densities of 2×10^5 cells/Petri dish in a 2 ml culture medium. The Petri dishes were covered and incubated for 5 h to allow adherence to coverslips before dye loading commenced. When not used immediately, sufficient culture medium was added to Petri dishes and cells were incubated overnight.

4.3.2. Dye loading. Culture medium was replaced with normal DMEM containing 500 nM of the fluorescent potentiometric indicator, tetramethylrhodamine methyl ester (TMRM, Molecular Probes, the Netherlands), and incubated for 40 min at 37 °C to allow complete dye equilibration.[12–14] Dye containing medium was replaced with standard recording solution containing 126 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, and 15 mM glucose of which the pH was adjusted to 7.3 with NaOH and osmolarity to 320 mOsmol/L with sucrose. One hundred nanomoles of TMRM was added to minimize dye leakage from cells.

4.3.3. Imaging and analysis. All experiments were conducted at room temperature. Pentacycloundecane derivatives were dissolved in dimethylsulfoxide (DMSO) and diluted to a 100 μM concentration in normal recording solution (final concentration DMSO in all solutions = 0.1%). Glass coverslips were mounted in a recording chamber during optical imaging and cells were incubated with the selected pentacycloundecane derivative for 2 min. Application of a high concentration potassium solution (containing KCl 100 mM, NaCl 40 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, Hepes 10 mM, and glucose 15 mM to which pH was adjusted to 7.3 with NaOH and osmolarity adjusted to 320 mOsmol with sucrose) was used to initiate membrane depolarization. Control experiments, conducted in the absence of test compounds, were composed of a 2 min incubation in a normal recording solution followed by the application of high concentration potassium solution.

A time series of 30 optical scans were recorded at 30 s intervals on a laser scanning confocal microscope (Nikon PCM 2000). Cells were exposed to the 505 nm line of a Spectra-Physics He/Ne laser and emission was detected at 568 nm. A 60 × 1.4 NA Apo Planar (Nikon) oil immersion objective was used for imaging. A 5 μm pinhole together with a neutral density filter at 10% was employed to prevent dye bleaching as well as light scattering. A scan speed of 3 μs/scan was used, and laser power and gain settings were kept constant in comparative experiments to enable quantitative analysis. Image processing and analysis were performed using supplied Nikon software.

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