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Full Paper

Synthesis and Biological Evaluation of Pentacycloundecylamines and Triquinylamines as Voltage-Gated Calcium Channel Blockers

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Preclinical studies for neurodegenerative diseases have shown a multi-targeted approach to be successful in the treatment of these complex disorders with several pathoetiological pathways. Polycyclic compounds, such as NGP1-01 (**7a**), have demonstrated the ability to target multiple mechanisms of the complex etiology and are referred to as multifunctional compounds. These compounds have served as scaffolds with the ability to attenuate Ca^{2+} overload and excitotoxicity through several pathways. In this study, our focus was on mitigating Ca^{2+} overload through the L-type calcium channels (LTCC). Here, we report the synthesis and biological evaluation of several novel polycyclic compounds. We determined the IC_{50} values for both the pentacycloundecylamines and the triquinylamines by means of a high-throughput fluorescence calcium flux assay utilizing Fura-2/AM. The potential of these compounds to offer protection against hydrogen peroxide-induced cell death was also evaluated. Overall, 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01, **7a**) had the most favorable pharmacological profile with an IC_{50} value of 86 μM for LTCC inhibition and significant reduction of hydrogen peroxide-induced cell death. In general, the triquinylamines were more active as LTCC blockers than the oxa-pentacycloundecylamines. The aza-pentacycloundecylamines were potent LTCC inhibitors, with 8-hydroxy-*N*-phenylethyl-8,11-azapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**8b**) also able to offer significant protection in the cell viability assays.

Keywords: L-type calcium channel (LTCC) blockers / Multifunctional drugs / Neurodegeneration / Pentacycloundecylamine / Triquinylamine

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Introduction

Perturbation of Ca^{2+} homeostasis and subsequent Ca^{2+} overload have been implicated in acute neurological disorders such as ischemia, trauma and epilepsy, and in chronic neurological disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [1]. For these conditions, one common key mediator in the neuronal death is calcium (Ca^{2+}). Although physiological elevation of intracellular Ca^{2+} is part of normal cell function and homeostatic mechanisms exist to maintain the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), excessive influx of Ca^{2+} together with Ca^{2+} release from intracellular compartments can overwhelm Ca^{2+}-regulatory mechanisms and lead to cell

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death [2]. Apart from the *N*-methyl-D-aspartate receptor (NMDAR), voltage-gated Ca^{2+} channels (VGCCs), such as the L, N, T, and P/Q-type calcium channels have been implicated as a major gateway for Ca^{2+} entry from the extracellular environment [3]. One approach in the treatment of neurodegenerative diseases would be to block Ca^{2+} influx through the L-type Ca^{2+} channels (LTCCs). Blockers of these channels have been shown to be protective in models of acute neurological disorders such as ischemia [4]. The ability of known LTCC blockers such as the 1,4-dihydropyridines (DHP) nimodipine and nitrendipine, the phenylalkylamine (PAA) verapamil, and the benzothiazepine (BTZ) diltiazem to attenuate excitotoxicity caused by an increase in $[Ca^{2+}]_i$ is well established [5].

Multifunctional or multimechanistic drugs [6] hold an advantage in cases such as ischemic stroke where a combination of therapies has been shown to be beneficial as shown with the combination of memantine, a polycyclic compound, and clenbuterol [7]. Such an approach retains the beneficial therapeutic effect of combining multiple drugs, while simultaneously limiting the side-effect profile to that of only one drug [8], and it is this multimechanistic action that gives these compounds a unique advantage in the treatment of neurodegenerative disorders [9]. An example of a multimechanistic drug is the polycyclic compound 8-benzylamino-8,11-oxapentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01). Initially characterized as a LTCC blocker [10], NGP1-01 was later found to also be a potent uncompetitive NMDAR channel blocker [11] and NGP1-01 (**7a**) thus has the ability to modulate Ca^{2+} influx through both ion channel types. In addition, NGP1-01 (**7a**) has also been shown to be neuroprotective *in vivo* using the middle cerebral artery occlusion mouse model of stroke [12], and also offer protection in a transient model of stroke following reperfusion [13]. Although much is known about pentacycloundecylamines such as **7a** (reviewed in Refs. [14] and [15]), further assessment as LTCC blockers and the ability of these compounds to offer protection against induced cell death is necessary. The aim of this paper is to present the synthesis and biological evaluation for derivatives of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane-8-11-dione (**4**) scaffold, such as oxa- and aza-bridged pentacycloundecylamines (**7a–e** and **8a–c**), and the tricyclo-[6.3.0.0^{2,6}]undecane-3,11-dione scaffold, such as the aza-triquinylamines (**14a–f**). The latter was obtained by reductive amination of the flash vacuum pyrolysis product of **4**. The IC_{50} values for these compounds as LTCC blockers were determined and the ability of these compounds to attenuate peroxide-induced cell death in PC12 cells by means of the LDH (lactate dehydrogenase) and Trypan blue staining assays was evaluated.

Results and discussion

Chemistry

A series of aza-pentacycloundecylamines (**8a–c**) and aza-triquinylamines (**14a–f**) to supplement the series of oxa-pentacycloundecylamines (**7a–e**) was synthesized. The pentacycloundecylamine **7a** served as a lead compound and R substitution was guided by structure–activity relationship (SAR) data from previous studies [16]. In short, pentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**4**), also called Cookson's "cage" compound, was synthesized by photochemically initiated intramolecular [2 + 2] cycloaddition of the Diels–Alder adduct (**3**) obtained from the reaction of 1,3-cyclopentadiene (**2**) with *p*-benzoquinone (**1**) [17]. The synthesis of the oxa-pentacycloundecylamine derivatives (**7a–e**), illustrated in Scheme 1, has been described previously by our group [16c, 18]. These compounds can be obtained by the reductive amination of **4** with the desired primary amine. To obtain the oxa-bridge compound, reduction of the Schiff base/imine intermediate (**6**) was performed with sodium borohydride, to facilitate ring closure through a transannular reaction. In order to obtain the aza-bridged derivatives (**8a–c**), reductive amination was done with sodium cyanoborohydride.

One of the required pharmacophoric features of polycyclic LTCC blockers is a bulky, lipophilic scaffold [10a]. The *cis*,*syn*,*cis* triquinane scaffold (**10**), which is a thermal-fragmented derivative of the pentacycloundecane (**4**), retains the bulkiness (±10%) of **4** as expressed in molecular volume (**4**: 498 Å^3 and **10**: 552 Å^3) and solvent accessible surface area (**4**: 325 Å^2 and **10**: 351 Å^2) [16a]. The triquinane scaffold, in addition to contributing a larger surface area to the geometric conformation, was also found to be a less-strained asymmetric compound with greater flexibility [19]. The *cis*,*syn*,*cis* triquinane system can only be derived through thermal fragmentation and we aimed to optimize and simplify the method for the synthesis of the triquinylamines by redesigning the apparatus (Fig. 1) needed to perform the thermal fragmentation (step a, Scheme 2). The thermal fragmentation was performed through flash vacuum pyrolysis (FVP), also known as flash thermolysis. The synthesis for the aza-triquinylamines (**14a–f**) is shown in Scheme 2. We utilized a combination of methods described by several authors for the design and synthesis of the apparatus [16a, 20]. In short, the thermal fragmentation of the saturated four-membered ring (**4**) afforded the *cis*,*syn*,*cis* triquinane system: tricyclo[6.3.0.0^{2,6}]-undecane-4,9-diene-3,11-dione (**9**). FVP was carried out in a quartz vigreux column connected to a substrate tube and a vacuum line provided with a liquid nitrogen cold trap. Sublimation of the substrate **4**, which was contained in a borosilicate glass tube, was achieved at 150°C under vacuum of 1 Torr. The sublimate traveled through the quartz vigreux column, which was heated to a temperature of 650°C under vacuum of 1 Torr. The condensate was deposited in a specially designed freeze fall, that was cooled with liquid nitrogen, and afforded the tricyclo[6.3.0.0^{2,6}]undecane-4,9-diene-3,11-dione (**9**). We designed and built the tube furnaces to heat the sublimation tube, and all the glass components and quartz column were custom made. Our improvements on the design attributed to a more safe and effective synthesis, increasing in yield from 64.7% previously reported [16a] to 81.7% and delivered a throughput rate of 1 g every 30 min.

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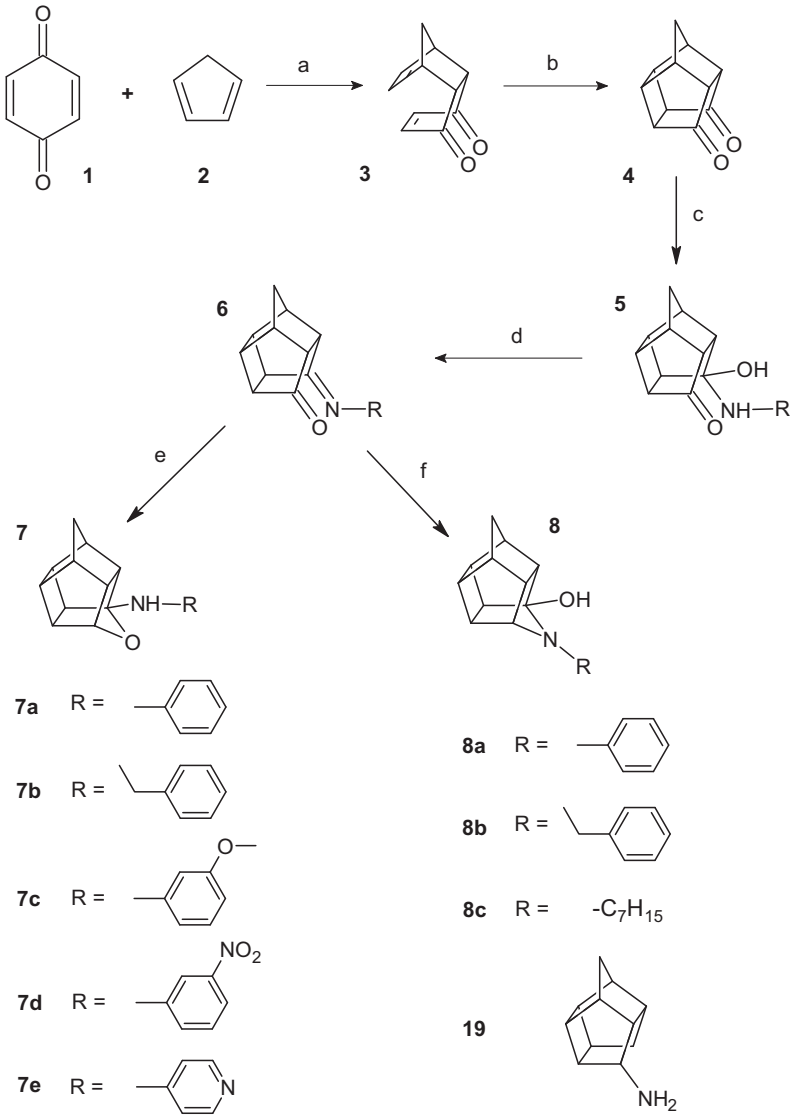


Scheme 1. Synthesis of compounds **7** and **8**. Reagents and conditions: (a) benzene, 5°C; (b) acetone, UV, 6 h; (c) THF, 5°C, NH_2–R; (d) benzene, Δ, $–H_2O$, 1 h; (e) MeOH, THF, $NaBH_4$; (f) HOAc, MeOH, $NaBH_3CN$, 12 h. The synthesis of **19** was achieved by selective decarboxylation of (**4**) by means of Huang–Minlon reaction followed by reductive amination with $NaBH_4$. Compound (**19**) was previously synthesized by Geldenhuys et al., and the synthesis is described in the original paper [19].

The temperature was measured with K-type thermocouples and regulated with a Shinko Ramp/Soak Auto tune PID controller (Wika Instruments, Johannesburg, South Africa). Before pyrolysis commenced, the entire apparatus was flushed with nitrogen gas and then evacuated to 1 Torr by a high-capacity rotary vane oil pump. This step is essential for successful sublimation and to eliminate tar formation.

The product of thermolysis (**9**) was hydrogenated over 10% Pd-C catalyst to yield tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (**10**). The aza-triquinylamine compounds (**14a–f**) were obtained by reductive amination (Scheme 2, steps d–f) of **10** with the desired primary amine side chains. The aza-bridge compound was obtained by reduction of the imine intermediate (**13**) with sodium cyanoborohydride. To obtain the oxa-bridge 3-hydroxy-3,11-oxatricyclo[6.3.0.0^{2,6}]undecane (**11**), reduction of the diketone (**10**) was accomplished with sodium borohydride to facilitate ring closure through a transannular reaction. The aim was to compare the two scaffolds (triquinane vs. pentacycloundecane) by adding similar substituents. For both series, we included compounds where the aromatic moiety was replaced with an aliphatic side chain (**8c**, **14d**, and **19**) to explore the functional role of the aromatic moiety in isolation, since it is reasoned that activities for these compounds as LTCC blockers are dominated by the lipophilic amine substituent with the polycyclic scaffold only contributing a larger surface area to the geometric conformation [16c]. Several compounds that contain electron withdrawing or donating moieties (**7c**, **7d**, and **14e**) were also included and the chain length was altered to assess binding within the channel pore (**7b**, **8b**, **14b**, and **14c**). Detailed descriptions of

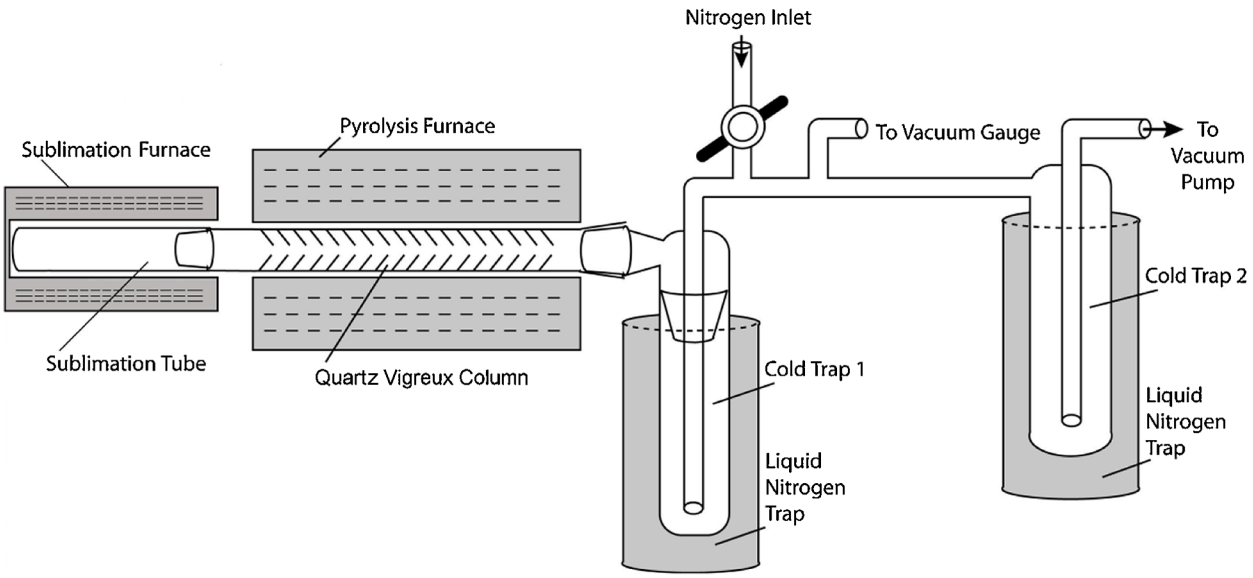
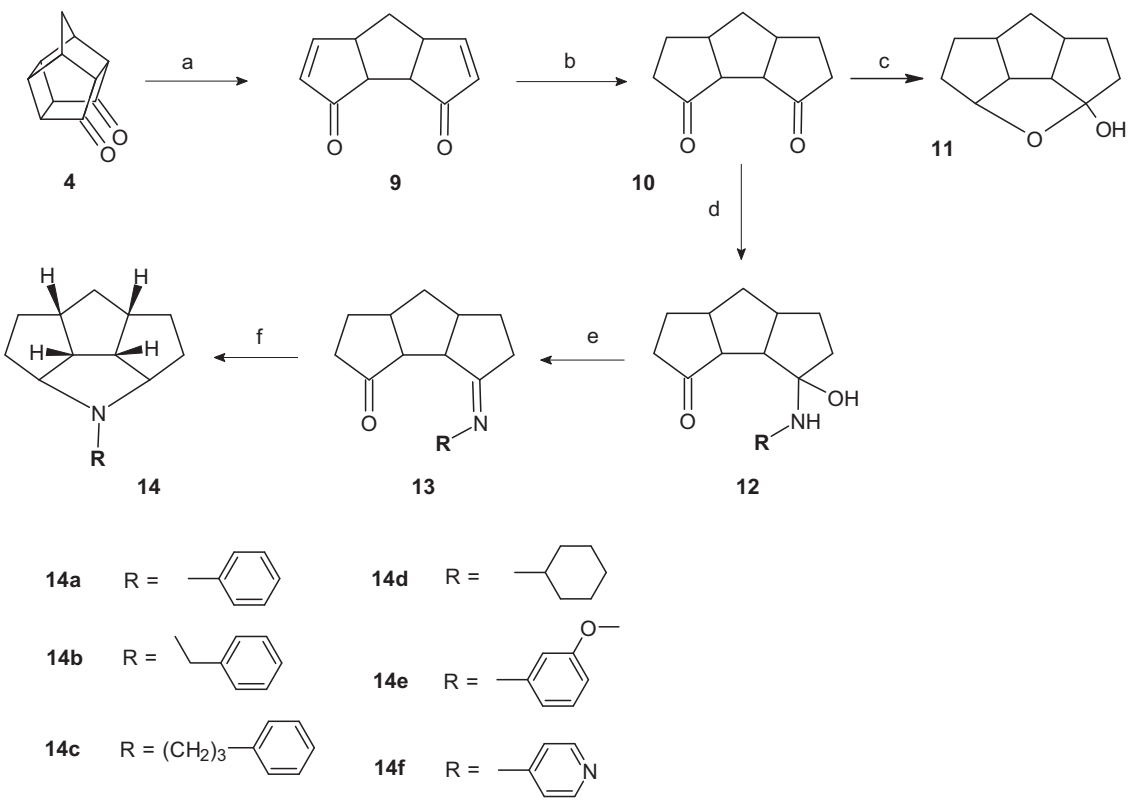


Figure 1. Schematic representation of the apparatus for flash vacuum pyrolysis (FVP).



Scheme 2. Synthesis of compounds **11** and **14**. Reagents and conditions: (a) Δ, 650°C, 1 Torr, 45 min; (b) EtOAc, 2 atm H_2, 10% Pd/C, 40 min; (c) MeOH, THF, $NaBH_4$; (d) THF, 5°C, NH_2–R, 6 h; (e) benzene, Δ, $–H_2O$, 1 h; (f) MeOH, THF, $NaBH_3CN$, 18 h.

all the synthesis performed and structure elucidation are given in the Experimental section.

Fluorescence measurement of calcium influx

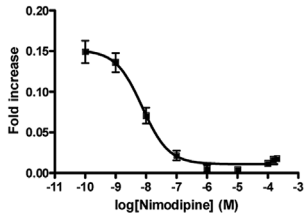
To examine the ability of these compounds to modulate calcium influx through the VGCC, and to determine their IC_{50} values, we developed a high-throughput fluorescence Ca^{2+} flux assay in undifferentiated rat pheochromocytoma cells (PC12 cells) loaded with Fura-2/AM. Depolarizing the cell membrane by increasing the extracellular $[K^+]_0$ is known to increase the influx of Ca^{2+} through the VGCC [21]. PC12 cells responded to the addition of 50 mM KCl, with a rapid increase in $[Ca^{2+}]_i$ (Supporting Information Fig. SA.1(a–t)). Depolarization with 50 mM KCl in the absence of any test compound served as the control for normal physiological function and experiments were done in triplicate. Several known LTCC blockers were evaluated to serve as references for the amplitude of Ca^{2+} influx suppression and to confirm that LTCC were involved. The reference compounds gave a concentration-dependent suppression of Ca^{2+} influx (Supporting Information Fig. SA.1(a–d)). For this assay, the IC_{50} values for reference compounds were determined to be 0.002 μM (nitrendipine), 0.008 μM (nimodipine), 0.328 μM (verapamil), and 0.449 μM (diltiazem). We then proceeded to evaluate the ability of the polycyclic amine compounds, which included the oxa-pentacycloundecylamines (**7a–e**), aza-pentacycloundecylamines (**8a–c**), and aza-triquinylamines (**14a–f**), to suppress Ca^{2+} influx after depolarization with 50 mM KCl (Supporting Information Fig. SA.1(e–t)) and calculated their IC_{50} values. The IC_{50} values are summarized in Table 1 and the log concentration–response graphs are presented in Fig. 2a–t. According to their inhibitory potency, the test compounds fell into three categories, with **14c** and **7d** (IC_{50} value of 0.398 and 0.580 μM, respectively) being the most potent and matching the IC_{50} values of the reference compounds. Compounds **8b**, **8c**, **14e**, and **14f** fell into the second category with IC_{50} values ranging from 5 to 11 μM; and the remainder fell into the third category with IC_{50} values ranging from 35 to 86 μM. All of the compounds tested had higher potency as LTCC blockers than the lead compound **7a** (NGP1-01, $IC_{50} = 86$ μM).

When comparing the dose–response profiles of the synthesized compounds to the reference compounds (Fig. 2a–q), several observations can be made. All of the compounds were able to block K^+-induced Ca^{2+} influx in a dose-dependent manner. Compound **14c** ($IC_{50} = 0.398$ μM), an aza-triquinylamine with a phenyl propyl substituent had a similar inhibition profile to nimodipine and nitrendipine. Compounds **7d** and **14f**, with the second and third lowest IC_{50} values (0.580 and 5 μM, respectively) had similar inhibition profiles to verapamil and diltiazem. For compounds **7e**, **11**, and **19**, we observed an agonist-like sigmoidal dose–response profile (Fig. 2r–t). These were compounds that demonstrated a potentiating effect. A possible explanation could be that neither compound **11** nor **19** had substituents that could extend to interact with binding sites within the channel pore; therefore these compounds might act in a similar manner as (S)-Bay K8644 and FPL 64176 (LTCC agonist or activators) [22], by prolonging Ca^{2+} channel current activation and deactivation and/or increasing the probability of channel opening and the mean channel open time. This observation was also made when we evaluated the lipophilic scaffolds **4** and **10** (data not shown). The pentacycloundecane (**4**) had no activity, while the triquinane-dione (**10**) had a slight potentiating effect but no antagonistic activity. For compound **7e**, a possible explanation for lack of activity could be drawn from an observation made in a study by Malan et al. [16c], in which the author concluded that compounds such as **7e** with low-calculated energy minima where the oxa-bridged pyridin-4-ylmethylamine side chain folds back onto the polycyclic structure displayed poor inhibition of calcium current in a whole cell patch clamp experiment. Thus, the geometric orientation could be unfavorable for binding especially since its counterpart, the triquinane, **14f** with the aza-bridged pyridin-4-ylmethyl substituent showed favorable activity ($IC_{50} = 5$ μM). We also observed that for most compounds, the graphs illustrate an inhibitory potency which increased in a dose-dependent manner between 10 and 200 μM. However, between 0.0001 and 1 μM we observed a slight potentiating effect which could be due to Ca^{2+}-induced Ca^{2+} release (CICR). These compounds may not have the ability at lower concentrations to block KCl-induced increases in $[Ca^{2+}]_i$, which has been shown to release Ca^{2+} from intracellular stores [23]. We will attempt to further elaborate on this

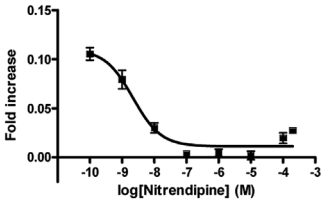
Table 1. The IC_{50} (μM) values for the inhibition of K^+-induced Ca^{2+} influx as determined experimentally and predicted logP for the series of 8,11-oxapentacycloundecylamines (7a–d); 8,11-azapentacycloundecylamines (8a–c), and 3,11-azatricycloundecylamines (14a–f).

Compound	IC_{50} (μM)	logP
7a	86	2.11
7b	51	2.4
7c	42	1.95
7d	0.580	–
8a	35	1.85
8b	8	2.14
8c	7	2.78
14a	74	3.67
14b	310.9	3.96
14c	0.398	3
14d	68	4.06
14e	11	3.51
14f	5	2.45

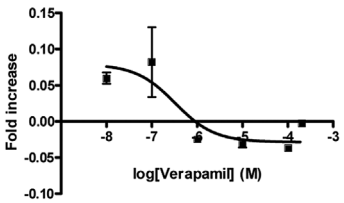
Experimental values are means of at least three experiments ($n = 3$). IC_{50} values of reference compounds were calculated to be: nimodipine 0.008 μM ($n = 6$), nitrendipine 0.002 μM ($n = 3$), verapamil 0.328 μM ($n = 3$), and diltiazem 0.449 μM ($n = 3$).



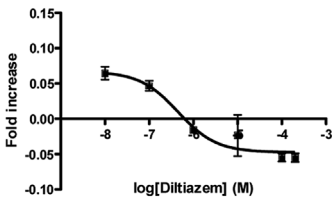
(a)



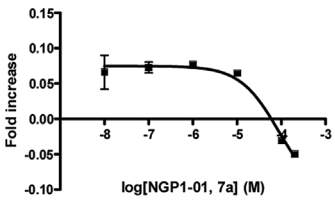
(b)



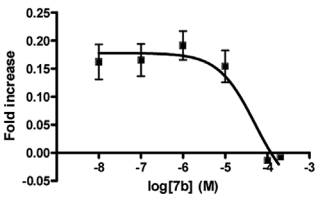
(c)



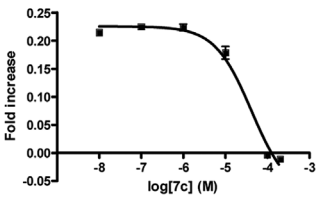
(d)



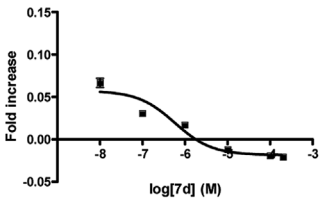
(e) **7a** (NGP)



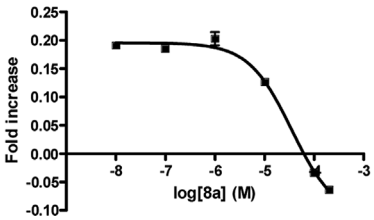
(f) **7b**



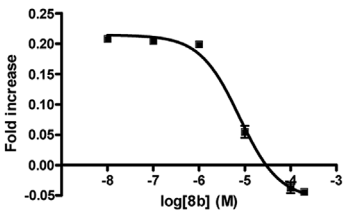
(g) **7c**



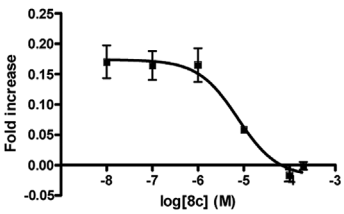
(h) **7d**



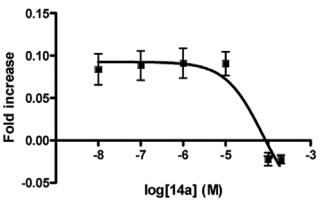
(i) **8a**



(j) **8b**

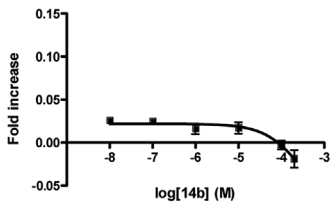


(k) **8c**

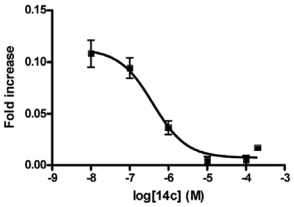


(l) **14a**

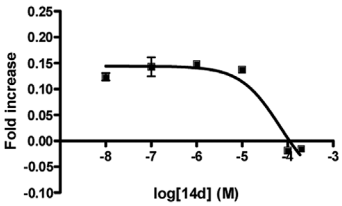
Figure 2. The dose–response relationship of (a–q) indicates antagonist inhibition of K^+-induced Ca^{2+} influx. The dose–response relationship of (r–t) indicates a potentiating effect after K^+-induced depolarization. Results are presented as the mean ± STDEV (n = 3–6). Where error bars are not shown, these are covered by the point itself.



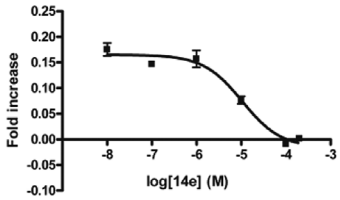
(m) **14b**



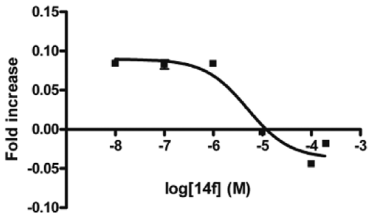
(n) **14c**



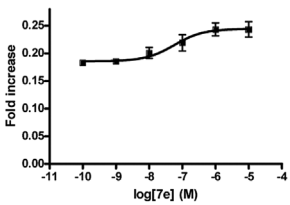
(o) **14d**



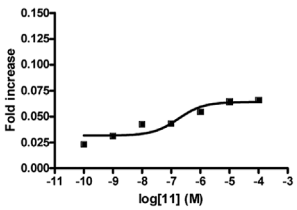
(p) **14e**



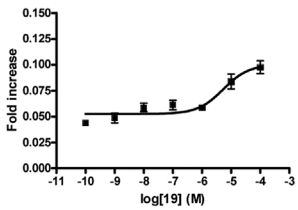
(q) **14f**



(r) **7e**



(s) **11**



(t) **19**

Figure 2. Continued.

observation in future experiments. This biphasic profile was also described by another group that evaluated the 1,2,4-oxadiazol-5-one derivatives, and also attributed their observations to CICR from the sarcoplasmic reticulum [24].

For all three series of compounds, the unsubstituted benzylamine derivatives (**7a**, **8a**, and **14a**) were found to be the least active. Increases in chain length (**7b**, **8b**, and **14c**) increased the inhibition of Ca^{2+} influx and the aza-triquinylamine derivative (**14c**), with a phenylpropyl substituent, had the highest potency. This correlated with previous findings that attributed this increase in activity to an increase in volume and lipophilicity [16c]. Overall the aliphatic derivatives were more active than their aromatic counterparts. The aliphatic heptylamine (**8c**) derivative had the highest potency of the aza-pentacycloundecylamines and for the aza-triquinylamines, the aliphatic derivative (**14d**) was slightly more active than the unsubstituted benzylamine (**14a**). In general, it was observed that substitution in the *meta* position (**7c**, **7d**, and **14e**) increased the activity when compared to the unsubstituted derivatives (**7a** and **14a**). Structures with substitution in the *meta* position with an electron-withdrawing moiety such as the nitro group (**7d**) were more active than the methoxy-substituted compound. The electron deficient 4-substituted pyridine (**14f**) was

observed to be more active for the aza-triquinanes when compared with (**14a**) and also more active than the *meta*-methoxy substituent.

Measurement of cell viability

It is well known that an increase in $[Ca^{2+}]_i$ plays an important role in the mechanism of apoptosis and necrosis, which can be induced by oxidative stress [25]. We performed cell viability studies utilizing the LDH and Trypan blue staining assays to investigate whether our compounds, which have the ability to regulate calcium influx through the LTCC, would offer protection against H_2O_2-induced cell death.

Estimation of LDH release

Plasma membrane damage in PC12 cells was evaluated by measuring the amount of intracellular LDH released in the media (Fig. 3). To assess inherent toxicity, compounds were evaluated in the absence of H_2O_2. Compound **14c** caused

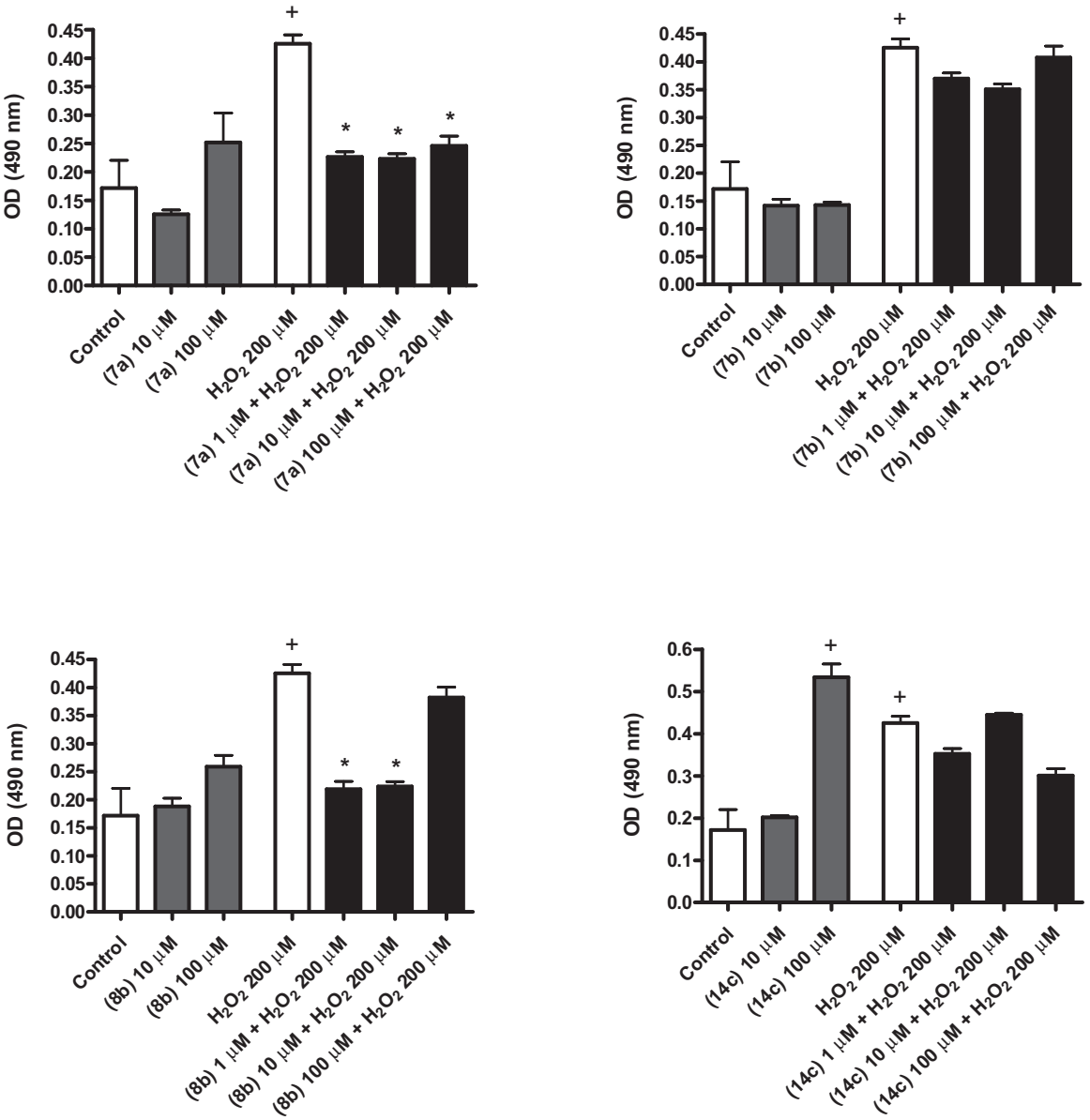


Figure 3. The effect of selected compounds on LDH release after cell injury induced by 200 μM H_2O_2. Data are mean ± SEM (n = 3). Data were subjected to an ANOVA analysis, followed by Dunn's post-test and significance was defined as $^{+}p < 0.05$ compared to control-untreated cells and $^{*}p < 0.05$ compared to 200 μM H_2O_2.

significant LDH release ($p < 0.05$), but only at the highest concentration tested (100 μM). The aberrant cytotoxicity observed for compound **14c**, which also had the lowest IC_{50} value ($IC_{50} = 0.398$ μM) as a LTCC blocker, was more than what was observed for the control of H_2O_2 treatment alone. Compounds **7a** and **8b** caused some LDH release at 100 μM, although this was found not to be significant. In general, H_2O_2-induced LDH release was attenuated in the presence of selected compounds. Compound **7a** showed significant attenuation of LDH release at concentrations (1, 10, and 100 μM, $^*p < 0.05$) as did compound **8b** at concentrations (1 and 10 μM, $^*p < 0.05$). Compound **7a** was the most active, displaying no significant inherent toxicity, and demonstrating the ability to significantly reduce H_2O_2-induced LDH release at all three concentrations evaluated.

Trypan blue exclusion

Trypan blue staining assays were performed to verify results from the LDH assays. Cell viability was assessed by criterion of Trypan blue exclusion (Fig. 4). The failure to exclude Trypan blue dye reflects a loss of plasma membrane integrity associated with necrosis. The results corresponded well to

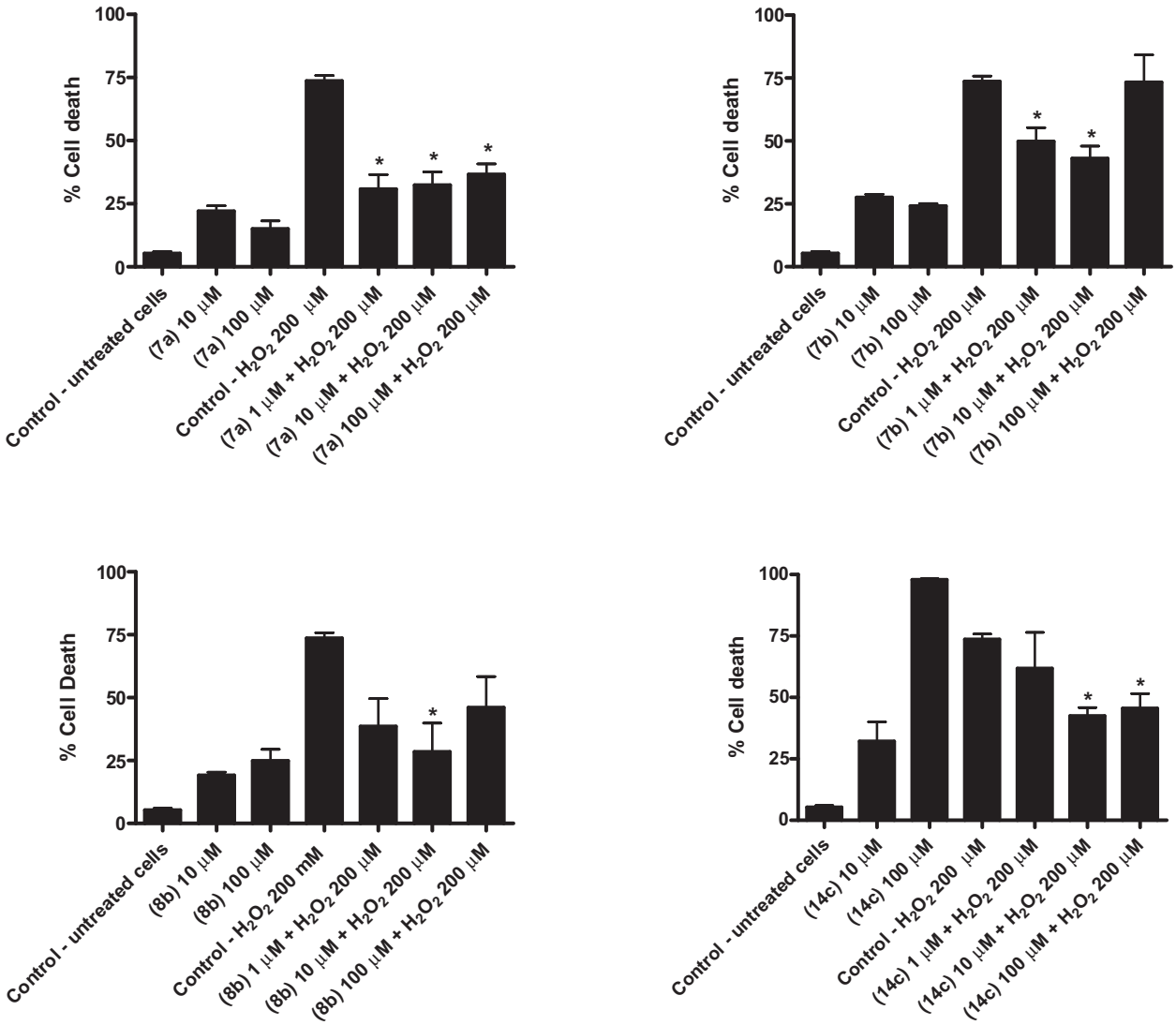


Figure 4. Assessment of percentage cell viability by means of Trypan blue uptake and exclusion. The effect of compounds alone was assessed by counting viable cells and comparing to control of untreated viable cells. The ability of selected compounds to offer protection against cell injury induced by 200 μM H_2O_2 was assessed by counting stained non-viable cells and comparing to the control of cells treated with 200 μM H_2O_2. Data are mean ± SEM (n = 3, counted two fields per repeat). Data were subjected to an ANOVA analysis, followed by Dunn's post-test and significance was defined as $^*p < 0.05$ compared to 200 μM H_2O_2.

results obtained with the LDH assay (compare Fig. 3 with 4). When compounds were evaluated in the absence of H_2O_2 to assess inherent toxicity, compounds **7a**, **7b**, and **8b** caused a slight decrease in cell viability at 10 and 100 μM, compared to the control of viable untreated cells. Compound **14c** showed considerable reductions (97.91%) in cell viability at 100 μM, compared to the control of viable untreated cells ($^*p < 0.05$), which indicates cytotoxicity for this compound at higher concentrations. This observation also correlates with results from the LDH assay for this compound at the same concentration. Treatment with 200 μM H_2O_2 caused significant (73.6%) reduction in cell viability compared to the control of viable untreated cells. Pretreatment with selected test compounds generally attenuated H_2O_2-induced cell injury when compared to H_2O_2 treatment alone. Of the compounds evaluated, **7a** demonstrated the most favorable pharmacological profile, with significant reduction ($^*p < 0.05$) of H_2O_2-induced toxicity at all concentrations evaluated (1, 10, and 100 μM). The same observation was made for this compound with the LDH assay.

Conclusion

We evaluated the ability of polycyclic compounds such as the pentacycloundecylamine and triquinylamine derivatives to modulate Ca^{2+} influx through the LTCC by means of a high-throughput fluorescence assay utilizing Fura-2/AM. This forms part of an ongoing study into their potential as novel multifunctional drug candidates in the treatment of neurodegenerative disease. For compounds **14c** and **7d**, the IC_{50} values (0.398 and 0.580 μM, respectively) and dose–response profiles were comparable to that of verapamil ($IC_{50} = 0.328$ μM) and diltiazem ($IC_{50} = 0.449$ μM). However, **14c** which had the lowest IC_{50} value (0.398 μM) showed toxicity at 100 μM when evaluated in the absence of H_2O_2 in both the LDH and Trypan blue staining cell viability assays. The IC_{50} value for NGP1-01 (**7a**) was determined to be 86 μM and this compound had the most favorable pharmacological profile with the ability to attenuate cell death in both cell viability assays. This compound also displayed no inherent toxicity. Compounds **7b** and **8b**, oxa- and aza-pentacycloundecylamines which both have phenylethyl substituents, had favorable activity as LTCC blockers ($IC_{50} = 51$ and 8 μM, respectively). These compounds were also able to offer protection in the cell viability assays.

In general, the aza-pentacycloundecylamines (**8a–c**) were the most potent LTCC blockers. In the cell viability assays, compound **8b** offered protection at lower concentrations but showed some toxicity at 100 μM. For future studies, it would be worthwhile to synthesize more compounds in the aza-pentacycloundecylamines series and more extensively evaluate these compounds for activity as possible neuroprotective drugs [26]. Calcium effects obtained through the interactions of these structures on σ-receptors and intracellular calcium channels such as inositol-1,4,5-triphosphate receptors (IP_3Rs) or ryanodine receptors (RyRs), also need to be investigated [27].

Concerning the SAR, it is interesting to note that the compounds evaluated in this study are structurally unrelated to established LTCC blockers and based on the IC_{50} values obtained we were able to draw meaningful conclusions regarding the SAR. Calculated IC_{50} values also allowed for direct comparison of the triquinylamines with the pentacycloundecylamines. For this series of compounds, SAR as LTCC blockers were influenced by geometric and steric effects. Modifying the "cage" moiety of the pentacycloundecane skeleton (**4**) by thermal fragmentation to obtain the *cis*,*syn*,*cis* triquinane system (**9**) did not diminish activity and, in general, the triquinane compounds were more active as LTCC blockers. Previous studies demonstrated that altering the geometric conformation of the polycyclic scaffold by increasing the size and volume leads to an increase in activity [16c]. This could explain why the triquinane compounds were more active, as the triquinane scaffold has a larger size and solvent accessible surface area (SASA) [19]. The triquinane scaffold shows promise in the design of novel LTCC blockers. Increase in activity was also observed for compounds (**7b**, **8b**, and **14c**) with an increase in chain length. As expected activity was diminished for compounds such as **11** and **19** that did not have the substituted amino side chain moiety, for activity as a LTCC blocker resides primarily in the combination of the secondary amino side chain and the bulky hydrocarbon polycycle [16a, c]. We found that compounds with aliphatic side chains (**8c** and **14d**) were more active than their aromatic counterparts (**7a**, **8a**, and **14a**). As shown by calculated logP values (Table 1), the aliphatic compounds are more lipophilic, which has been shown to be a contributing factor to an increase in activity [16b]. Since these compounds lack the aromatic group necessary for interaction with the DHP binding site [28], we could speculate that these compounds are pore-blocking drugs that occlude the transmembrane pore or act as allosteric modulators. Known LTCC blockers are neither structurally nor pharmacologically homogeneous, thus our findings serve as a valuable guide for future optimization efforts.

Experimental

Chemistry

Chemicals and instrumentation

Reagents were obtained from Sigma–Aldrich (UK and USA), Merck (Germany), D.H. Chemicals (UK), Acros Organics (USA), Fluka (Switzerland and USA), and Saarchem (South Africa). Reaction and elution solvents were purchased from commercial sources. Column chromatography was performed using either flash chromatography with nitrogen gas on glass columns [29], or conventional glass columns packed with silica gel, 60 Å pore size, mesh 70–230, purchased from Separations (South Africa) or by flash chromatography utilizing the Versa Flash station with VersaPak columns, silica cartridge 40 × 75 mm, purchased from Supelco (Pretoria, South Africa). Preparative thin-layer chromatography was

performed on precoated silica gel glass plates, 60 Å pore size, diameter 20 × 20 cm, and layer thickness 2 mm with fluorescent indicator for 245 nm, purchased from Separations, and also on C-18 preparative chromatography glass plates, 60 Å pore size, diameter 20 × 20 cm, and layer thickness 0.25 mm, purchased from Whatman Chemical Separation Inc. (USA). Commonly used abbreviations are DCM (dichloromethane), EtOAc (ethyl acetate), EtOH (ethanol), Et_2O (diethyl ether), HOAc (acetic acid), MeOH (methanol), PE (petroleum ether), and THF (tetrahydrofuran).

Differential scanning calorimetry (DSC) thermograms were recorded with a Shimadzu DSC-50 instrument (Shimadzu, Kyoto, Japan). The measurement conditions were as follows: sample weight, approximately 2 mg; sample holder, aluminum crimp cell; gas flow, nitrogen at 35 mL/min; heating rate, 10°C/min. Melting points (m.p.) were measured using a Gallenkamp and Stuart SMP10 melting point apparatus. Infrared (IR) spectra were recorded on a Nicolet 470 FT-IR spectrophotometer. Oily products were applied on the KBr disks as a thin film. Mass spectra (MS) were recorded on an analytical VG 7070E mass spectrometer. Ionization was induced by means of electron impact at 70 eV. HS-MS were recorded on a Thermo Scientific LTQ Orbitrap XL. Nuclear magnetic resonance (NMR) spectra were acquired on a Varian Gemini 300 with ^1H 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 were used to indicate multiplicities of the respective signals: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

Synthesis

A general method for the synthesis of aza-pentacycloundecylamines (**8a–c**) and aza-triquinylamines (**14a–f**) to supplement a series of oxa-pentacycloundecylamines (**7a–e**) is described in the text and is illustrated in Schemes 1 and 2.

*Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**4**)*

The pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**4**) was synthesized according to the well-established method previously described [17]. The physical characterization, data not presented, correlated with the physical characteristics described by Cooksen et al. [17].

*Tricyclo[6.3.0.0^{2,6}]undecane-4,9-diene-3,11-dione (**9**)*

We found, also reported in literature [20b], that optimal yield was achieved when the starting compound (**4**) was sublimated using a small sample spread as a thin film over the entire inner surface of the sublimation tube. Pentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**4**) (1 g, 0.006 mol) was dissolved in a minimal amount of DCM and spread over the surface of the sublimation tube using a heat gun to evaporate the DCM. The sublimation tube was connected to the vigreux quartz column by interspersing Teflon joint tape. Nitrogen gas (can also use argon) was flushed through the system with the vacuum pump turned on. The nitrogen inlet was then closed and the whole apparatus evacuated to 1 Torr by a high-capacity, rotary vane oil pump. Sublimation was achieved by sliding the specially designed ceramic thermal unit over the sublimation tube and heating the sublimation tube to 150°C, under 1 Torr vacuum. The substrate slowly migrated through the quartz column which was preheated to a temperature of 650°C. The condensate that yielded **9** was deposited as white waxy crystals mixed with black tar in the liquid nitrogen freeze fall. The pyrolysis was carried out at a throughput rate of 1 g every 30 min and repeated several times. The custom-designed apparatus utilized to perform pyrolysis is illustrated in Fig. 1. The condensate (**9**) was purified by recrystallization in cyclohexane to produce white needle-like crystals (yield: 17.15 g, 0.098 mol for 20.98 g pyrolyzed, 81.7%). $C_{11}H_{10}O_2$; m.p. (DSC) 97.23°C. ^1H NMR (300 MHz, $CDCl_3$): δ 7.51–7.30 (m, 2H, H_5, H_9), 6.17–5.50 (m, 2H, H_4, H_{10}), 3.67–3.35 (m, 2H, H_6, H_8), 3.28–3.03 (m, 2H, H_1, H_2), 2.45–2.10 (m, 1H, H_{7b}), 2.06–1.75 (m, 1H, H_{7a}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 207.35 (s, 2C, C_3, C_{11}), 165.93 (d, 2C, C_4, C_{10}), 133.35 (d, 2C, C_5, C_9), 53.05 (d, 2C, C_1, C_2), 50.29 (d, 2C, C_6, C_8), 31.36 (t, 1C, C_7). IR (KBr, cm^{-1}): ν_{max} 1720, 1639. MS (EI, 70 eV) *m/z*: calc. for $C_{11}H_{10}O_2$: 174.1959, found: 174 (M^+), 146, 131, 117, 91, 66, 39, 28. HR-MS *m/z*: calc. for $C_{11}H_{10}O_2$: 174.1959, found: 174.0759. As discussed by Young et al. [19], this compound was also characterized by means of single crystal X-ray analysis.

*Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (**10**)*

Tricyclo[6.3.0.0^{2,6}]undecane-4,9-diene-3,11-dione (3.09 g, 0.0177 mol) was dissolved in 500 mL of dry EtOAc and reduction was done with 10% Pd-C (300 mg) at a pressure of 2 atm (H_2) for 40 min. The catalyst was removed by filtration through Celite® and the solvent was removed *in vacuo*. Recrystallization from cyclohexane produced **10** as needle-like white/colorless crystals (yield: 3.01 g, 0.0169 mol, 95.42%). Physical characterization for this product correlate with that reported in literature [16a, 20a]. $C_{11}H_{14}O_2$; m.p. (DSC) 95.82°C. ^1H NMR (300 MHz, $CDCl_3$): δ 2.97–2.70 (m, 4H, H_1, H_2, H_6, H_8), 2.49–2.12 (m, 5H, H_{4a}, H_{4b}, H_{7b}, H_{10a}, H_{10b}), 2.12–1.94 (m, 2H, H_{5b}, H_{9b}), 1.72–1.54 (m, 2H, H_{5a}, H_{9a}), 1.30–1.10 (m, 1H, H_{7a}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 218.59 (s, 2C, C_3, C_{11}), 55.97 (d, 2C, C_1, C_2), 43.44 (d, 2C, C_6, C_8), 38.54 (t, 1C, C_7), 37.02 (t, 2C, C_4, C_{10}), 26.01 (t, 2C, C_5, C_9). IR (KBr, cm^{-1}): ν_{max} 1736, 1417. MS (EI, 70 eV) *m/z*: calc. for $C_{11}H_{14}O_2$: 178.2277, found: 178 (M^+), 122, 96, 79, 66, 55, 39, 28. HR-MS *m/z*: calc. for $C_{11}H_{14}O_2$: 178.2277, found: 178.1072. As discussed by Young et al. [19], this compound was also characterized by means of single crystal X-ray analysis.

*General method for the synthesis of compounds **8a–c** as derivatives of azahexacyclo[5.4.1.0^{2,6}.0^{5,9}.0^{8,11}]dodecan-8-ol (**8**) by reductive amination of pentacyclo-[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**4**)*

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**4**) was dissolved in anhydrous THF (50 mL) and cooled to 5°C on an external ice bath. An equimolar quantity of the primary amine

was slowly added stoichiometrically, while magnetically stirring the solution. The reaction was monitored on TLC till completion. The carbinol amine formed as a white precipitate, which was retrieved by means of filtration. This product was dehydrated under Dean–Stark conditions for approximately 1 h using 100 mL anhydrous benzene. The solvent was removed *in vacuo* to afford the Schiff base (**5**) as yellow oil, which was dissolved in a mixture of HOAc (15 mL) and MeOH (250 mL). Reduction was carried out by slowly adding $NaBH_3CN$ (2 g, 0.032 mol), in one molar excess, over a period of 5 min. The reaction mixture was allowed to stir overnight at room temperature. The solvent was removed *in vacuo* and H_2O (100 mL) was added. While stirring, solid $NaHCO_3$ was added portion-wise until the evolution of carbon dioxide ceased. Excess $NaHCO_3$ (2 g) was added and the mixture was extracted with DCM (4 × 50 mL). The combined organic phases were washed with H_2O (2 × 100 mL), dried with anhydrous $MgSO_4$, and filtered. The solvent was removed *in vacuo*. The compounds were purified by means of flash column chromatography or crystallization.

*N-Benzyl-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecane (**8a**)*

This compound was prepared according to the general method for derivatives of **8** described in above text. Equimolar quantities of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5 g, 0.029 mol) and benzylamine (3.08 g, 0.029 mol) were used. The resulting product was purified by means of crystallization from benzene to yield white crystals (yield: 0.882 g, 0.003 mol, 11.58%). $C_{18}H_{19}NO$; m.p. 159°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.70–7.00 (m, 5H, H_{14}, H_{15}, H_{16}, H_{17}, H_{18}), 5.10–4.30 (bs, 1H, OH), 3.90–3.40 (AB-q, 2H, J = 13.4, H_{12a}, H_{12b}), 3.35–3.20 (t, 1H, J = 5.3, H_{11}), 2.85–2.20 (m, 8H, H_1, H_2, H_3, H_5, H_6, H_7, H_9, H_{10}), 1.90–1.37 (AB-q, 2H, J = 10.5 Hz, H_{4a}, H_{4b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 138.87 (s, 1C, C_{13}), 128.61 (d, 2C, C_{15}, C_{17}), 128.36 (d, 2C, C_{14}, C_{18}), 128.26 (d, 1C, C_{16}), 126.89 (s, 1C, C_{11}), 64.78 (d, 1C, C_8), 54.95 (d, 1C, C_{10}), 51.63 (t, 1C, C_{12}), 50.72 (d, 1C, C_1), 45.58 (t, 1C, C_4), 44.60 (d, 1C, C_9), 42.99 (d, 1C, C_5), 42.20 (d, 2C, C_2, C_6), 41.69 (d, 1C, C_3), 41.31 (d, 1C, C_7). IR (KBr, cm^{-1}): ν_{max} 3130, 2961, 2834, 1454, 1322, 1108, 921, 729. MS (EI, 70 eV) *m/z*: 265 (M^+), 173, 131, 91, 77, 28. HR-MS *m/z*: calc. for $C_{18}H_{19}NO$: 265.3496, found: 265.1545.

*N-Phenylethyl-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecane (**8b**)*

This compound was prepared according to the general method for derivatives of **8** described in above text. Equimolar quantities of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (4.065 g, 0.023 mol) and phenylethylamine (2.83 g, 0.023 mol) were used. The resulting product was purified by means of flash column chromatography (SiO_2, PE/EtOAc/DCM/EtOH 1:1:1:2 as eluent) to yield white crystals (yield: 2.064 g, 0.007 mol, 32.13%). $C_{19}H_{21}NO$; m.p. 150°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40–7.10 (m, 5H, H_{15}, H_{16}, H_{17}, H_{18}, H_{19}), 5.90–4.60 (bs, 1H, OH), 3.70–3.40 (t, 1H, J = 5.3, H_{11}), 3.20–2.30 (m, 12H, H_{12a}, H_{12b}, H_{13a}, H_{13b}, H_1, H_2, H_3, H_5, H_6, H_7, H_9, H_{10}), 1.93–1.42 (AB-q, 2H, J = 10.5 Hz, H_{4a}, H_{4b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.34 (s, 1C, C_{14}), 128.55 (d, 2C, C_{16}, C_{18}), 128.38 (d, 2C, C_{15}, C_{19}), 128.29 (d, 1C, C_{17}), 126.03 (s, 1C, C_{11}), 65.12 (d, 1C, C_8), 55.07 (d, 1C, C_{10}), 50.76 (d, 1C, C_1), 48.98 (t, 1C, C_{12}), 45.56 (t, 1C, C_4), 44.73 (d, 1C, C_9), 43.10 (d, 1C, C_5), 42.36 (d, 1C, C_2), 41.66 (d, 1C, C_3), 41.59 (d, 1C, C_6), 41.52 (t, 1C, C_{13}), 35.13 (d, 1C, C_7). MS (EI, 70 eV) *m/z*: 279 (M^+), 246, 188, 131, 91, 77, 28. HR-MS *m/z*: calc. for $C_{19}H_{21}NO$: 279.3761, found: 279.1701.

*N-Heptyl-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecane (**8c**)*

This compound was prepared according to the general method for derivatives of **8** described in above text. Equimolar quantities of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (3 g, 0.017 mol) and heptylamine (1.98 g, 0.017 mol) were used. The resulting product was purified by means of flash column chromatography (SiO_2; PE/EtOAc/EtOH 5:4:1 as eluent) to yield white crystals (yield: 2.031 g, 0.007 mol, 43.13%). $C_{18}H_{27}NO$; m.p. 100°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.30–5.70 (bs, 1H, OH), 3.65–3.42 (t, 1H, J = 5.3, H_{11}), 3.00–2.80 (m, 1H, H_{12a}), 2.80–2.10 (m, 9H, H_1, H_2, H_3, H_5, H_6, H_7, H_9, H_{10}, H_{12b}), 1.87–1.40 (AB-q, J = 10.5 Hz, 2H, H_{4a}, H_{4b}), 1.60–1.40 (m, 2H, H_{13a}, H_{13b}), 1.36–1.10 (m, 8H, H_{14a}, H_{14b}, H_{15a}, H_{15b}, H_{16a}, H_{16b}, H_{17a}, H_{17b}), 0.96–0.70 (t, 3H, J = 6 Hz, H_{18a}, H_{18b}, H_{18c}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 179.86 (s, 1C, C_{11}), 64.69 (d, 1C, C_8), 54.56 (d, 1C, C_{10}), 50.51 (t, 1C, C_{12}), 46.59 (d, 1C, C_1), 45.75 (t, 1C, C_4), 44.44 (d, 1C, C_9), 43.26 (d, 1C, C_5), 41.91 (d, 2C, C_2, C_6), 41.64 (d, 1C, C_3), 41.56 (d, 1C, C_7), 31.74 (t, 1C, C_{16}), 29.06 (t, 1C, C_{15}), 27.76 (t, 1C, C_{13}), 27.59 (t, 1C, C_{14}), 22.56 (t, 1C, C_{17}), 14.01 (q, 1C, C_{18}). IR (KBr, cm^{-1}) ν_{max}: 3194, 2961, 2868, 1475, 1333, 1296, 1149. MS (EI, 70 eV) *m/z*: 273 (M^+), 271, 256, 242, 228, 213, 200, 156, 129, 91, 77, 29. HR-MS *m/z*: calc. for $C_{18}H_{27}NO$: 273.4131, found: 273.2171.

*General method for the synthesis of compounds **14a,c–f** as derivatives of azatricyclo[6.3.0.0^{2,6}]undecane (**14**) by amination of tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (**10**)*

Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione was dissolved in anhydrous THF (30 mL), stirred and cooled to 5°C on an external ice bath. An equimolar quantity of the desired primary amine was slowly added stoichiometrically, while the mixture was stirred for approximately 6 h at low temperature. The reaction was monitored on TLC till completion. The solvent was removed *in vacuo* to yield the Schiff base (**12**) as a yellow oil. This product was dehydrated under Dean–Stark conditions for approximately 1 h using 40 mL anhydrous benzene. Benzene was removed *in vacuo* to afford the imine (**13**). The resulting oil was dissolved in anhydrous MeOH (20 mL) and anhydrous THF (75 mL). Reduction was carried out by the addition of $NaBH_3CN$, in molar excess and stirred overnight (18 h) at room temperature. The solvent was removed *in vacuo* and the resulting dark-colored oil was suspended in approximately 50 mL of distilled water in a separation funnel. The product was extracted with DCM (4 × 20 mL) and the combined DCM fractions were washed with distilled water (2 × 50 mL). The

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organic phase was dried over anhydrous $CaSO_4$ and filtered. The solvent was removed *in vacuo*. The compound was purified with sequences of column chromatography, flash column chromatography, and/or preparative thin-layer chromatography.

*N-Benzyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (**14a**)*
This compound was prepared according to the general method for derivatives of **14** as described in the above text. Near equimolar quantities of the tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (2.0838 g, 0.0117 mol) and benzylamine (1.333 g, 0.0124 mol) were used. The resulting yellow/brown oil was purified with column and flash column chromatography sequences. The first purification step utilized column chromatography (SiO_2; PE/EtOAc/EtOH 5:4:2 as eluent). The second purification step utilized flash column chromatography (SiO_2; *n*-hexane/EtOAc/DCM 3:1:1 as eluent). The third purification step also utilized column chromatography (SiO_2; PE/Et_2O 5:1 as eluent) at lowered temperature (5°C). Purification afforded light yellow-colored oil (yield: 0.393 g, 0.002 mol, 13.27%). $C_{18}H_{23}N$; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40–7.09 (m, 5H, H_{14}, H_{15}, H_{16}, H_{17}, H_{18}), 3.77–3.57 (s, 2H, H_{12a}, H_{12b}), 3.27–3.05 (m, 2H, H_3, H_{11}), 2.88–2.69 (m, 2H, H_1, H_2), 2.60–2.35 (m, 2H, H_6, H_8), 2.00–1.47 (m, 8H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}), 1.37–1.16 (m, 2H, H_{7a}, H_{7b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 141.03 (s, 1C, C_{13}), 128.73 (d, 2C, C_{14}, C_{18}), 127.84 (d, 2C, C_{15}, C_{17}), 126.41 (d, 1C, C_{16}), 73.56 (d, 2C, C_3, C_{11}), 58.52 (t, 1C, C_{12}), 54.47 (d, 2C, C_1, C_2), 47.33 (d, 2C, C_6, C_8), 37.46 (t, 1C, C_7), 34.50 (t, 2C, C_4, C_{10}), 32.74 (t, 2C, C_5, C_9). IR (KBr, cm^{-1}): ν_{max} 2944, 1458. MS (EI, 70 eV) *m/z*: 253 (M^+), 224, 170, 162, 91, 65, 28. HR-MS *m/z*: calc. for $C_{18}H_{23}N$: 253.3819, found: 253.1908.

*N-Phenylpropyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (**14c**)*
This compound was prepared according to the general method for derivatives of **14** as described in the above text. Equimolar quantities of the tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (3.44 g, 0.0193 mol) and phenylpropylamine (2.613 g, 0.0193 mol) were used. The resulting dark brown oil was purified with column, flash column, and preparative plate chromatography sequences. The first purification step utilized column chromatography (SiO_2; EtOAc/EtOH 9:1 as eluent). The second purification step utilized flash column chromatography (SiO_2; PE/Et_2O 3:1 as eluent) at lowered temperatures (5°C). The third purification step utilized column chromatography (SiO_2; DCM as eluent) at lowered temperature (5°C). The fourth purification step utilized preparative thin-layer chromatography (stationary phase was SiO_2; DCM/PE 9:1 as eluent). Purification afforded red/brown-colored oil (yield: 0.1871 g, 0.0007 mol, 3.44%). $C_{20}H_{27}N$; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37–7.08 (m, 5H, H_{16}, H_{17}, H_{18}, H_{19}, H_{20}), 3.40–3.27 (m, 2H, H_3, H_{11}), 3.25–3.14 (t, 2H, $J = 7.5$ Hz, H_1, H_2), 3.01–2.43 (m, 6H, H_6, H_8, H_{13a}, H_{13b}, H_{14a}, H_{14b}), 2.19–2.06 (m, 2H, H_{12a}, H_{12b}), 1.99–1.62 (m, 8H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}), 1.56–1.39 (m, 2H, H_{7a}, H_{7b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 141.90 (s, 1C, C_{15}), 128.34 (d, 2C, C_{16}, C_{20}), 125.81 (d, 2C, C_{17}, C_{19}), 73.11 (d, 1C, C_{18}), 61.73 (d, 2C, C_3, C_{11}), 53.55 (d, 2C, C_1, C_2), 51.05 (t, 1C, C_{12}), 47.11 (d, 2C, C_6, C_8), 40.26 (t, 1C, C_{14}), 37.07 (t, 1C, C_7), 34.35 (t, 1C, C_{13}), 33.83 (t, 2C, C_4, C_{10}), 32.62 (t, 2C, C_5, C_9). IR (KBr, cm^{-1}): ν_{max} 2944, 1444. HR-MS *m/z*: calc. for $C_{20}H_{27}N$: 281.4351, found: 281.2222.

*N-Cyclohexylmethyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (**14d**)*
This compound was prepared according to the general method for derivatives of **14** as described in the above text. Equimolar quantities of the tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (2.721 g, 0.0153 mol) and cyclohexylmethylamine (1.7304 g, 0.0153 mol) were used. The resulting light brown oil was purified with column chromatography sequences. The first purification step utilized column chromatography (SiO_2; PE/$CHCl_3$/EtOAc 1:1:1 as eluent). The second purification step utilized column chromatography (SiO_2; PE/THF 5:1 as eluent) at lowered temperature (5°C). Purification afforded compound as white waxy substance (yield: 0.106 g, 0.0004 mol, 2.67%). $C_{18}H_{29}N$; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.65–3.37 (t, 2H, $J = 7.5$ Hz, H_3, H_{11}), 3.25–3.00 (t, 2H, $J = 7.5$ Hz, H_1, H_2), 2.95–2.64 (m, 2H, H_{12a}, H_{12b}), 2.52–2.25 (m, 2H, H_6, H_8), 2.22–1.60 (m, 19H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}, H_{13}, H_{14a}, H_{14b}, H_{15a}, H_{15b}, H_{16a}, H_{16b}, H_{17a}, H_{17b}, H_{18a}, H_{18b}), 1.60–1.47 (m, 2H, H_{7a}, H_{7b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 63.08 (d, 2C, C_3, C_{11}), 60.44 (d, 2C, C_1, C_2), 47.40 (d, 2C, C_6, C_8), 45.88 (d, 1C, C_{13}), 40.39 (t, 1C, C_{12}), 40.38 (t, 2C, C_{15}, C_{17}), 39.51 (t, C, C_{16}), 37.22 (t, 2C, C_{14}, C_{18}), 32.36 (t, 2C, C_4, C_{10}), 32.13 (t, 2C, C_5, C_9), 26.91 (t, 1C, C_7). HR-MS *m/z*: calc. for $C_{18}H_{29}N$: 259.4296, found: 259.2378.

*N-(3-Methoxybenzyl)-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (**14e**)*
This compound was prepared according to the general method for derivatives of **14** as described in the above text. In addition to the spectroscopic analysis, this compound was characterized by means of single crystal X-ray analysis. We reported the synthesis and characterization for this compound in Young et al. [19].

*N-(Pyridin-4-ylmethyl)-3,11-azatricyclo[6.3.0.0^{2,6}]-undecane (**14f**)*
This compound was prepared according to the general method for derivatives of **14** as described above. Equimolar quantities of the tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (3.87 g, 0.0217 mol) and pyridinylamine (2.351 g, 0.0217 mol) were used. The resulting dark red/brown oil was purified with column and preparative thin-layer chromatography sequences. The first purification step utilized column chromatography (SiO_2, Et_2O as eluent). The second purification step utilized preparative thin-layer chromatography (stationary phase was SiO_2 and EtOAc as eluent). The third purification step utilized preparative thin-layer chromatography (reverse phase with C-18 absorbent layer; MeOH/ACN 2:1 as eluent). Purification afforded red/brown-colored oil (yield: 0.22 g, 0.0009 mol, 4%). $C_{17}H_{22}N_2$; ^{1}H NMR (300 MHz, $CDCl_3$): δ

8.73–7.07 (dd, 4H, H_{14}, H_{15}, H_{16}, H_{17}), 3.74–3.52 (s, 2H, H_{12a}, H_{12b}), 3.23–3.12 (m, 2H, H_3, H_{11}), 2.86–2.67 (m, 2H, H_1, H_2), 2.66–2.40 (m, 2H, H_6, H_8), 2.12–1.61 (m, 8H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}), 1.57–1.41 (m, 2H, H_{7a}, H_{7b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 151.00 (s, 1C, C_{13}), 149.40 (d, 2C, C_{15}, C_{16}), 122.80 (d, 2C, C_{14}, C_{17}), 74.33 (d, 2C, C_3, C_{11}), 58.05 (t, 1C, C_{12}), 54.57 (d, 2C, C_1, C_2), 47.28 (d, 2C, C_6, C_8), 37.36 (t, 1C, C_7), 34.57 (t, 2C, C_4, C_{10}), 32.70 (t, 2C, C_5, C_9). IR (KBr, cm^{-1}): ν_{max} 2944, 1611. MS (EI, 70 eV) *m/z*: 254 (M^+), 225, 180, 162, 120, 91, 67, 41, 28. HR-MS *m/z*: calc. for $C_{17}H_{22}N_2$: 254.3700, found: 254.1861.

*Method for the synthesis of N-phenylethyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (**14b**)*

Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione was dissolved in anhydrous THF (50 mL), stirred and cooled down to 5°C on an external ice bath. Equimolar quantities of the tricyclo-[6.3.0.0^{2,6}]undecane-3,11-dione (0.179 g, 0.001 mol) and phenylethylamine (0.12 g, 0.001 mol) were slowly added stoichiometrically, while the mixture was stirred for approximately 1 h at reduced temperature. The reaction was allowed to stir for an additional hour at room temperature. The solvent was removed *in vacuo* to yield the carbinol amine (**12**) as yellow oil. For this reaction, the Schiff base formed spontaneously from the carbinol amine and we did not perform Dean–Stark dehydration. The resulting oil was dissolved in ice cold MeOH (50 mL). Reduction was carried out by the addition of $NaBH_3CN$ in molar excess. The reaction mixture was allowed to stir at room temperature for 1 h and the solvent was removed *in vacuo*. The resulting oil was suspended in approximately 20 mL distilled water in a separation funnel. The product was extracted with DCM (5 × 20 mL) and the combined DCM fractions were washed with distilled water (2 × 50 mL). The organic phase was dried over anhydrous $MgSO_4$ and filtered. The subsequent *in vacuo* removal of the solvent afforded a yellow oil. The compound was purified by means of flash column chromatography (SiO_2; PE/EtOAc/EtOH 5:4:1 as eluent), which afforded the product as a light yellow oil (yield: 0.072 g, 0.0003 mol, 26.9%). $C_{19}H_{25}N$; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30–7.10 (m, 5H, H_{15}, H_{16}, H_{17}, H_{18}, H_{19}), 3.30–3.10 (m, 2H, H_3, H_{11}), 2.90–2.70 (m, 4H, H_1, H_2, H_{13a}, H_{13b}), 2.60–2.40 (m, 2H, H_6, H_8), 2.00–1.60 (m, 8H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}), 1.50–1.30 (m, 2H, H_{12a}, H_{12b}), 1.30–1.20 (m, 2H, H_{7a}, H_{7b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.60 (s, 1C, C_{14}), 128.69 (d, 2C, C_{16}, C_{18}), 128.20 (d, 2C, C_{15}, C_{19}), 125.66 (d, 1C, C_{17}), 72.96 (d, 2C, C_3, C_{11}), 55.00 (t, 1C, C_{12}), 54.74 (d, 2C, C_1, C_2), 47.36 (d, 2C, C_6, C_8), 34.94 (t, 1C, C_{13}), 34.68 (t, 1C, C_7), 32.68 (t, 2C, C_5, C_9), 32.66 (t, 2C, C_4, C_{10}). IR (KBr, cm^{-1}) ν_{max}: 2949, 1453, 699. MS (EI, 70 eV) *m/z*: 267 (M^+), 190, 176, 162, 151, 91, 67, 27. HR-MS *m/z*: calc. for $C_{19}H_{25}N$: 267.4085, found: 267.2065.

*3-Hydroxy-3,11-oxatricyclo[6.3.0.0^{2,6}]undecane (**11**)*

Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (0.5 g, 0.0028 mol) was dissolved in MeOH (50 mL), stirred and cooled down to 5°C on an external ice bath. The reducing agent $NaBH_4$ (0.425 g, 0.0112 mol) was slowly added in molar excess. The reaction proceeded at room temperature for 1 h. The solvent was removed *in vacuo* and the remaining content was suspended with distilled H_2O (20 mL) in a separation funnel. The product was extracted with DCM (2 × 20 mL) and the combined DCM fractions were washed with distilled water (1 × 20 mL). The organic phase was dried over anhydrous $MgSO_4$ and filtered. Subsequent *in vacuo* removal afforded 0.475 g of the crude material, which was purified by means of column chromatography (SiO_2; EtOAc/benzene 3:17 as eluent). The hemiacetal was obtained as white crystals (yield: 0.081 g, 0.0004 mol, 16%). $C_{11}H_{16}O_2$; m.p. 95°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.85–4.70 (m, 1H, H_{11}), 3.60–3.10 (bs, 1H, OH), 3.10–2.95 (m, 1H, H_1), 2.95–2.82 (t, 1H, $J = 7.5$, H_2), 2.82–2.65 (m, 1H, H_6), 2.65–2.40 (m, 1H, H_8), 2.20–1.40 (m, 10H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{7a}, H_{7b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}). ^{13}C NMR (75 MHz, $CDCl_3$): δ 118.22 (s, 1C, C_3), 87.750 (d, 1C, C_{11}), 60.70 (d, 1C, C_1), 56.38 (d, 1C, C_2), 47.14 (d, 1C, C_6), 46.02 (d, 1C, C_8), 39.63 (t, 1C, C_7), 37.85 (t, 1C, C_4), 34.52 (t, 1C, C_{10}), 32.57 (t, 1C, C_5), 32.26 (t, 1C, C_9). IR (KBr, cm^{-1}) ν_{max}: 3382, 2918, 1463, 1013. MS (EI, 70 eV) *m/z*: 180 (M^+), 163, 120, 96, 91, 79, 67, 53, 41, 27. HR-MS *m/z*: calc. for $C_{11}H_{16}O_2$: 180.2435, found: 180.1228.

Biological evaluation

Cell culture

Undifferentiated rat pheochromocytoma (PC12) cells obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were used in this study. The PC12 cell line can be utilized to study Ca^{2+} influx through the LTCC and can serve as a model for Ca^{2+} regulation in the nervous system [30]. The cells were cultured in 75 cm^2 tissue culture-treated flasks containing RPMI-1640 media (Hyclone, Fisher Scientific, USA) supplemented with 5% fetal bovine serum, 10% horse serum, 0.5% penicillin/streptomycin/amphotericin B, and 1% (2.05 mM) L-glutamine. The medium was formulated for use with 5% CO_2 at 37°C. Culture media were changed every 3 days and cells were sub-cultivated twice a week.

Evaluation of calcium influx

PC12 cells were plated (1 × 10^5 cells/well) in a black clear-bottom 96-well plates (Costar, Corning, NY, USA) 24 h before experiments. Experimental techniques were similar to those in published studies [31]. Cultures were washed and then loaded with 5 μM Fura-2/AM (Invitrogen™, Molecular Probes™, Eugene, OR, USA) in Hanks balanced salt solution (HBSS) containing Ca^{2+}, Mg^{2+}, and 10% bovine serum albumin (BSA). Preincubation with compounds to be evaluated was conducted in HBSS at increasing concentrations (0.0001–200 μM) over a 10 min period before the application of 50 mM KCl. Fluorescence was read on a BioTech Synergy 4 microplate reader (Winooski, VT, USA) with the excitation wavelengths set at 340 and 380 nm and emission wavelength set at 510 nm. After 10 min of recording, cells were depolarized with a high concentration of KCl (50 mM). Fluorescence intensity (ratio of 340/380 nm) was normalized for each graph to start at 1 and

plotted as a function of time (sec). The graphs illustrate Ca^{2+} influx through the LTCC ($n = 3$) and the inhibitory potency of the compounds in a dose-dependent manner. Amplitude of suppression for each concentration was plotted as a dose–response curve from which the IC_{50} values could be calculated. The IC_{50} values are summarized in Table 1 and the log concentration–response graphs are presented in Fig. 2a–t.

Cell viability assays: Measurement of LDH release

Apoptosis can be assayed by evaluating the plasma membrane damage of PC12 cells and measuring the amount of intracellular lactate dehydrogenase (LDH) released in the media. LDH leakage was measured after a 24 h exposure to 200 μM H_2O_2. A 10 min preincubation with compounds to be evaluated (1, 10, and 100 μM) was done 30 min prior to treatment with 200 μM H_2O_2. We also evaluated the compounds in the absence of H_2O_2 at 10 and 100 μM to assess if these compounds had any inherent toxicity. The culture supernatant (100 μL) was collected from each well and the LDH activity was determined using a colorimetric LDH cytotoxicity assay kit obtained from Cayman® (Ann Arbor, MI, USA), according to the manufacturer's protocols. The absorbance was measured at 490 nm using a microplate reader (Molecular Devices SpectraMax® 340PC^{384} microplate reader, Sunnyvale, CA, USA).

Cell viability assays: Trypan blue exclusion

Conditions and treatments under which this assay was performed were the same as for the LDH assay and cell death was induced by 200 μM H_2O_2. Cultures were exposed to Trypan blue (5% v/v) for 30 min after a 24 h exposure to 200 μM H_2O_2. The same concentrations and treatment conditions were used for preincubation with test compounds as with the LDH assay. Cell injury was estimated by examining the cultures with a phase-contrast microscope (Vista Vision™, VWR, Bridgeport, NJ, USA) and counting the percentage of Trypan blue positive cells. Values represent means ± SEM ($n = 3$, counted two fields per repeat), and was presented as percentage of cells stained. Each well contained 1×10^5 cells and the microscopic field was assessed at magnification ×20. Triton X-100 (11% v/v) was used to induce 100% cell death as a control.

Statistical analysis

For the calcium influx assay, all experimental results are presented as the mean ± SDEV. Analyses were performed and graphs drawn in Excel (Microsoft® Office 2003, USA). For the calculation of IC_{50} values, the analyses were performed and graphs were drawn in Prism 3.0 (GraphPad, San Diego, CA), and results presented as the mean ± SDEV. For the LDH and Trypan blue staining assays, all experimental results are presented as the mean ± SEM and statistical significance of differences between the means was determined by one-way ANOVA analysis followed by Dunnett's *post hoc* test to compare relevant groups with controls. Where compounds were evaluated in the absence of H_2O_2, comparisons were done with control of untreated cells. Where compounds were evaluated in the presence of H_2O_2, the comparisons were done with control of 200 μM H_2O_2 treatment alone. The level of statistical significance was taken at $p < 0.05$. Prism 3.0 (GraphPad, San Diego, CA) statistical software was used to perform analyses.

Supplemental information available

Additional graphs illustrating fluorescence inhibition profiles are given in Appendix A. Spectroscopic graphs for compound characterization are given in Appendix B. The InChI codes of the new compounds are provided in a third Supporting Information file. This material is available free of charge at http://onlinelibrary.wiley.com/doi/10.1002/ardp.201500293/suppinfo.

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