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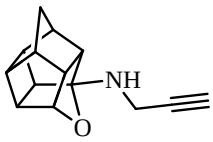
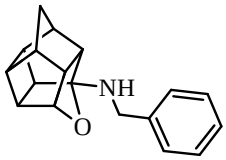
Graphical Abstract



P1

Neuroprotective

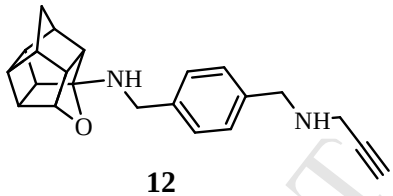


NGP1-01

Neuroprotective

VGCC blocker

NMDAr antagonist



12

Neuroprotective

VGCC blocker

NMDAr antagonist

MAO-B inhibitor

Design, synthesis and evaluation of pentacycloundecane and hexacycloundecane propargylamine derivatives as multifunctional neuroprotective agents

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Abstract

The multifactorial pathophysiology of neurodegenerative disorders remains one of the main challenges in the design of a single molecule that may ultimately prevent the progression of these disorders in affected patients. In this article, we report on twelve novel polycyclic amine cage derivatives, synthesized with or without a propargylamine function, designed to possess inherent multifunctional neuroprotective activity. The MTT cytotoxicity assay results showed the SH-SY5Y human neuroblastoma cells to be viable with the twelve compounds, particularly at concentrations less than 10 µM. The compounds also showed significant neuroprotective activity, ranging from 31% to 66% at 1 µM, when assayed on SH-SY5Y human neuroblastoma cells in which neurodegeneration was induced by MPP^+. Calcium regulation assays conducted on the same cell line showed the compounds to be significant VGCC blockers with activity ranging from 26.6% to 51.3% at 10 µM; as well as significant NMDAr antagonists, with compound **5** showing the best activity of 88.3% at 10 µM. When assayed on human MAO isoenzymes, most of the compounds showed significant inhibitory activity, with compound **5** showing the best activity (MAO-B: IC_{50} = 1.70 µM). Generally, the compounds were about 3 – 52 times more selective to the MAO-B isoenzyme than the MAO-A isoenzyme. Based on the time-dependency studies conducted, the compounds can be defined as reversible MAO inhibitors. Several structure activity relationships were derived from the various assays conducted, and the compounds' possible putative binding modes within the MAO-B enzyme cavity were assessed *in silico.*

Key words: Neurodegeneration, apoptosis, neuroprotection, multifunctional, polycyclic amine, propargylamine, excitotoxicity, monoamine oxidase.

Introduction

Neurodegenerative disorders (NDs) refers to a cluster of neuronal diseases that are characterised by transient and irreversible loss of neuronal cells in the brain due to apoptosis [1]. Examples of these diseases include Alzheimer's Disease (AD), Huntington's Disease (HD) and Parkinson's Disease (PD). Though the pathogenesis is yet to be fully understood, it is known that several factors are involved in the etiology of these disorders. Some of the established theories of disease causation include, but are not limited to, disturbances in

neurotransmitter systems such as the monoaminergic system [2,3], as well as excitotoxicity resulting from excessive calcium influx into neuronal cells [4,5].

Patients with PD and AD have been shown to have age-related elevated levels of the B-type monoamine oxidase isoenzyme (MAO-B) in the brain [6]. This enzyme does not only act indirectly as a trigger to the apoptotic process in neuronal cells, but together with the A-type isoenzyme (MAO-A), they form the major catabolic pathways of dopamine [7] and give rise to some of the signs and symptoms associated with these disorders [8]. Both these isoenzymes are therefore promising drug target sites for the treatment of NDs and as such, monoamine oxidase enzyme inhibitors (MAOI's) have been the mainstay therapy for the management of NDs. By inhibiting the activity of the MAO isoenzymes, MAOI's may exert neuroprotective effects by inhibiting the formation of toxic by-products of MAO-catalyzed oxidation of neurotransmitters [9]. Further to this, MAOI's enhance dopaminergic neurotransmission in the nigro-striatal pathway, thereby providing symptomatic relief in patients with PD [10].

The first generation of MAOI's such as tranylcypromine are nonspecific and irreversible inhibitors of both isoforms of MAO. While these dual inhibiting compounds produce a significant rise in dopamine levels and behavioural changes after administration [11], they present a drawback of causing clinically significant potentiation of the 'pressor response', a common adverse effect associated with ingesting tyramine containing foods together with nonspecific MAOI's. Potentiation of this adverse effect is mainly influenced by the irreversibility of the inhibitor and the degree of MAO-A inhibition [12]. This has been shown to be due to MAO-A being the major form of MAO in the liver and stomach. Reversible inhibitors of MAO-A are therefore preferred to their irreversible counterparts as they show a reduced hypertensive response [13].

A more widely explored strategy entails selectively inhibiting the MAO-B isoenzyme by inhibitors such as rasagiline and selegiline (Figure 1). This approach is based on the fact that the extrapyramidal region of the human brain has approximately 4-times more MAO-B isoenzyme than the MAO-A isoenzyme [14]. Rasagiline and selegiline are second generation propargylamine derivatives that irreversibly inhibit brain MAO-B, and have promising neuroprotective activities [15]. Their activity can be attributed to the propargyl moiety [8], that after oxidation, reacts with the flavin prosthetic group in the active site of the MAO-B enzyme, forming a covalent adduct at the *N*5 position of the flavin adenine dinucleotide (FAD) [16]. The propargyl moiety is also now known to play an important role in providing neuronal and mitochondrial protective properties [17] as well as anti-apoptotic properties [18]. This moiety has been particularly useful for incorporation into multi-target compounds with inherent MAO-B inhibitory capacity [19].

While inhibiting the oxidative deamination reaction catalysed by the MAO isoenzymes is a useful strategy in managing NDs, targeting a single enzymatic system or receptor has proved to be insufficient for the treatment of these multifactorial diseases [20]. A more effective therapy would result from the use of multi-target directed ligands (MTDLs) able to intervene in the different pathological events implicated in the etiology of neuronal disorders [21].

Calcium homeostasis in neuronal cells has also been implicated in the pathogenesis of NDs. Given the crucial and widespread role calcium signalling has in excitable cells, it is not surprising that alterations in calcium homeostasis is linked to several NDs [22,23]. Maintenance of intracellular calcium homeostasis is essential for the functioning and survival of neurons and is a fundamental component of synaptic transmission for both pre- and postsynaptic mechanisms [24]. Excessive influx of calcium can therefore overwhelm Ca^{2+}-regulatory mechanisms and lead to excitotoxicity and neuronal cell death [25]. The activation of the postsynaptic *N*-methyl-D-aspartate receptors (NMDAr), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)proprionate receptors and kainate receptors allows for opening of their associated ion channels to allow the influx of Ca^{2+} and Na^{+} ions into the neuronal cells. Calcium entry may also occur through *L*-type voltage gated calcium channels (VGCC) and result in both calcium overload and mitochondrial disruption [25]. This mechanism of cell death suggests these receptors and their associated calcium channels to be supplementary drug target sites for the treatment of NDs.

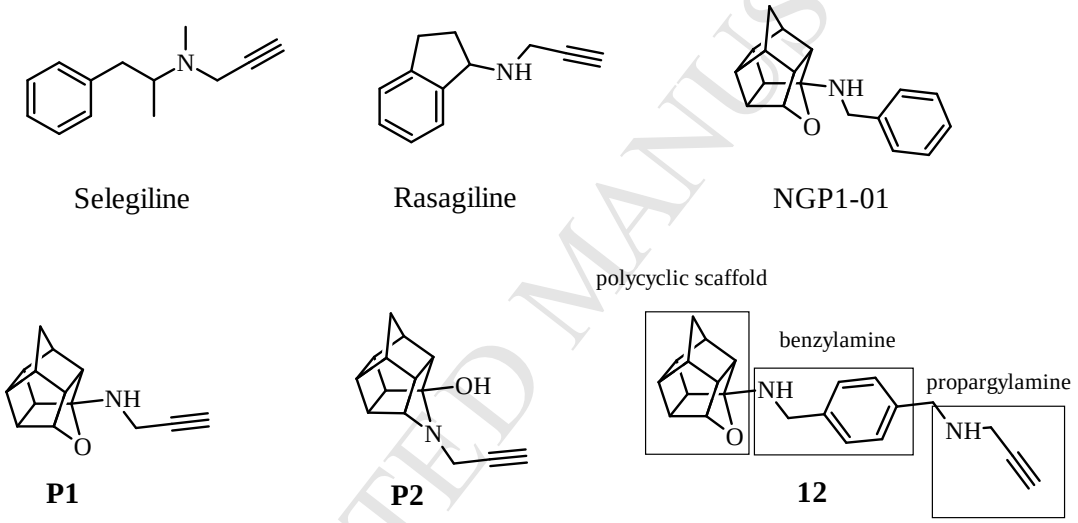


Figure 1. The molecular structures of the selective MAO-B inhibitors selegiline and rasagiline, the VGCC blocker and NMDAr antagonist NGP1-01, compounds from a previous study **P1** and **P2**, and the new series represented by compound **12**, with the polycyclic scaffold, benzylamine and propargylamine moieties highlighted.

In our previous work [26], we designed and synthesized polycyclic propargylamines, represented by **P1** and **P2** (Figure 1), to serve as multifunctional drug ligands by inhibiting MAO-B enzyme activity, and as regulators of cytosolic calcium entry predominantly mediated by NMDAr and VGCC. While these compounds showed good *in vitro* anti-apoptotic activity, they showed little to no MAO-B inhibitory activity and were inactive as NMDAr antagonist and VGCC blockers. These findings suggested that **P1** and **P2** exhibit their neuroprotective effect through some other mechanism(s) implicated in the complex etiology of neurodegeneration unexplored in the study. We further postulated that the lack of calcium regulatory activity in **P1** and **P2** was due to the absence of a benzylamine moiety within their structures. This moiety, which is present in structure of NGP1-01, a molecule known to have neuroprotective properties through VGCC blockade and NMDAr antagonistic activity [27], seems to be vital for calcium modulatory activity in such polycyclic analogues.

It is for this reason that we incorporated a benzylamine moiety in the design of a new series of compounds reported in this current study (represented by compound **12**, Figure 1).

Further investigation to clarify the lack of MAO-B inhibitory activity in these compounds was carried out by performing computer-assisted simulated docking in the MAO-B enzyme (PDB ID: 2V5Z) [28], utilizing Molecular Operating Environment (MOE) as previously described [29]. The best-ranked docking solutions showed that compound **P1** and **P2** occupy only the entrance cavity of the MAO-B enzyme and barely access the substrate cavity to form the necessary binding interactions with the FAD cofactor, which could have resulted in increased activity of these compounds (Figure 2). With the inclusion of a benzylamine moiety in the structures of the new series of compounds, as seen in compound **12** (Figure 1 and Figure 2), we envisaged better MAO-B inhibitory activity as this moiety significantly elongates the compound allowing the adjacent propargylamine moiety to come in close proximity to the FAD cofactor of the enzyme.

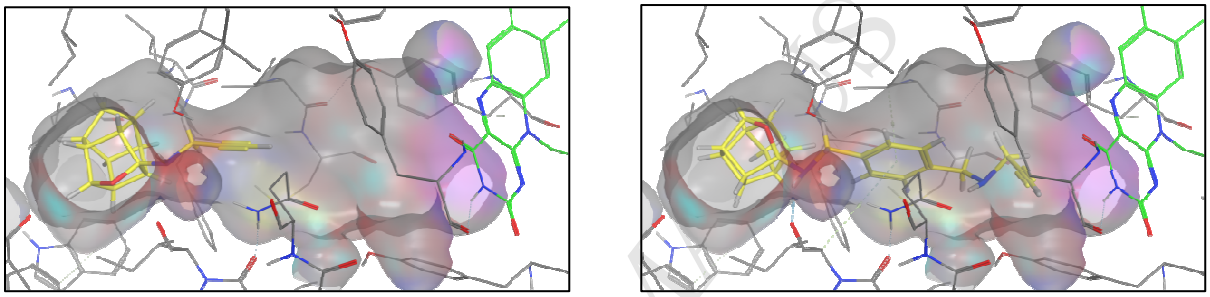


Figure 2. Schematics showing inactive compound **P1** (left) and a representative of the new polycyclic propargylamine derived series compound **12** (Right) in a computer simulated MAO-B enzyme cavity. The compounds are shown in yellow and the FAD co-factor in green. Note that compound **P1** merely occupies the entrance cavity of the enzyme pocket and remains distant from the FAD co-factor rendering it inactive, while the new series traverses deeper into the enzyme cavity to come in close proximity with the FAD co-factor allowing for potential binding interactions which may result in improved MAO-B activity.

The findings from our previous work have thus inspired the design, synthesis and evaluation of a new series of twelve pentacycloundecane and hexacycloundecane derivatives, synthesized with or without a propargylamine function. The compounds in this series, represented by compound **12** (Figure 1 and Figure 2), carry some or all of the following features; (a) a polycyclic cage scaffold - for side-chain attachment as well as for improving the drug's lipophilicity [30] and enhance drug transport across cellular membranes, including the selectively permeable blood–brain barrier, and also to increase drug affinity for lipophilic regions in target proteins [30,31]; (b) a benzylamine moiety - as present in the structure of NGP1-01, for improved VGCC blockade and NMDAr antagonism [27]; (c) a propargylamine moiety - for inherent MAO inhibitory capacity and to provide neuronal and mitochondrial protective properties [17], as well as anti-apoptotic properties [18]; (d) an elongated orientation – resulting from the inclusion of a benzylamine moiety, to promote molecular interaction between the propargylamine function and the FAD cofactor of the MAO enzyme. Compounds that show such multi-mechanistic activity may have promising potential to curtail the multifactorial etiology of neurodegenerative disorders.

The novel compounds were therefore synthesized and screened for cytotoxicity, neuroprotection, NMDAr antagonism and VGCC blockade by performing *in vitro* assays on

human neuroblastoma SH-SY5Y cells. Further to this, *in vitro* assays on the human MAO–A and MAO–B enzymes were performed in order to determine their inhibitory potential on the respective isoenzymes.

Chemistry

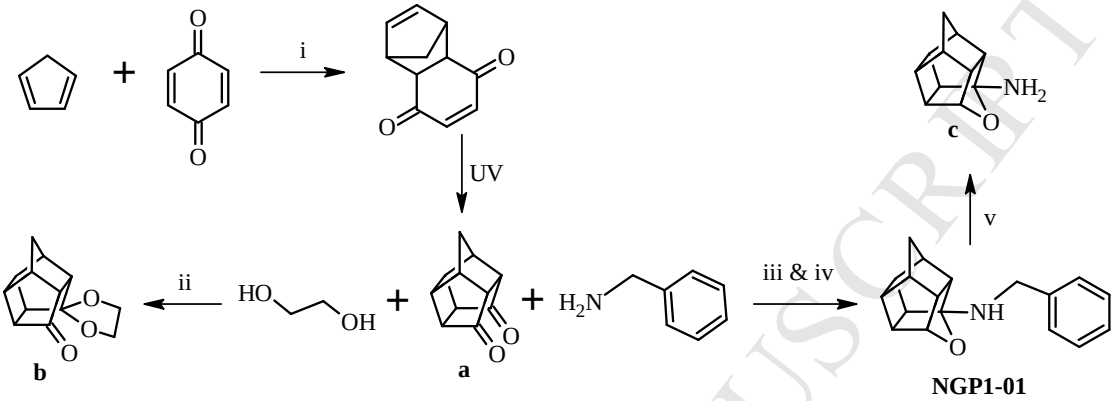
Each compound was synthesised to evaluate the activity and benefit of the presence of certain functional groups in the molecule. These groups included the following; a terminal propargylamine (**5**, **6**, **9** and **12**), a terminal secondary amine (**1**, **2**, **4** and **10**), as well as the ketal (**1 - 6**), aza (**7 - 9**) and oxa (**10 - 12**) variations of the polycyclic cage scaffold. The common starting Cookson's diketone (**a**), was prepared according to the method of Cookson *et al*. [32] From the Cookson's diketone, the monoprotected ketal polycyclic cage (**b**) was prepared according to the method of Dekker *et al*. [33] NGP1-01 was synthesized according to an adaptation of the microwave assisted method (MWAM) described by Joubert *et al*. [34**]** To synthesize the mono amine cage (**c**), debenzylation of NGP1-01 was carried out under high-pressure catalytic hydrogenation as reported by Marchand *et al*. [35] (Scheme 1).

Compounds **1** – **12**, were subsequently synthesized by direct conjugation of various analogues to the three polycyclic cage scaffolds (**a** – **c**) using microwave irradiation. MWAMs present several advantages which include; remarkable reduction of reaction time, improved yields, cleaner reactions and reduction or elimination of hazardous solvents compared to reactions performed under conventional thermal heating conditions [36,37]. As such, MWAMs have since been adopted by several researchers as the preferred strategy for the synthesis of these cage-derived organic compounds [34,38].

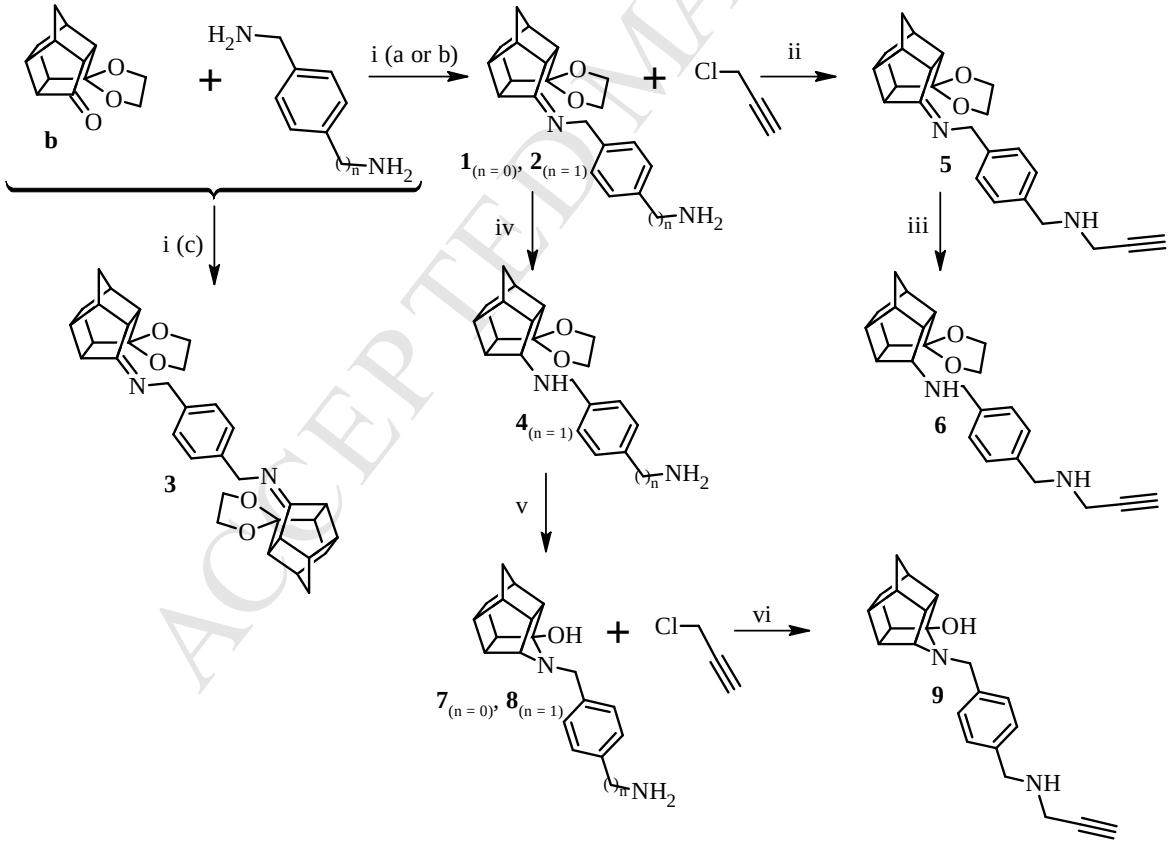
The synthesis of compounds **1**, **2** and **3** was performed by conjugating their respective primary diamines to the mono ketal polycyclic cage (**b**) through a direct amination reaction using microwave irradiation (Scheme 2). An excess amount of *p*-xylylenediamine (5.0 equiv) was used for the synthesis of compound **2** in order to promote the formation of a mono-substituted amine, however, a significant percentage of the di-substituted derivative **3** formed during the reaction and was successfully isolated giving respective yields of 32% and 13%. The serendipitously synthesized compound **3** was also included in the series of compounds for screening of potential activity. Propargyl chloride was conjugated to compound **2** using MWAMs through an S_N2 nucleophilic substitution reaction, in the presence of K_2CO_3, to produce compound **5**. To synthesize compounds **4** and **6**, reductive amination of the imines **2** and **5** was carried out using $NaBH_4$. A comparison of the biological activities of these four compounds provides insight on the influence of the imine bond on their activity. The reduction of the imines **1** and **2** with $NaBH_4$, followed by acid hydrolysed transanular cyclization using HCl, gave the desired aza-bridged compounds **7** and **8** respectively. Propargyl chloride was conjugated to compound **8** through a nucleophilic S_N2 substitution reaction using MWAMs to yield compound **9** (Scheme 2).

Compound **10**, which is an oxa analogue and structural isomer of **7**, was synthesized by conjugating 4-amino benzylamine to the Cookson's diketone (**a**), followed by reduction with $NaBH_4$ (Scheme 3). A microwave assisted nucleophilic S_N2 substitution reaction was employed to conjugate the mono amine cage (**c**) to *p*-xylylene dichloride to yield compound

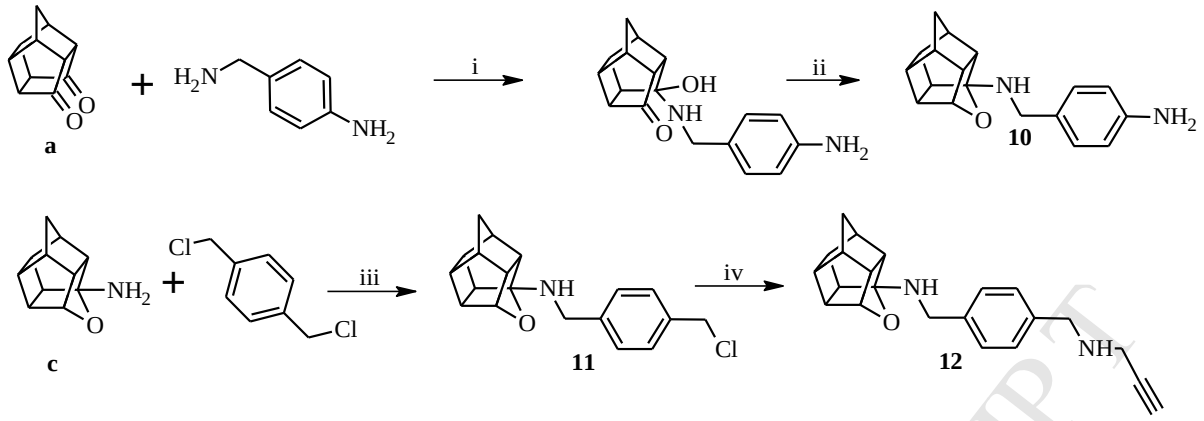
11 and to conjugate propargyl chloride to **11** and yield final compound **12**. (Scheme 3) Compounds **1**, **7** and **10** were synthesized to serve as intermediates to potential subsequent propargyl derivatives. However, due to the low reactivity of the aniline amine, reactions with halopropargyls in the form of propargyl chloride and propargyl bromide could not yield the desired derivatives. All compounds (**1** – **12**) were protected from light after synthesis and were appropriately stored at -84 °C to prevent degradation.



Scheme 1: Reagents and conditions for the synthesis of diketone (**a**), ketal (**b**) and monoamine (**c**) polycyclic scaffolds: (i) benzene, 0 °C, 1 h, 74%; (ii) benzene, *p*-TsOH (cat), Dean–Stark reflux, 5 h, 72%; (iii) ethanol, MW, 80 °C, 100 W, 100 psi, 2 h, quantitative yield; (iv) ethanol, $NaBH_4$, rt, 8 h, 62%; (v) ethanol, 10% Pd/C, H_2, 206 kPa, 50 °C, 14 h, 32%.



Scheme 2: Reagents and conditions for the synthesis of ketal- and aza-polycyclic derivatives (compounds **1** – **9)**: (i, a: n = 0) ethanol, MW, 80W, 150 psi, 100 °C, 30 min, 65%; (i, b: n = 1) ethanol, rt 1 h, then MW, 60 W, 80 psi, 100 °C, 3 h, 32%; (i, c) ethanol, rt 1 h, then MW, 60 W, 80 psi, 100 °C, 3 h, 13%; (ii) acetonitrile, K_2CO_3, 60 °C, 4 h, 21%; (iii) ethanol, $NaBH_4$, rt, 8 h, 72%; (iv) ethanol, $NaBH_4$, rt, 8 h, 69%; (v) acetone, 4 M HCl, rt, 12 h, 42% and 78%; (vi) acetonitrile, K_2CO_3, MW, 60 W, 60 °C, 2.5 h, 18%.



Scheme 3: Reagents and conditions for the synthesis of oxa-polycyclic derivatives (compounds **10** – **12)**: (i) ethanol, MW, 100 W, 100 psi, 80 °C, 2 h; (ii) ethanol, $NaBH_4$, rt, 8 h, 32%; (iii) acetonitrile, K_2CO_3, MW, 150 W, 20 psi, 70 °C, 1 h, 19%; (iv) acetonitrile, K_2CO_3, MW, 150 W, 20 psi, 70 °C, 1 h, 21%.

Characterization of all the compounds was carried out by means of ^{1}H-, ^{13}C-NMR, IR and HREI-mass spectra. A common structural feature in all the synthesized compounds was the polycyclic cage moiety. This structural moiety showed characteristic signal peaks on the ^{1}H-NMR spectra which included a characteristic AB quartet signal, due to the two unsymmetrical protons on the bridgehead. This signal appeared at a chemical shift in the range of δ 1.10–1.91 ppm, with a coupling constant in the range of 10.6–10.8 Hz. The rest of the protons making up the polycyclic cage appeared as multiplets or groups of multiplets, apparent quartets, and apparent triplets in the range of δ 2.30–3.00 ppm. These signals confirmed the presence of the polycyclic scaffold in the compound structures [38].

All ketal derivatives (**1** – **6**) showed a characteristic multiplet signal in the range of δ 3.70–3.95 ppm, owing to the four protons on the ketal carbons. In compounds were the polycyclic cage scaffold was linked to the rest of the molecule *via* a rigid imine bond (**1**, **2**, **3** and **5)**, the ketal moiety seemed to influence the signal of the two protons of the -N-CH_2- linker by virtue of long-range coupling. Due to this coupling, the two protons appeared on the ^{1}H-NMR spectra as apparent doublet of doublets at about δ 4.30–4.60 ppm, with a coupling constant of 3.2 Hz. When the imine bond was reduced to a rotatable amine bond, in the case of **4** and **6**, this coupling effect was lost and the signal appeared as a multiplet at an upfield shift of about δ 3.95 ppm.

A typical signal for the presence of the aza-cage moiety (**7** – **9**), was a triplet at a chemical shift in the range of δ 3.21–3.95 ppm, with coupling constants in the range of 4.8–5.2 MHz. This triplet corresponded to the single proton at the -CH-N- position, and its multiplicity is attributed to the presence of two protons at the adjacent carbons atoms within the polycyclic cage [38]. In the case of the oxa-cage moiety (**10** – **12**), this triplet signal was at a range of about δ 4.65–4.73 ppm with a coupling constant in the range of 4.8–5.0 Hz. The downfield shift of this methine hydrogen resulted from the deshielding effect of the adjacent oxygen atom. This downfield shift, compared with that of its aza analogues, is attributed to the electronegativity difference between nitrogen and oxygen, and thus, an increased deshielding effect compared to the aza derivatives.

The presence of a propargyl moiety in the structures of compounds **5**, **6**, **9** and **12** was confirmed by a characteristic ^{1}H-NMR triplet at a chemical shift in the range of δ 2.20–2.30 ppm, with a coupling constant of about 2.4 Hz. This triplet corresponds to the terminal acetylene proton and its multiplicity is attributed to the neighbouring methylene protons which influence it *via* long-range coupling. The terminal acetylene proton equally influenced the two symmetrical methylene protons, which form part of the propargyl moiety, to appear as a doublet in the range of δ 3.40–3.50 ppm, with a coupling constant of about 2.4 Hz.

Further characterization of these compounds was carried out by means of ^{13}C-NMR, IR and HREI-mass spectra, which all supplemented the ^{1}H-NMR spectra findings, further confirming the structures of the compounds.

Cytotoxicity

Compounds **1**-**12** were assayed for cell viability on SH-SY5Y human neuroblatoma cells using a standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay method [39]. The results are depicted in Figure 3 and Table 1. This assay, which measures cell metabolic activity, was used to determine the cytotoxicity of the test compounds at 10 µM, 50 µM and 100 µM concentrations. The cells were exposed to test compounds for 48 hours, after which cell viability was determined spectrophotometrically. Vehicle control cells were treated with dimethyl sulfoxide (DMSO, solvent for dissolving test compounds) and served as a reference for 100% cell viability. NGP1-01, known to have neuroprotective properties [27], was also used as a reference control for relative comparison.

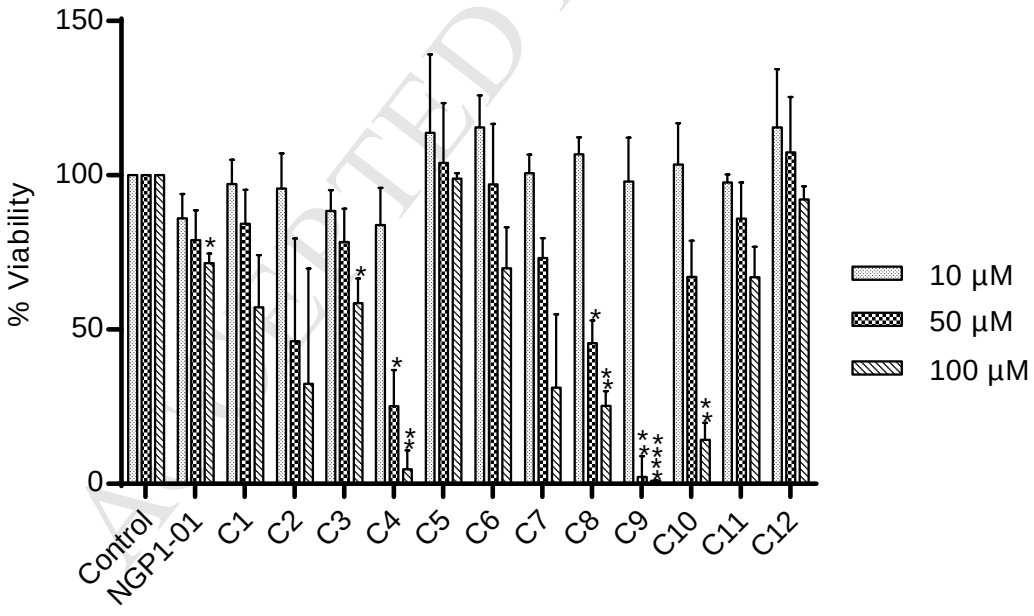


Figure 3. Percentage cell viability of compounds **1**-**12** assessed by means of measuring the metabolic activity of SH-SY5Y neuroblatoma cells, after 48 h exposure to test compound, relative to a control of untreated viable cells. Data are mean ± SEM (n = 3, four fields per repeat). Data were subjected to an ANOVA statistical analysis and significance was defined as [(*) $p < 0.05$, (**) $p < 0.001$, (***) $p < 0.0001$].

The results from this assay suggested the SH-SY5Y human neuroblastoma cells to be viable with the test compounds, particularly at 10 µM, as none of the compounds showed

statistically significant cytotoxicity on the cell line at this concentration (Figure 3). Percentage cell viability at this concentration ranged from 84% to 116% relative to the untreated control. This was comparable to NGP1-01, which was found to be 86% viable at the same concentration. The influence of the propargyl moiety was evident at higher concentrations of test compound as seen when comparing the propargyl containing compound **5**, which was 99% viable at 100 μM, with its precursor devoid of this moiety, compound **2**, which was only 32% viable at the same concentration. This observation suggests compound **2** to be 3-times more toxic than its propargyl containing derivative. The same observation was made when comparing compound **6**, which was 70% viable at 100 μM, to its precursor compound **4**, which was only 5% viable at the same concentration, suggesting the inclusion of a propargyl moiety to be responsible for a 14-fold decrease in cytotoxicity.

Neuroprotection

The SH-SY5Y cytotoxicity assay results were used to determine the test concentration of compounds **1**-**12** which would insignificantly affect the viability of the SH-SY5Y cells. Based on the SH-SY5Y cytotoxicity analysis, it was decided to conduct the neuroprotection studies at test concentrations between 1 μM and 10 μM, in order to maintain cell viability.

In this study, we employed a widely used cellular model in which neurotoxicity was induced by 1-methyl-4-phenyl pyridinium (MPP^+) in SH-SY5Y neuroblastoma cells [40,41]. MPP^+ is highly toxic to neurons and has been widely used to induce neurodegeneration in various *in vitro* and *in vivo* models [40,41]. Several signaling pathways have been suggested to be responsible for MPP^+-mediated neurotoxicity in SH-SY5Y cells, for instance, trigger of oxidative stress [42], induction of apoptosis [43], and inactivation of pro-survival phosphoinositide 3-kinase (PI3-K)/Akt cascade [44,45]. This assay was deemed appropriate to test for initial neuroprotective ability of the compounds because of the multitude of pathways involved in MPP^+ mediated neurotoxicity and the potential multifunctional inhibitory abilities of the test compounds.

Briefly, the assay entailed treating the SH-SY5Y cell line with different concentrations (1 μM, 5 μM, 10 μM) of test compounds two hours prior to MPP^+ treatment. After 48 hours of incubation, the neuroprotective effect of the compounds was assessed by means of the MTT mitochondrial function assay which measured cell viability. Vehicle control cells were treated with DMSO (solvent for dissolving test compounds) and served as a reference for 100% cell viability. Percentage neuroprotection values were calculated as the difference between the final percentage cell viability of the test compound treated cell line and that of the MPP^+ only treated cell line. These values are presented in Figure 4 and Table 1. NGP1-01, known to have neuroprotective properties [27], was also used as a reference control for relative comparison.

As illustrated in Figure 4, after the exposure to 1000 µM MPP^+ for 48 hours, the cell viability declined significantly to around 45%. However, its cytotoxic effects were significantly ameliorated in the presence of the test compounds at 1 µM, 5 µM and 10 µM concentrations. All compounds in the series exhibited significant neuroprotective effects at a 1 µM concentration as they managed to improve cell viability to values between 86% and 110%. This was expected from these compounds as they all share molecular similarities with NGP1-01 which is known to have neuroprotective properties as confirmed in this assay [27]. At 5 µM and 10 µM a slight decrease in cytoprotection was observed for most of the compounds. This may indicate that the neurotoxin challenged state of the SH-SY5Y cells may be more sensitive to the cytotoxic effect of the test compounds at higher concentrations.

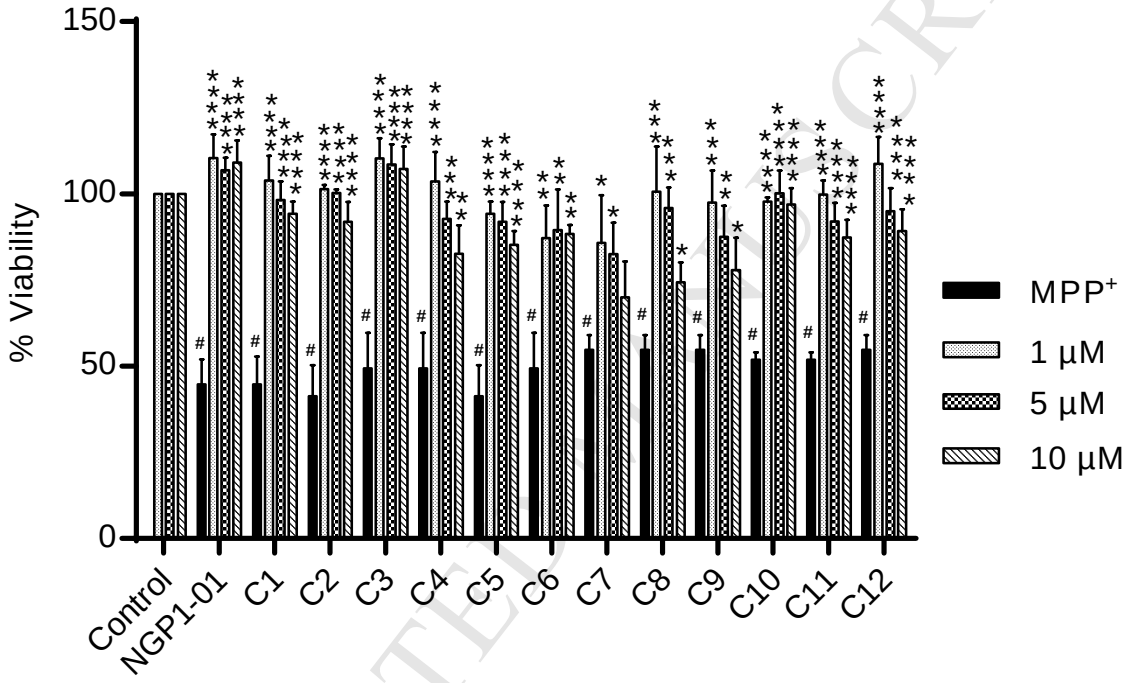


Figure 4. The effects of compounds **1-12** on MPP^+-induced (1000 µM) cytotoxicity in SH-SY5Y cells. The viability of the untreated control was defined as 100%. MPP^+ without test compound showed a significant decrease in cell viability relative to the control (#, $p < 0.05$). Data are mean ± SEM ($n = 3$, four fields per repeat). The level of statistical significance for the test compounds is set at * $p < 0.05$ compared to the MPP^+ only treated control. Tukey's multiple comparisons statistical analysis was performed on all raw data and significant neuroprotective effect was defined as [(*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$].

VGCC assay

To investigate the test compounds' potential VGCC blocking effect, we developed an assay based on the methods described by Young *et al*. [46] and León *et al*. [47] Briefly, SH-SY5Y human neuroblastoma cells loaded with the ratiometric fluorescent calcium indicator, Mag-Fura-2/AM, were incubated at 37 ºC in the presence of the test compounds at a 10 µM concentration for 30 mins, then stimulated with a concentrated solution of KCl to allow opening of the calcium channels. The assay was performed at a 10 µM concentration as determined by the cytotoxicity assay performed on the same cell line. Changes in fluorescence as a consequence of an increase in cytosolic calcium elicited by high K^+

concentrations were measured in a Biotek® fluorescent microplate reader. Two positive controls were included in the VGCC assay; nimodipine, a commercially available dihydropyridine *L*-type calcium channel blocker [48], and the prototype polycyclic amine cage compound, NGP1-01 [49]. The percentage VGCC blockade of each compound was calculated relative to the activity of nimodipine and the data is presented in Table 1.

Based on the assay results, all compounds, except **3**, **6** and **10**, showed significant VGCC inhibitory activity; ranging from 26.6% to 51.3%, relative to a 10 µM solution of nimodipine. This activity is comparable to that of NGP1-01, which inhibited calcium influx by 25.7% at the same concentration. This general improvement in activity, compared to **P1** and **P2** which were inactive as VGCC blockers at 100 µM [26], emphasizes the need to include a benzylamine moiety in the structure of polycyclic amine cage derived compounds designed for VGCC blockade as we previously postulated [26].

It seems that a primary terminal amine imparts the molecules with increased VGCC inhibition. This was seen in compounds **1**, **2**, **4**, **7** and **8**, which showed activities of 40%, 35%, 43%, 36% and 51% respectively, a significant improvement to the activity noted for NGP1-01. When this primary amine terminal was converted to a secondary amine, by conjugating a propargyl moiety, in the case of compounds **5**, **6** and **9**, the VGCC inhibitory activity notably decreased to 26%, 22.9% and 39% respectively, when compared to their primary amine carrying precursors (**2**, 35%; **4**, 43% and **8**, 51%). A terminal primary amine could be necessary for the formation of productive binding interactions between the respective compounds and the binding site of the VGCC resulting in their blockade.

NMDA assay

To assess the compounds' ability to block Ca^{2+} influx *via* the NMDAr channels, a similar method as in the VGCC assay was used. However, a concentrated NMDA/Glycine solution, instead of KCl, was used to stimulate calcium flux through the NMDAr. Two positive controls were included in this assay; MK-801, a commercially available non-competitive antagonist of the NMDAr [50], and NGP1-01 [49]. All compounds were assayed at 10 µM and the percentage NMDAr inhibition of each compound was calculated relative to the activity of MK-801. The calculated percentage inhibition values are presented in Table 1.

Several compounds showed great NMDAr antagonism, with compound **5** showing the highest activity of 88.3%. Compounds **1**, **3** and **6** also showed good activity with percentage inhibition values of 63.1%, 70.5% and 63.9% respectively. These four compounds, as well as compound **2**, which showed significant activity of 49.5%, showed NMDAr antagonism comparable to NGP1-01, which exhibited 32.5% inhibition at the same concentration. A common structural feature of these five compounds is a ketal moiety which seemed to be important for increased NMDAr antagonism. This observation was more compelling when considering that compounds **9** and **10**, which were devoid of this functional moiety, were both inactive; as well as compounds **7** and **11**, which also lacked the ketal moiety, showed statistically insignificant activity of 11.9% and 26.1% respectively. The ketal moiety could be providing necessary bulk and charge which could allow for productive ligand-NMDAr

binding interactions. Further mechanistic studies could provide further insight on the putative binding mode of these compounds.

The inclusion of a propargyl moiety in the compound structures seemed to contribute to improved NMDAr antagonism. This was evident when comparing the propargyl carrying compounds **5** and **6**, which were 88.3% and 63.9% active respectively, to their precursor compounds **2** and **4**, devoid of this moiety, which were 49.5% active and inactive respectively. An imine bond between the polycyclic cage and the benzylamine moiety, as seen in compounds **2** and **5**, seemed to be ideal for NMDAr inhibitory activity compared to the simple amine bond contained in the structures of compounds **4** and **6**. When the imine bond in compound **2** is reduced to a simple amine bond in compound **4**, the NMDAr antagonistic activity was completely lost. Similarly, there was about 25% decrease in activity when the imine bond in compound **5** was reduced to afford compound **6**. The rigid imine bond seemed to be ideal for NMDAr inhibition. A probable explanation for this could be that the rigid ligand confirmation allows the compounds to adequately traverse the ligand-recognition region of the NMDAr subunit which is defined by two polypeptide segments, S1 and S2 [51].

Generally, these compounds seem to possess calcium regulatory potential comparable to the prototype NGP1-01 by acting on both VGCC and NMDAr. However, the compounds appeared to be more active as NMDAr antagonists than they are VGCC blockers.

MAO-A and MAO-B inhibition studies

The target compounds were investigated for their inhibitory activity against human MAO (hMAO) by measuring the extent to which the test compounds reduce the oxidative catalysis of kynuramine, a mixed MAO-A/B substrate, by the respective MAO enzymes [52]. The fluorescence of the MAO generated 4-hydroxyquinoline in the supernatant fractions were measured using a Biotek® fluorescent microplate reader at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. IC_{50} values were calculated as the compound concentration that produces 50% enzyme activity inhibition. The inhibition potencies of the test compounds and reference compounds, selegiline and clorgyline, are presented in Table 1.

Based on the results, most of the active compounds displayed higher inhibitory potencies against the MAO-B compared to the MAO-A isoenzyme as suggested by the selectivity index (SI) values. The only exception was compound **10,** which showed selectivity to the MAO-A isoenzyme. The ketal moiety seemed to be influential in conferring the molecules MAO-B selectivity as observed from the high SI values of compounds **1**, **2**, **5** and **6**, which all carry this moiety. These compounds were about 3 to 52 times more selective to the MAO-B compared to the MAO-A isoenzyme. Conversely, compounds **9** and **12**, though devoid of the ketal moiety, also showed selectivity to MAO-B with SI values of 14.8 and 3.6 respectively.

Several structure activity relationships could be derived from comparing the MAO IC_{50} values of the various compounds in the series. Interesting to note, was that the propargylamine carrying compounds **5**, **6**, **9** and **12** showed MAO-B inhibition values which ranged between 1.7 µM and 36.31 µM (IC_{50}). This was a significant improvement from the activities shown by the previously reported compounds **P1** and **P2**, which were inactive as

MAO-B inhibitors[26]. This improved activity can be attributed to the increased molecular length of these compounds as a result of the incorporation of a benzylamine moiety into their structures.

Table 1: The biological activity profiles of compounds **1-12** and related reference compounds.

Compound	Cell Viability %	Neuro-protection %[a]	VGCC %	NMDA %	MAO- A	MAO-B	SI MAO-B[b]
	10 µM	1 µM	10 µM	10 µM	IC_{50} µM	IC_{50} µM	
Selegiline	-	-	-	-	-	<10 nM	-
Clorgyline	-	-	-	-	<10 nM	-	-
MK-801	-	-	Inactive	100.0	-	-	-
Nimodipine	-	-	100.0	Inactive	-	-	-
NGP1-01	86 ± 7.8	66 ± 6.9****	25.7 ± 9.2*	32.5 ± 9.9*	>100	>100	-
P1[26]	-	-	Inactive	Inactive	-	Inactive	-
P2[26]	-	-	18	4	-	6%	-
1	97 ± 7.9	59 ± 7.2****	40.1 ± 3.1***	63.1 ± 11.3****	>100	1.91**	>52.4
2	96 ± 11.4	60 ± 1.3****	35.0 ± 10.2**	49.5 ± 6.0**	>100	13.80*	>7.2
3	88 ± 6.8	61 ± 5.9****	24.3 ± 15.7	70.5 ± 4.2***	>100	100	-
4	84 ± 12.0	54 ± 8.5****	43.2 ± 3.9***	Inactive	>100	>100	-
5	114 ± 25.3	53 ± 3.5****	26.6 ± 3.9*	88.3 ± 7.9****	>100	1.70*	>58.8
6	116 ± 10.4	38 ± 9.4**	22.9 ± 5.5	63.9 ± 13.4**	>100	36.31*	>2.8
7	101 ± 6.0	31 ± 13.0*	36.5 ± 8.2***	11.9 ± 7.8	>100	>100	-
8	107 ± 5.5	46 ± 13.2***	51.3 ± 10.4****	59.0 ± 4.7****	>100	>100	-
9	98 ± 14.2	43 ± 9.2***	39.4 ± 8.0***	0.6 ± 8.1	56.23	3.80*	14.8
10	104 ± 13.4	46 ± 1.2****	7.6 ± 1.3	Inactive	2.37**	100	0.02
11	98 ± 2.7	48 ± 4.1****	31.6 ± 1.7**	26.1 ± 2.0	>100	14.13**	7.08
12	116 ± 18.8	54 ± 7.8****	29.1 ± 5.8**	31.4 ± 6.8*	>100	28.18**	3.55

[a]Percentage neuroprotection values calculated as the difference between the final percentage cell viability of the test compound treated cell line and that of the MPP^{+} only treated cell line. Experimental values are a mean of at least 9 independent experiments (n = 9); (b) = hMAO-B selectivity index calculated as: IC_{50}(hMAO-A)/IC_{50}(hMAO-B); Statistical analysis was performed on raw data, with asterisks indicating significant activity [(*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$].

The data from this study suggests the juxtapositioned benzylamine and propargyl moieties are imperative in rendering the compounds better MAO-B inhibitory activity. This observation was noted by comparing the activity of compounds **4** and **8**, which both lacked the propargyl moiety but carried the benzylamine moiety, to the activity of compounds **6** and **9**, with both moieties. Compounds **4** and **8** showed IC_{50} values of more than 100 µM, while compounds **6** and **9** showed values of 36.31 µM and 3.89 µM respectively. It is important to note that when the benzylamine moiety is absent in the molecular structure of these compounds, as in the case of **P1** and **P2**, the propargylamine moiety alone fails to render significant activity on the MAO-B enzyme, thus further supporting the need for both moieties in the structures of these type of compounds for activity.

By comparing derivatives **5** and **6**, whose IC_{50} values were 1.70 µM and 36.31 µM respectively, the influence of the imine bond between the polycyclic scaffold and the benzylamine moiety could be appreciated. This bond seemed to confer compound **5** a 16-fold increase in activity when compared to compound **6**, in which the imine bond was reduced to a secondary amine. The same was true for the precursors of compounds **5** and **6**, compounds **2** (imine) and **4** (amine), whose IC_{50}'s were 13.8 µM and >100 µM respectively. Perhaps this is

because the imine bond, present in compounds **2** and **5**, is more rigid than the rotatable amine bond present in compounds **4** and **6**, thus allowing compounds **2** and **5** to be confined to a fixed region of the MAO-B enzyme active site allowing for more productive binding interactions.

When compound **2** is di-substituted with the ketal-polycyclic moiety to give compound **3**, MAO-B inhibitory activity is lost. The di-substituted molecule appears to be bulky and probably fails to make access into the enzyme active pocket, rendering it inactive. The absence of a propargyl function within the structure of compound **3** further explains the lack of MAO inhibition observed from this compound.

NGP1-01 showed little activity on both MAO-A and MAO-B enzymes, IC_{50} >100 µM on both isoenzymes, but when para-substituted with a primary amine to give compound **10**, MAO-A inhibition improved (IC_{50} = 2.37 µM)**.** This improved activity may be due to the involvement of the amine group in the formation of binding interactions with amino acid residues found in the substrate cavity of the MAO-A enzyme. Contrary to this, compound **4**, which was also para-substituted with a secondary amine, showed little activity on the MAO-A isoenzyme. This could be due to the presence of the bulk ketal moiety attached to the polycyclic cage scaffold, which may be hindering the entrance of the molecule into the enzyme substrate cavity. It is important to note that all ketal derivatives **1 – 6** showed little activity on the MAO-A isoenzyme of IC_{50} > 100 µM.

In general, compounds **1**, **2**, **5**, **6, 9** and **12** showed the best overall MAO inhibitory activity and appear to be selective for the MAO-B isoenzyme. Although the MAO-B inhibitory activity herewith reported was a significant improvement in comparison to the previously reported compounds **P1** and **P2**, it is important to note that this activity was still about 1000-fold weaker than selegiline. Further structural modifications of these compounds may therefore be necessary to afford molecules which may potentially show improved MAO-B inhibition.

MAO reversibility studies

To determine the binding mode of the studied compounds on MAO-A and/or -B, a time dependency of enzyme inhibition was measured. If the compounds form a covalent adduct with the enzyme, a time-dependent reduction of enzyme activity would be expected. In this regard, the time dependent inhibition of MAO-A and-B by the active compounds **1, 5, 6, 9** and **12**, were evaluated (Figure 5). Briefly, recombinant human MAO-A and MAO-B was preincubated with the test compounds for periods of 0, 15, 30 and 60 min prior to starting the enzyme reaction and the residual rates of the MAO-A and -B catalysed oxidation of kynuramine were measured. For this purpose, the concentrations of the test compounds chosen were approximately twofold the measured IC_{50} values for the inhibition of the respective enzymes. Clorgyline, with known irreversible inhibition for MAO-A [53], and selegiline, with known irreversible inhibition for the MAO-B [54], were used as a reference compounds.

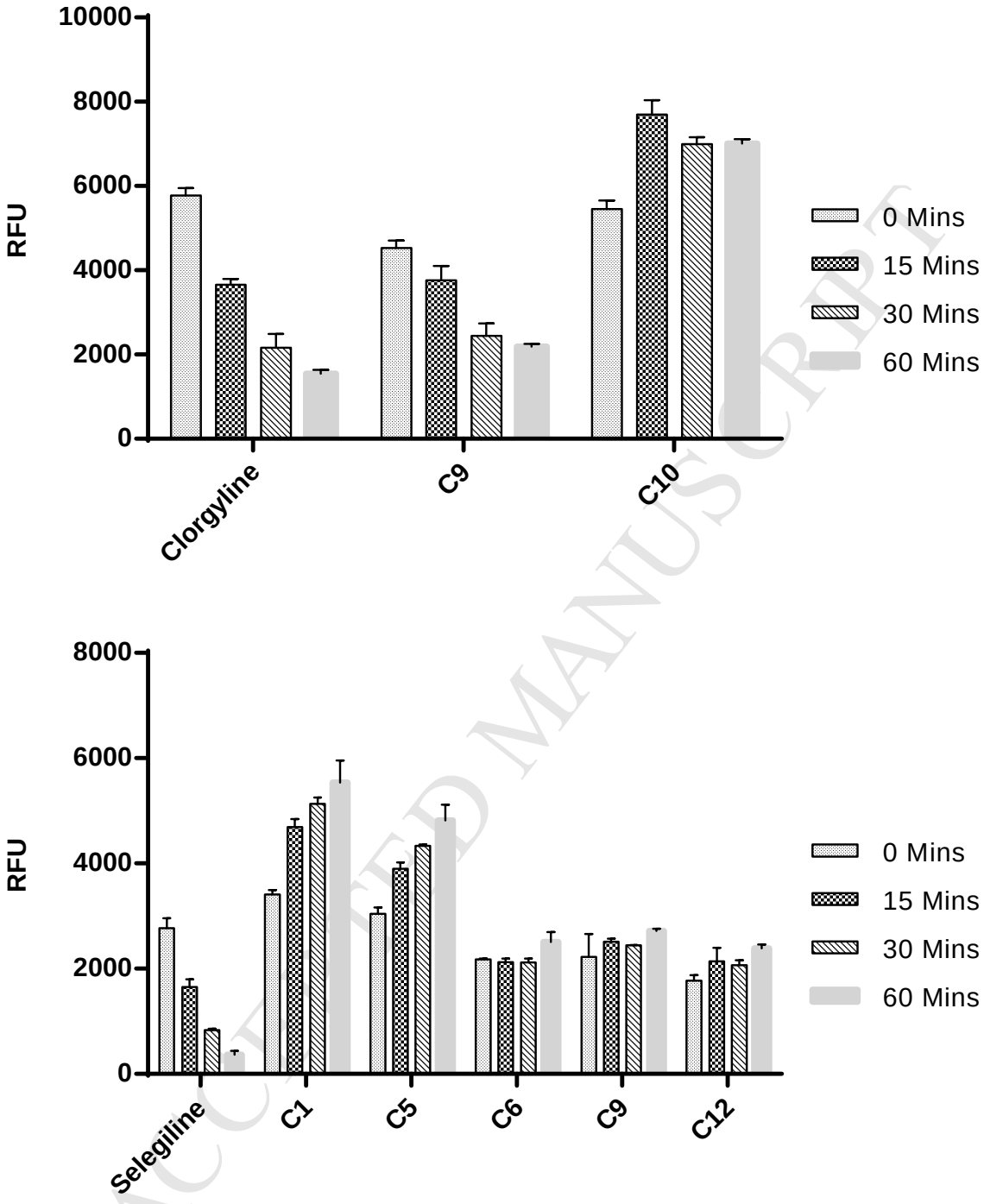


Figure 5. Time-dependent inhibition of the recombinant human MAO-A (top) and MAO-B (bottom) catalysed oxidation of kynuramine by the selected active compounds. The enzymes were preincubated for various periods of time (0–60 min, x-axis) Data are expressed as the mean RFU (relative fluorescent units) ± SEM of three independent experiments.

As shown in Figure 5, only compound **9** showed a significant reduction in enzymatic activity with increased preincubation time when assayed on the MAO-A isoenzyme, suggesting compound **9** to be an irreversible inhibitor of MAO-A. MAO-B enzyme activity was not reduced by compounds **1, 5, 6, 9** and **12** with increased preincubation time. This indicates

that the selected compounds were reversible inhibitors of MAO-B. Preliminary MAO-A molecular modelling studies performed on compound **9** (data not shown) showed potential involvement of the free hydroxyl group attached to the polycyclic moiety in forming covalent binding interactions with amino acid residue Gln215 of the MAO-A substrate cavity. This interaction could be the explanation for the observed irreversible MAO-A inhibition exhibited by compound **9**. No covalent interactions were observed between compound **9** and the MAO-B isoenzyme (see Figure 7), perhaps explaining the reversible MAO-B inhibition observed for compound **9**. It also was interesting to note that compounds **1** and **5** showed a notable increase in enzymatic activity over time. This might be due to test compound degradation in the aqueous medium as both compounds possess a ketal moiety as well as an imine bond. The imine function is known to undergo hydrolysis in aqueous medium [55], however, further degradation studies on these compounds are necessary to validate this notion.

MAO-B molecular modelling studies

With most compounds showing selectivity for the MAO-B isoenzymes, the binding modes of all twelve compounds in the MAO-B substrate cavity were examined *in silico*. Docking simulations were performed on the human MAO-B crystal structure (PDB ID: 2V5Z) [28], using the Molecular Operating Environment (MOE) software [29]. In general, the amino acid residues between 120 and 220, as well as the FAD cofactor, are important in conferring substrate selectivity of MAO-B [56], most importantly residues Ile-199, situated in the entrance cavity, and Gln-206, situated in the substrate cavity. Interaction with these amino acid residues may confer a compound MAO-B inhibitory activity as shown by safinamide (Figure 6), a known MAO-B inhibitor.

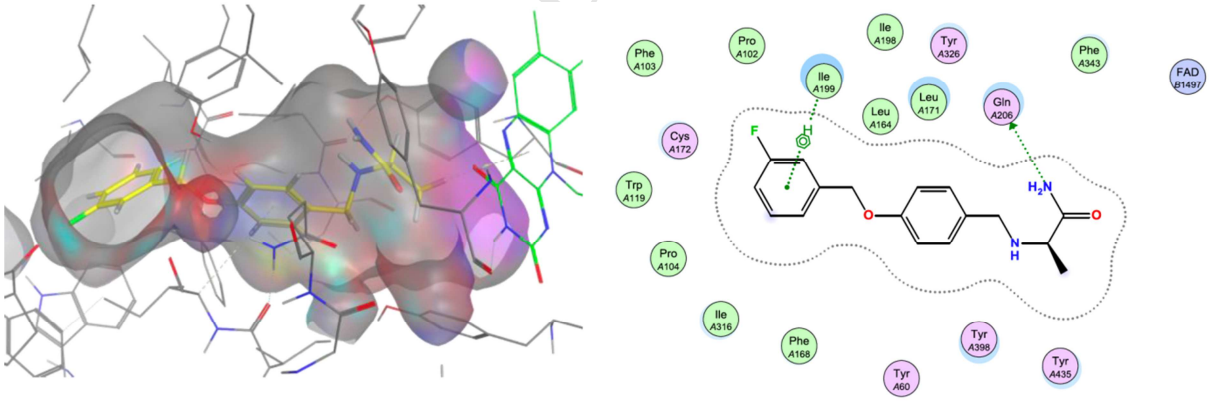


Figure 6. The putative binding modes of safinamide within the human MAO-B enzyme active site. Safinamide and the FAD cofactor are shown in the docking simulation on the left as bold lines (indicated in yellow and green respectively), and the binding interactions with the amino acid residues shown on the right. Safinamide shows productive interactions with amino acid residues Ile-199 and Gln-206.

The docking simulation results generally showed that the lipophilic polycyclic cage unit, carried by all the test compounds, stabilized within the hydrophobic environment of the enzymes entrance cavity, while the rest of the molecular structure traversed deep into the substrate cavity (see Figure 7). Further analysis of the best-ranked docking solutions showed all active compounds to be forming productive binding interactions with the MAO-B substrate cavity. Interestingly, most of the active compounds, particularly compounds **1**, **2**, **5**,

9, **11** and **12**, seemed to be forming binding interactions specifically with Ile-199, an observation which could explain their noted *in vitro* activity. The electron-rich π system, presented by the aromatic ring within these compounds' structures, appeared to be vital for the formation of 'arene-hydrogen' interactions with the amino acid Ile-199.

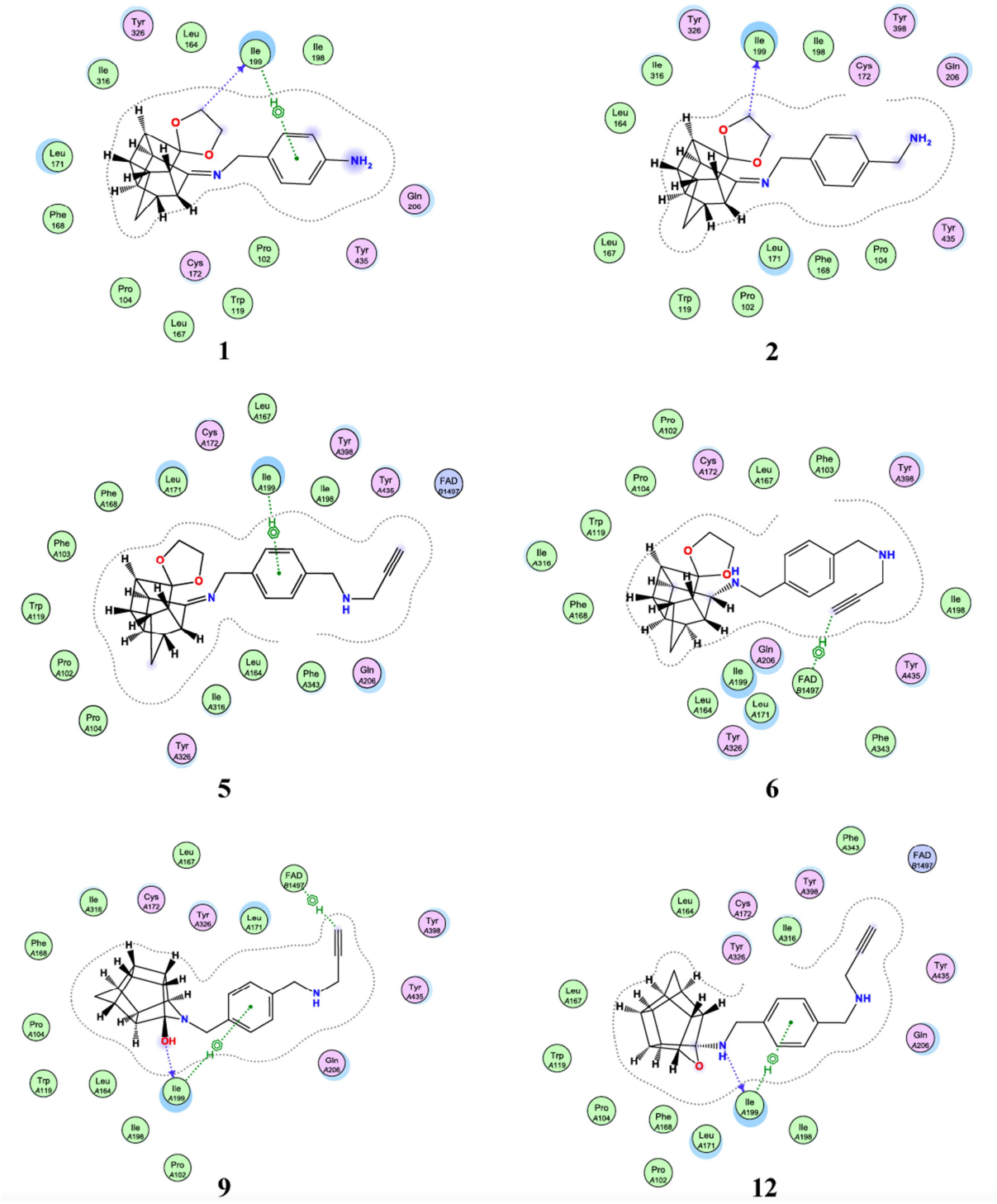


Figure 7: MOE generated binding interactions of compounds **1**, **2**, **5**, **6**, **9** and **12** with the respective amino acid residues within the human MAO-B enzyme active site.

In the case of compounds **1** and **2**, the ketal moiety also seemed to be involved in the formation of binding interactions with Ile-199. This interaction may be due to the electronegative oxygen atom which creates a slightly positive environment on the adjacent -CH_2 group causing it to serve as a proton donor to the amino acid residue. While the aromatic system and ketal moiety seemed to be important for the formation of productive interactions with the enzyme cavity, it appeared as though these interactions were dependent on the presence of an imine bond between the polycyclic cage and the benzylamine moiety. This was evident when comparing binding interactions observed for compounds **1**, **2** and **5**, which all carried this bond and showed interactions with Ile-199, to the binding interactions observed for compounds **4** and **6**, which lacked this bond and hence failed to interact with Ile-199. This observation suggests the imine bond to be important for stabilizing the molecules' benzylamine moiety within the MAO-B enzyme cavity, thus allowing for the formation of productive binding interactions. The observation correlates with the observed *in vitro* activity of compounds **1**, **2** and **5**, which all showed significant MAO-B inhibition of IC_{50}: 1.91 µM, 13.80 µM and 1.70 µM, respectively. While compound **6,** shows no interaction with Ile-199 due to the absence of an imine linkage, the compound remains active due to the presence of a propargylamine group which forms interactions with the FAD cofactor.

The inactive compounds **7**, **8** and **10**, the oxa- and aza-type derivatives with a terminal primary amine group, showed no interaction with any amino acid residues within the MAO-B active site. This observation may be the explanation to these compounds' lack of activity. Interesting to note was that when the secondary amine group was conjugated to a propargyl function, as seen in compound **9**, the resultant compound showed interactions with Ile-199 and the FAD cofactor. The role of the propargyl moiety seems to be particularly important for MAO-B inhibitory activity as suggested by the *in vitro* activities of compounds **5**, **6**, **9** and **12** (IC_{50}: 1.70 µM, 36.31 µM, 3.80 µM and 28.18 µM, respectively). Molecular modelling findings showed the propargyl moieties of these four compounds to either be in close proximity to the FAD cofactor, in the case of compounds **5** and **12**, or to be forming binding interactions with the FAD cofactor, in the case of compounds **6** and **9**.

Conclusion

We have successfully designed and synthesized twelve novel pentacycloundecane and hexacycloundecane derived compounds which showed promising neuroprotective potential. Regarding cytotoxicity, the SH-SY5Y human neuroblastoma cells seemed to be viable with all the test compounds, particularly at 10 µM, as none of the compounds showed statistically significant cytotoxicity at this concentration. At a 1 µM concentration, the compounds significantly improved the viability of SH-SY5Y neuroblastoma cells, previously exposed to the neurotoxin MPP^+, from about 45% to values between 86% and 110%.

Most of the compounds, except **3**, **6** and **10**, showed statistically significant VGCC inhibitory activity ranging from 26.6% to 51.3%, relative to a 10 µM solution of nimodipine. This was comparable to that of NGP1-01, which inhibited calcium influx by 25.7% at the same concentration. Compounds **1**, **2**, **3**, **5**, **6**, **8** and **12**, showed significant NMDAr inhibitory activity ranging between 31.4% and 88.3%, relative to a 10 µM solution of MK-801.

When assayed for MAO inhibition, most of the active compounds (**1**, **2**, **5**, **6**, **9**, **11** and **12**) displayed selectivity to the MAO-B isoenzyme with IC_{50} values ranging from 1.70 µM to 36.31 µM, with the only exception being compound **10**, which showed selectivity to the MAO-A isoenzyme. The most active MAO-A inhibitor, compound **10**, as well as the MAO-B inhibitors, compounds **1**, **5**, **6**, **9** and **12**, all appeared to be reversible MAO inhibitors as defined by the time-dependency studies conducted. Compound **9** however, seemed to be an irreversible inhibitor of the MAO-A isoenzyme, an undesirable type of inhibition which has been shown to cause the unwanted hypertensive response in patients [13]. Further structural modifications to compound **9** are therefore necessary in order to afford a resultant molecule with the desired reversible MAO-A inhibitory potential.

Molecular modelling studies gave some insight on the compounds' potential binding interactions with the MAO-B isoenzyme. Based on the *in silico* studies, the benzylamine moiety seemed to be a very important structural component in affording this series MAO-B inhibitory activity. Not only was it important for elongating the molecules to allow for the propargylamine moiety to interact with the isoenzyme's FAD cofactor, but it also seemed to be involved in most of the binding interactions observed *in silico* with the MAO-B substrate cavity, particularly with Ile-199.

Various other structure-activity relationships were derived from the series of compounds, and these include the influence of; (a) the propargyl moiety, in reduced cytotoxicity, improved NMDAr antagonism and improved MAO-B inhibition, (b) a terminal primary amine, which improved VGCC blockade (c) a benzylamine moiety for improved VGCC blockade as well as MAO-B inhibition, (d) an imine bond between the polycyclic cage and the benzylamine moiety, which was ideal for NMDAr antagonism and MAO-B inhibition, (e) a ketal moiety, which seemed to be important for increased NMDAr antagonism and conferring the compounds MAO-B selectivity.

When considering multifunctionality, compounds **1**, **2**, **5** and **12** showed the best overall activity as viable neuroprotective agents with inherent calcium regulatory potential, by blocking VGCC and NMDAr, and MAO-B inhibitory capacity. More importantly, due to the inclusion of a propargyl moiety within their structures, compounds **5** and **12** are promising leads to multi-mechanistic compounds the may be useful as drug agents for the treatment of neurodegenerative disorders. Further *in vitro* and *in vivo* studies, which may include mechanistic studies as well as elucidation of the actual binding mode of these compounds, would provide better insight on their drugabilty. Also, these analogues may be screened for potential neuroprotective activity on various other drug targets implicated in neurodegeneration.

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Supplementary material

Supplementary data to this article including experimental procedures, additional molecular modelling data and NMR spectra can be found online at xxx.

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Highlights

- *In silico* studies guided the design of twelve new polycyclic cage compounds.
- All compounds showed significant neuroprotection between 31% and 61% at 1 μM.
- The compounds have VGCC and NMDAr Ca^{2+} regulatory potential similar to or better than NGP1-01.
- *In vitro* and *in-silico* studies suggest the compounds to be reversible MAO-B inhibitors.
- The inclusion of the propargylamine led to multi-mechanistic neuroprotective agents.