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Design, synthesis and evaluation of indole derivatives as multifunctional agents against Alzheimer's disease

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A series of indole derivatives was designed and synthesised to improve on activity and circumvent pharmacokinetic limitations experienced with the structurally related compound, ladostigil. The compounds consisted of a propargylamine moiety (a known MAO inhibitor and neuroprotector) at the *N*1 position and a ChE inhibiting diethyl-carbamate/urea moiety at the 5 or 6 position of the indole ring. In order to prevent or slow down the *in vivo* hydrolysis and deactivation associated with the carbamate function of ladostigil, a urea moiety was incorporated into selected compounds to obtain more metabolically stable structures. The majority of the synthesised compounds showed improved MAO-A inhibitory activity compared to ladostigil. The compounds possessing the propargylamine moiety showed good MAO-B inhibitory activity with **6** and **8** portraying IC₅₀ values between 14–20 fold better than ladostigil. The ChE assay results indicated that the compounds have non-selective inhibitory activities on eeAChE and eqBuChE regardless of the type or position of substitution (IC₅₀: 2–5 μM). MAO-A and MAO-B docking results showed that the propargylamine moiety was positioned in close proximity to the FAD cofactor suggesting that the good inhibitory activity may be attributed to the propargylamine moiety and irreversible inhibition as confirmed in the reversibility studies. Docking results also indicated that the compounds have interactions with important amino acids in the AChE and BuChE catalytic sites. Compound **6** was the most potent multifunctional agent showing better inhibitory activity than ladostigil *in vitro* on all enzymes tested (hMAO-A IC₅₀ = 4.31 μM, hMAO-B IC₅₀ = 2.62 μM, eeAChE IC₅₀ = 3.70 μM, eqBuChE IC₅₀ = 2.82 μM). Chemical stability tests confirmed the diethyl-urea containing compound **6** to be more stable than its diethyl-carbamate containing counterpart compound **8**. Compound **6** also exerted significant neuroprotection (52.62% at 1 μM) against MPP⁺ insult to SH-SY5Y neural cells and has good *in silico* predicted ADMET properties. The favourable neuronal enzyme inhibitory activity, likely improved pharmacokinetic properties *in vivo* and the potent neuroprotective ability of compound **6** make it a promising compound for further development.

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1. Introduction

Alzheimer's disease (AD) is an age related neurodegenerative disorder characterised by progressive memory loss and cognitive impairment occurring as a result of a process of programmed cell death known as apoptosis.^{1,2} Over the years several pathways have been indicated in the pathology of the disease. The cholinergic hypothesis states that there is an extensive loss of cholinergic neurons in the central nervous system that contributes to impairment in the cognitive and

memory symptoms of the affected person.³ Oxidative stress and amyloid β (Aβ) plaque formation have been shown to be involved in the pathophysiology of the disease.^{4,5} Acetylcholinesterase (AChE) inhibitors in AD act by increasing the endogenous levels of acetylcholine (ACh) in the brain and thereby enhance cholinergic transmission and improve cognitive functions (Fig. 1).^{6,7} They however do not halt the process of apoptosis nor improve the depressive symptoms of the disease due to the multifactorial nature of AD. Butyrylcholinesterase (BuChE) also has the capacity to hydrolyse acetylcholine. Excess ACh levels in the brain cause saturation of AChE and in turn increase the activity of BuChE towards the neurotransmitter.⁸ Even though AChE hydrolyses more ACh than BuChE, the latter contributes more to AD because of the decreased levels of the true cholinesterase, hence its inhibition is of value to AD.^{9,10} Accordingly, a compound that inhibits

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Fig. 1 Structures of previously described cholinesterase inhibitors.

this enzyme, in addition to AChE, would prove beneficial for treatment of AD.

The monoamine oxidase (MAO) enzymes natively metabolise amine neurotransmitters such as dopamine and 5-hydroxytryptamine¹¹ and have been identified as attractive targets for the treatment of neurological disorders.¹² The enzyme, occurring in two isoforms, MAO-A and MAO-B, produces peroxides that cause oxidative stress alongside the depletion of neurotransmitters.¹³ MAO-B constitutes about 80% of the total MAO activity in the human brain and is the predominant form of the enzyme in the striatum, while MAO-A is mainly distributed peripherally.^{13,14} Inhibition of MAO-A and MAO-B permits accumulation of neurotransmitters and reduces the formation of oxidative free radicals to confer neuroprotection. Inhibitors of the enzyme are thus expected to protect from neurodegeneration due to their ability to reduce the formation of peroxides and radical species from amine catalysis.^{15–18} Although MAO-B is present in higher concentrations than MAO-A in the human basal ganglia, MAO-A inhibitors have also been shown to enhance dopamine levels in this region.^{19,20} In order to conserve dopamine in the basal ganglia, mixed MAO-A/B inhibitors may therefore be more efficacious than selective inhibitors and may be of value in the treatment of AD and other neurodegenerative disorders.¹² Propargylamine derived MAO inhibitors such as rasagiline and ladostigil (Fig. 2) have also shown anti-apoptotic activity unrelated to their MAO inhibitory activity.^{21,22}

All of the drugs presently approved for AD treatment offer transient symptomatic relief only. Current treatment options have moved towards including multiple therapies to address the varied pathological aspects of AD.²³ The multi-target-directed ligand (MTDL) approach is a promising strategy that yielded ladostigil.²⁴ Ladostigil or TV3326 is a neuroprotective

bifunctional analogue with the aminoindan structure of rasagiline (Fig. 2) and the carbamate cholinesterase inhibitory moiety of rivastigmine and physostigmine (Fig. 1).^{24,25} It was initially designed for AD in high doses but failed to meet its endpoint in a Phase 2b clinical trial in 2012. It is currently under investigation for mild cognitive impairment due to its neuroprotective, anti-oxidant and microglial activation abilities.²⁶ Various MTDL combinations featuring two or more of the following properties; MAO-, cholinesterase- and A β inhibition, anti-inflammatory activity, metal and iron homeostasis and anti-apoptotic activity have been described in the literature.^{23,27}

Recent observations have suggested that dual ChE and MAO inhibition, as is the case with ladostigil, is likely to be more effective against AD.²⁷ However only the *R*-enantiomer of ladostigil is active, raising issues with synthesis and quality control. The carbamate on ladostigil is also open to acid hydrolysis and enzymatic metabolism, and once this metabolism takes place, it leaves a non-enzyme inhibiting neuroprotective molecule TV3279.^{28,29} This loss of enzymatic ability may account for one of the reasons why ladostigil failed in Phase 2b AD clinical trials. In order to overcome these unfavourable properties of ladostigil a series of compounds structurally similar to ladostigil, but based on an indole structure was designed (1–8, Fig. 3 and 4). The propargylamine moiety has been shown to inhibit MAO as well as confer neuroprotection²² and was incorporated at the N1 position of compounds 5–8 to eliminate the chirality associated with rasagiline and ladostigil. The ChE inhibiting carbamoyl moiety in the form of a diethylcarbamate/urea was conjugated at the 5 or 6 position with the aim of inhibiting ACh hydrolysis by AChE and BuChE.³⁰ The diethyl moiety was selected for conjugation over the methyl-ethyl moiety of ladostigil and rivastigmine. Preliminary molecular modelling studies utilising the ChE enzymes during the initial design phase of this study suggested that the diethyl moiety will show improved binding in the ChE active site pockets and stabilise the molecules leading to optimal activity. In order to prevent or slow down the *in vivo* hydrolysis associated with the carbamate function of ladostigil a urea moiety was incorporated into compounds 1, 2, 5 and 6. It is expected that the urea containing compounds will be more metabolically stable

Fig. 2 Structure of the MAO inhibitor rasagiline and the MTDL ladostigil.

Fig. 3 Design strategy for the novel indole derivatives incorporating the carbamoyl/urea and propargylamine moieties.^{22,30–32} X = NH or O.

Fig. 4 Synthetic pathway of the novel compounds 1–8. Reagents and conditions: (i) CH₃CN, K₂CO₃, rt, 2 h; (ii) DMF, NaH, microwave (2–4 h, 95 °C, 150 W).

than their carbamate counterparts (3, 4, 7 and 8) and lead to improved enzymatic inhibition *in vivo*. In the context of the discovery of neuroprotective agents an indole scaffold was selected because it possesses efficiency in scavenging free radicals and as an antioxidant.^{31,32} The indole heterocycle has also been investigated in the design of AChE-induced A β aggregation and neuronal nitric oxide synthase (nNOS) inhibitors³² and MAO-B inhibitors.^{31,33}

The objective of this study was to improve the inhibitory, neuroprotective and stability profile and eliminate the stereochemistry associated with rasagiline and more recently, ladostigil. It is expected that these novel compounds will serve as dual AChE/BuChE and MAO-A and MAO-B inhibitors and possess similar activity with improved pharmacokinetic profiles compared to ladostigil and other related anti-AD/neuroprotective agents.

2. Results and discussion

2.1. Chemistry

The synthetic strategy developed to obtain the novel MTDLs provides several advantages, including the elimination of the chiral centre on the indane ring of rasagiline and ladostigil,

easy accessibility of the final product by one step or two step processes using commercially available starting materials and simple scale-up from grams to larger quantities. The synthesis utilises the commercially available 5- or 6-hydroxy/amino indole (1a–1d) that was conjugated to a diethyl-carbamoyl chloride moiety at position 5 or 6 *via* a nucleophilic addition-elimination reaction using acetonitrile as solvent in the presence of potassium carbonate (1–4, Fig. 4). These intermediates were then treated with sodium hydride in dimethylformamide and propargyl bromide and reacted under optimized microwave irradiation conditions (150 W, 95 °C, 2–4 h) to give the final compounds 5–8 in high yields (>87%).

In order to characterise the novel compounds, analytical instruments and techniques as described in the experimental section were used. Compounds with similar substitutions showed similar signal patterns on their NMR and IR spectra. The five aromatic protons of the indole were observed between 7.70 and 6.30 ppm on the ¹H NMR, with the carbamate/urea portion showing a triplet at around 1.25 ppm (CH₃) and a multiplet at around 3.40 ppm (CH₂). The presence of the propargyl moiety in compounds 5–8 was confirmed by a doublet and triplet peak at around 4.50 ppm (CH) and 2.50 ppm (CH₂) respectively. The ¹³C NMR of the

compounds showed the distinct peak of the carbonyl carbon of the carbomoyl or urea moiety in the vicinity of 158 ppm. The molecular mass of each compound was confirmed using MS and HR-MS.

2.2. Monoamine oxidase inhibition studies

The compounds were investigated for inhibition of human MAO-A and MAO-B using recombinant human enzymes. Enzyme inhibition tests of reference and test compounds were performed by a fluorescence based assay with the substrate kynuramine measuring the production of 4-hydroxyquinoline formed as a by-product of the enzymatic deamination reaction.³⁴ The selective inhibitors clorgiline ($IC_{50} = 1.06$ nM) and selegiline ($IC_{50} = 20$ nM) were used as the positive controls for the MAO-A and MAO-B assays respectively. IC_{50} values of the test compounds are presented in Table 1. The assay results showed that all the compounds, except 1, had the ability to inhibit the MAO-A enzyme at concentrations below 100 μ M. As observed from the IC_{50} values the compounds that did not possess the propargylamine moiety (1–4) had, in general, lower activity when compared to their propargylamine containing counterparts (5–8). Literature confirms that the propargylamine moiety is responsible for the MAO inhibitory properties of compounds containing this functional group.²⁹ The results obtained agree with this, as can be seen by the more potent IC_{50} values upon inclusion of this moiety. The presence of a urea moiety (compounds 5 and 6) resulted in compounds that have comparably better inhibitory abilities than the carbamate compounds 7 and 8. Compound 6 had the lowest IC_{50} within the series ($IC_{50} = 4.31$ μ M) towards MAO-A. The comparable compound 8 with similar orientation, except for a carbamate instead of the urea, is 12 times less potent ($IC_{50} = 52.64$ μ M) towards the enzyme. This may indicate that the hydrogen donating ability of the secondary amine in the urea moieties may contribute to important interactions with amino acids in the active site of the enzyme. Overall the compounds were moderately good inhibitors of the MAO-A enzyme showing better activity compared to ladostigil *in vitro*.

The results of the MAO-B assay indicated that compounds 1–4 had MAO-B IC_{50} values above 100 μ M. These compounds did not possess the propargylamine substitution at position

N1 and may lack vital interactions with the flavin adenine dinucleotide (FAD) co-factor as observed for the propargylamine containing compounds, rasagiline and selegiline.²⁹ The conjugation of the propargylamine moiety (5–8) led to a significant improvement in MAO-B inhibition. This is in line with literature that suggests that the propargylamine moiety is a good contributor towards MAO-B inhibitory capacity.²⁹

The compounds that contain a carbamate showed, in general, slightly better MAO-B activity (although not significantly, $p > 0.05$) compared to their urea counterparts. This is observed when compound 5 is compared with 7 and 6 with 8. This observation may indicate that the electron accepting abilities of the oxygen molecule contributes to some interaction in the MAO-B active site. Compounds 6 and 8 had the lowest MAO-B IC_{50} values of 2.62 μ M and 1.84 μ M, respectively. These compounds had the carbamate/urea at the 6 position and the N1 propargylamine moiety similar to ladostigil. This orientation of the propargylamine to the carbamoyl/urea moiety seems to be favourable for good MAO inhibitory activity of these compounds.

2.3. MAO-reversibility studies

Compound 6 was selected for MAO reversibility studies because it showed the most promising activities on both enzymes. This study aimed to investigate if the interaction between the propargyl moiety of 6 and the FAD co-factor is reversible or irreversible. For this purpose the time dependence of MAO-A and -B inhibition by compound 6 was evaluated. Recombinant human MAO-A and MAO-B was preincubated with the test compounds for periods of 0, 15, 30 and 60 min and the residual rates of the MAO-A and -B catalysed oxidation of kynuramine were measured. The concentration of compound 6 used was approximately twofold the measured IC_{50} value for the inhibition of the respective enzymes. Clorgiline and selegiline, known irreversible inhibitors of MAO-A and MAO-B respectively, were used as a reference compounds. As shown in Fig. 5 and 6, both clorgiline and selegiline showed a time-dependent reduction in the rates of MAO-A and -B catalysed oxidation of kynuramine, confirming irreversible inhibition. Compounds 6 also showed

Table 1 *In vitro* IC_{50} values (μ M) of test compounds for hMAO-A, hMAO-B, eeAChE and eqBuChE

Compound	MAO-A	MAO-B	SI MAO-B ^a	AChE	BuChE	SI AChE ^b
1	>100	>100	n.d.	1.97	3.48	1.77
2	20.37	>100	<0.20	2.28	4.55	2.0
3	13.15	>100	<0.13	2.78	2.29	0.82
4	88.11	>100	<0.88	3.72	3.18	0.86
5	33.42	28.84	1.16	2.77	3.96	1.43
6	4.31	2.62	1.65	3.70	2.82	0.76
7	99.08	16.52	6.00	2.57	3.01	1.17
8	51.64	1.84	28.06	2.41	5.01	2.08
Ladostigil ^c	>100	37.1	>2.69	31.80	1.98	16.10

^a hMAO-B selectivity index = $IC_{50}(\text{hMAO-A})/IC_{50}(\text{hMAO-B})$. ^b AChE selectivity index = $IC_{50}(\text{eeBuChE})/IC_{50}(\text{eqAChE})$. ^c IC_{50} values of ladostigil obtained from ref. 21 and 35; n.d. = not determined.

time-dependent reduction in the rates of MAO-A and -B catalysed oxidation of kynuramine. From these results it can be concluded that the inhibition of MAO-A and -B by compound **6** is irreversible, at least for the time period (60 min) and at the inhibitor concentrations ($2 \times \text{IC}_{50}$) evaluated. This irreversible MAO inhibition of compound **6** indicates that the interaction between the propargyl moiety and the FAD cofactor of MAO-A and MAO-B is also present in these structures as is the case for the irreversible MAO inhibitor ladostigil.^{24,25} This finding also shows that the propargylamine can be incorporated within the heterocycle and retain its irreversible MAO inhibition.

2.4. Cholinesterase inhibition studies

The compounds were also evaluated for inhibitory activity on eeAChE (electric eel) and eqBuChE (equine serum) according to Ellman's method,³⁶ with tacrine (AChE $\text{IC}_{50} = 0.11 \mu\text{M}$; BuChE $\text{IC}_{50} = 0.017$) as positive control. All the compounds were able to inhibit AChE with IC_{50} values between $1 \mu\text{M}$ and $4 \mu\text{M}$ (Table 1). This illustrates that both the carbamate and urea functional groups contribute to inhibition of the AChE enzyme. Also, the compounds exerted good activity regardless of the position of the carbamate or urea function. Addition of the propargylamine moiety did not affect the inhibitory capacity of the compounds indicating that the carbamate and urea moieties are highly involved in activity. Comparing the IC_{50} values of the test compounds to that of ladostigil shows that the test compounds more active AChE inhibitors.

The role that BuChE plays in the progression of AD is its ability to hydrolyse acetylcholine in the event of AChE saturation.⁸ Accordingly, a compound that inhibits this enzyme, in addition to AChE, would prove beneficial for the treatment of AD. Carbamoyl containing compounds have also been shown to be BuChE enzyme substrates³⁷ and it is postulated that the diethylcarbamoyl moiety interacts with the enzyme to block its catalytic activity. The results showed that the synthesised compounds had good activity in inhibiting the

BuChE enzyme with IC_{50} values between 2 and $5 \mu\text{M}$ comparable to that of ladostigil.

When the different substitutions of the test compounds are compared, it is observed that the 5 substituted compounds yielded similar results compared to the 6 substituted compounds. Selectivity indices were close enough for the compounds to be classified as non-selective ChE inhibitors *in vitro* with a good balance between AChE and BuChE activities. Importantly, the compounds portray non-selective inhibitory activities compared to ladostigil which is 16 times more selective towards BuChE over AChE *in vitro*.^{21,35} Moreover, literature has reported that AChE promotes the assembly of A β into amyloid fibrils hence these compounds, with the improved AChE inhibitory activity compared to ladostigil, may also prove to reduce the conglomeration of amyloid fibrils into plaques in addition to reducing ACh hydrolysis.^{38,39}

2.5. Molecular modelling

To provide insight, the binding mode of compounds 1–8 in hMAO-A, hMAO-B, eeAChE and eqBuChE were examined using molecular docking. The structures of human MAO-A co-crystallized with clorgiline (PDB entry: 2BXS),⁴⁰ human MAO-B co-crystallized with safinamide (PDB entry: 2V5Z)⁴¹ and eeAChE co-crystallized with donepezil (PDB entry: 1EVE)⁴² were retrieved from the Brookhaven Protein Data Bank (www.rcsb.org/pdb) and the docking was carried out with the Dock application of the Molecular Operating Environment (MOE) software.⁴³ In the absence of a X-ray structure of eqBuChE, a homology model was used to rationalize the experimental data. The modeling of the 3D structure was performed by an automated homology-modeling program (SWISS-MODEL).^{44,45} The putative 3D structure of eqBuChE has been created based on the crystal structure of hBuChE (PDB: 2 PM8) as these two enzymes exhibited 89% sequence identity. The docking of the compounds into this homology model of eqBuChE was carried out with Autodock Vina.⁴⁶ The docking results were analyzed with MOE.

Fig. 5 Time-dependent inhibition of the recombinant human MAO-A catalysed oxidation of kynuramine by clorgiline and **6**. The enzyme was preincubated for various periods of time (0–60 min, x-axis) with clorgiline and **6** at concentrations of $0.002 \mu\text{M}$ and $8.62 \mu\text{M}$. The concentration of kynuramine used was $45 \mu\text{M}$.

Fig. 6 Time-dependent inhibition of the recombinant human MAO-B (panel B) catalysed oxidation of kynuramine by selegiline and **6**. The enzymes were preincubated for various periods of time (0–60 min, x-axis) with selegiline and **6** at concentrations of 0.040 μM and 5.24 μM . The concentration of kynuramine used was 30 μM .

2.5.1. MAO molecular modelling studies. Docking results showed that as expected compounds **1–4** lacked interactions with vital residues in the active site of MAO-A (Fig. 7). The indole heterocyclic ring of **1–4** was best orientated towards the FAD cofactor in the substrate cavity with the diethyl-carbamoyl/urea moiety occupying the entrance cavity. In the compounds that contained the propargylamine moiety (**5–8**), this moiety is found within close proximity to the FAD cofactor with the rest of the molecule occupying the active site (Fig. 7). This orientation has been reported to be essential for MAO inhibitory capacity.⁴⁷ The indole heterocyclic ring did not portray any significant interactions with the MAO-A active site, but the compounds were well accommodated within in the active site which may account for the good inhibitory activities observed for some of these compounds.

The MAO-B molecular docking experiments showed that compounds **1–4** were not able to reach the substrate cavity but rather maintained interactions in the entrance cavity (Fig. 8). This inability of **1–4** to reach the substrate cavity may indicate the reason why these compounds did not show

MAO-B activity. The propargylamine containing compounds (**5–8**) were able to reach the substrate cavity and compounds **6** and **8** showed π -H interactions between the propargylamine moiety and the FAD co-factor (Fig. 8). Compounds **5** and **7** adopted an inverted orientation compared to **6** and **8**, and lacked the π -H interaction. This observation may account for the improved MAO-B inhibitory activity of the 6-substituted indole derivatives, **6** and **8** (Table 1).

2.5.2. ChE molecular modelling studies. The assay results indicated that all the compounds were able to inhibit ChE enzymes with IC_{50} values between 1 μM and 5 μM and molecular docking was employed to gain insight into the binding modes of all the compounds in the active site of AChE and BuChE.

The docking results, Fig. 9, showed that compounds **1–8** mainly interact within the catalytic anionic site (CAS) of AChE. The alkyl component of the diethyl-carbamate/urea of compounds **1–4** showed π -H interactions with Trp84 in the CAS. This interaction has been reported to be crucial for AChE inhibitory activity.⁴⁸ The indole heterocycle of **1–4** mainly sits in the

Fig. 7 The MAO-A active site cavity displaying the binding and interactions of compounds **1–4** (left) and compounds **5–8** (right). Compound **1** and **5** are shown in green, compound **2** and **6** in light blue, compound **3** and **7** in red and compound **4** and **8** in magenta. The FAD co-factor is shown to the right, in magenta, in the outside of the binding pocket.

Fig. 8 The MAO-B active site cavity displaying the binding and interactions of compounds 1–4 (left) and compounds 5–8 (right). Compound 1 and 5 are shown in green, compound 2 and 6 in light blue, compound 3 and 7 in red and compound 4 and 8 in magenta. The FAD co-factor is shown to the right, in red, in the outside of the binding pocket.

active site gorge and does not occupy the peripheral anionic site (PAS). The indole moiety of compounds 5–8 possesses π – π stacking interactions with Trp334 and Phe330 (a key residue involved in ligand binding in the active gorge).⁴⁹ The interaction with Trp84 was also observed for compounds 5–8. This may explain why the good inhibitory activities were retained across the series. It is important to note that the propargylamine moiety does not show any significant interactions with amino acids in the active site but occupied the gorge facing the PAS.

The binding mode of the non-propargyl containing compounds, 1–4, at the active site of the eqBuChE homology model is illustrated in Fig. 10. The docking results showed that the lowest energy binding orientation all four compounds adopted a similar conformation with the indole moiety forming π – π stacking interactions with Phe329 in the anionic site of the enzyme. The diethylcarbamate/urea moiety binds in the CAS region of the enzyme,⁵⁰ establishing π – π stacking interactions with Trp82 and a hydrogen bond interaction with His438 of the catalytic-triad. The propargylamine containing compounds, 5–8, were all well accommodated inside the active site gorge showing similar orientations. The lowest energy binding orientations enabled the indole moiety to interact with Trp82 and His438 in the CAS through π – π

stacking interactions. The diethyl-carbamate/urea moiety is orientated away from the CAS region and forms an interaction with Trp231. The N1-propargyl substitution caused these molecules to adopt an inverted orientation compared to their non-propargyl containing counterparts. However, even though the adopted orientation is significantly different important interactions were still retained and may account for the similar BuChE activity observed for compounds 5–8 compared to 1–4.

2.6. Chemical stability study

Chemical stability of pharmaceutical molecules is a matter of great importance as it ultimately affects the stability and efficacy of the final drug product. One of the drawbacks of the carbamate of ladostigil is its liability to rapid hydrolysis in acidic conditions. *In vivo* ladostigil undergoes rapid acid and enzyme hydrolysed metabolism into a non-enzyme inhibiting neuroprotective molecule TV3279.^{27,28} Considering that compounds 1–8 contain a carbamate or urea that may affect stability and ultimately metabolism, it was decided to employ a forced chemical degradation test to study the stability of these moieties in both acidic and alkaline conditions.

Fig. 9 The AChE active site cavity displaying the binding and interactions of compounds 1–4 (left) and compounds 5–8 (right). Compound 1 and 5 are shown in green, compound 2 and 6 in light blue, compound 3 and 7 in red and compound 4 and 8 in magenta.

Fig. 10 Overlays of compounds 1–4 (left) and compounds 5–8 (right) in complex with the eqBuChE homology model. Compound 1 and 5 is shown in green, compound 2 and 6 in light blue, compound 3 and 7 in red and compound 4 and 8 in magenta.

Compounds 6 and 8 were selected for stability testing because they contained both the carbamoyl/urea and propargylamine moiety and portrayed good activity as either a urea (6) or carbamate (8) derivative. The forced degradation study was done according to the slightly modified method of Blessy *et al.*, 2014.⁵¹ The test compound (1 mg mL^{-1}) were dissolved in aqueous 0.1 M HCl or 0.1 M NaOH with DMF (5%) as co-solvent. The test solutions were kept at 37°C in an incubator for 7 days and sampled on days 0, 1, 3, 4 and 7. UV/vis absorbance of the sample was measured at maximal wavelengths of 232 nm for 6 and 234 nm for 8.

It is important to note that both the urea and carbamate functional groups undergo hydrolysis but at different rates.⁵² Fig. 11 shows the results of the compounds 6 and 8 after 7 days in 0.1 M HCl and 0.1 M NaOH. Compound 6 displayed an almost linear degradation rate compared to 8 that portrayed a more rapid decay. From these results, it is evident that the carbamate derivatives are less stable than their urea containing counterparts. Ladostigil is a carbamate compound and could act similarly to 8 in acidic conditions. Following this logic, urea (6) should prove to be more stable than the carbamate derived ladostigil. This should minimise the metabolic effects that induce rapid hydrolysis to prevent limiting factors such as a short half-life and the rapid formation of non-inhibiting metabolites. The compounds behaved

in a similar manner in alkaline conditions. At this stage we are not informed as to the activity of the metabolites of the compounds, but like rasagiline and ladostigil whose metabolites portray neuroprotective activity;^{21,27} the compounds described in this paper may act in a similar manner.

2.7. SH-SY5Y cell viability studies

In order to investigate the influence of the test compounds on neuronal cell viability, SH-SY5Y cells were treated with various concentrations ($10 \mu\text{M}$, $50 \mu\text{M}$ and $100 \mu\text{M}$) of compounds 2 and 6 for 48 hours and evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay.⁵³ Compound 6 was chosen for this assay as it showed the most promising inhibitory activity on all enzymes tested and contained both the more stable urea functional group and the *N1*-propargyl moiety. Compound 2 was included to explore the influence of the *N1*-propargyl moiety. Exposure of SH-SY5Y cells to compounds 2 and 6 lead to a reduction in cell viability in a concentration-dependent manner (Fig. 12). At $10 \mu\text{M}$ the compounds did not show any significant ($p > 0.05$) effect on cell viability. In general, based on this preliminary data, compound 6 seems to be slightly less toxic than compound 2 at the three concentrations tested. Another finding from these studies is that the CC_{50} values of both

Fig. 11 Forced degradation results of compounds 6 (blue) and 8 (red) in acidic (left) and alkaline (right) conditions.

Fig. 12 Cellular viability of SH-SY5Y cells treated with compounds 2 and 6 at 10 μ M, 50 μ M and 100 μ M for 48 hours. Data is presented as mean $\pm$ SEM. Significance is set at a level of * p < 0.05 compared to vehicle treated control cells (taken as 100% cell viability).

compounds 2 and 6 on SH-SY5Y human neuroblastoma cells are more than 100 μ M. This indicates that at the concentrations where especially compound 6 inhibit ChE and MAO (IC_{50} values less than 5 μ M, see Table 1) it will have very limited toxicity towards human cells.

Based on the SH-SY5Y cytotoxicity analysis it was decided that the MPP⁺ neuroprotection studies would be done at compound concentrations between 1 μ M and 10 μ M in order not to significantly affect the viability of the cells. MPP⁺ is highly toxic to neurons and has been widely used to induce neurodegeneration in various *in vitro* and *in vivo* models.^{54,55} Several signaling pathways have been suggested to be responsible for MPP⁺-mediated neurotoxicity in SH-SY5Y cells, for instance, trigger of oxidative stress,⁵⁶ induction of apoptosis,⁵⁷ and inactivation of pro-survival phosphoinositide 3-kinase (PI3-K)/Akt cascade.^{58–60} This assay was deemed appropriate to test for initial neuroprotective ability of compounds

2 and 6 because of the multitude of pathways involved in MPP⁺ mediated neurotoxicity and the multifunctional enzyme inhibitory abilities of the test compounds, especially 6.

SH-SY5Y cells were exposed to the neurotoxin MPP⁺ with or without test compound, and cell viability was evaluated using the MTT mitochondrial function assay.⁵³ As illustrated in Fig. 13, after the exposure to 1000 μ M MPP⁺ for 48 hours, the cell viability declined significantly to $52.78 \pm 7.35\%$. However, its cytotoxic effects were ameliorated in the presence of compounds 2 and 6 at 1 μ M, 5 μ M and 10 μ M concentrations. The compounds, at a 1 μ M concentration, restored cell survival to $84.32 \pm 4.62\%$ and $107.40 \pm 6.26\%$, respectively. At 5 μ M and 10 μ M a slight decrease in cytoprotection, although still significant (p < 0.05), was observed for 2 and 6. This may indicate that in the neurotoxin challenged state the SH-SY5Y cells may be more sensitive to the cytotoxic effect of the test compounds. Therefore, 2 and 6 might have enhanced the toxicity of the neurotoxin which resulted in lower neuroprotection values observed at higher concentrations. Compound 6 containing the propargyl substitution at the N1 position showed significantly improved cytoprotection (p < 0.05) at all three concentrations tested compared to compound 2 which is devoid of the propargyl substitution. This observation confirms that the propargyl function improves the neuroprotective ability of these compounds in this assay. Thus, in general and especially at a 1 μ M concentration, these compounds, and particularly 6, exerted highly significant cytoprotection against MPP⁺ insults to the SH-SY5Y neural cells.

2.8. *In silico* ADMET studies

Selected ADMET properties of compounds 6, 8 and ladostigil were calculated using web-based applications. Compounds 6 and 8 were selected because they contained both the propargylamine- and urea/carbamate moiety similar to ladostigil and showed the most promising activities within

Fig. 13 The effects of compounds 2 (left) and 6 (right) on MPP⁺-induced (1000 μ M) cytotoxicity in SH-SY5Y cells. The viability of untreated controls was defined as 100%. The level of significance for the test compounds is set at # p < 0.05 compared to the MPP⁺ only treated control (0). Statistical analysis (Tukey *post hoc* test) was performed on raw data, with asterisks signifying significant cytoprotection. * p < 0.05; ** p < 0.001; *** p < 0.0001. Data are expressed as means $\pm$ S.D. (n = 12).

Table 2 Predicted ADMET properties of selected compounds

Compound	BBB penetration ^a	HIA ^b (%)	Caco-2 permeability ^c	Log <i>P</i>	AMES toxicity ^d
6	2.10	96.13	42.12	3.18	None
8	0.47	99.55	53.80	2.95	None
Ladostigil	0.99	95.47	33.81	2.40	None

^a BBB penetration parameters; <0.1 = low penetration; 0.1–2 = medium penetration; >2 = high penetration. ^b Human intestinal absorption; values of >70% = high absorption. ^c Values between 4 and 70 indicates medium cell penetration. ^d Mutagenic potential of chemicals.

the series. Blood–brain barrier (BBB) penetration, human intestinal absorption (HIA), Caco-2 cell permeability, log *P* and AMES test were calculated using <https://preadmet.bmdrc.kr>. The predicted ADMET data is summarized in Table 2. Based on the predicted values for BBB penetration, both compounds 6 and 8 should be able to penetrate into the central nervous system. It is interesting to note that compound 6, the most active compound in the series, should show better BBB penetration than 8 and ladostigil based on the BBB penetration and log *P* values. The compounds were also predicted to be well absorbed based on the human intestinal absorption and Caco-2 permeability predictions. Moreover, the compounds did not show mutagenic effect with respect to the AMES test data. This data has significance indicating that the new compounds should have good ADMET profiles thus encouraging further drug design and development endeavours. The improved stability of compound 6 combined with the best overall predicted ADMET properties provide further evidence that it is a promising compound for further development.

3. Conclusion

The main objective of this study was to design a series of compounds with improved pharmacokinetic properties compared to ladostigil able to inhibit hMAO-A, hMAO-B, eeAChE and eqBuChE and provide neuroprotection. The results obtained indicate that the compounds act as dual ChE and MAO inhibitors. Molecular modelling showed significant interactions with vital residues in the active sites of the enzymes. Compound 6 was identified as a potent MTDL (IC₅₀; MAO-A = 4.31 μM, MAO-B = 2.62 μM, AChE = 3.70 μM, BuChE = 2.82 μM) with AChE and MAO-B inhibitory activity 9 and 14 times that of ladostigil respectively (Table 1). Compound 6 also showed irreversible inhibition of MAO, similar to that reported for ladostigil. The results of the chemical stability tests showed that 6 is also more stable than the carbamate compound 8 by virtue of possessing the urea functional group. Compound 6 would thus probably prove more chemically and metabolically stable than ladostigil and lead to better enzymatic inhibition *in vivo* which may provide improved ability to treat AD. Elimination of the stereochemistry associated with ladostigil was also achieved while maintaining and even improving neuronal enzyme inhibitory activities. Compound 6 also exerted highly significant cytoprotection towards MPP⁺ insults to the SH-SY5Y neural cells and can thus

be considered for optimisation and perhaps development as a multipotent drug molecule against Alzheimer's- and/or other neurodegenerative disease(s). Further investigations exploring the pharmacokinetics and *in vivo* activities as well as further mechanism of action studies are recommended for these compounds.

4. Experimental section

4.1. Chemistry

Unless otherwise specified, materials were obtained from commercial suppliers and used without further purification. All reactions were monitored by thin-layer chromatography on 0.20 mm thick aluminium silica gel sheets (Alugram® SIL G/UV₂₅₄, Kieselgel 60, Macherey-Nagel, Düren, Germany). Visualisation was achieved using UV light (254 nm and 366 nm), an ethanol solution of ninhydrin or iodine vapours, with mobile phases prepared on a volume-to-volume basis. Chromatographic purifications were performed on silica gel (0.063–0.2 mm, Sigma Aldrich) except when otherwise stated. The MS spectra were recorded on a Perkin Elmer Flexar SQ 300 mass spectrometer by means of direct injection with a syringe pump. High resolution electron spray ionisation (HREI) mass spectra for all compounds were recorded on a Waters API Q-ToF Ultima mass spectrometer at 70 eV and 100 °C. The IR spectra were recorded on a Perkin Elmer Spectrum 400 spectrometer, fitted with a diamond attenuated total reflectance (ATR) attachment. Melting points were determined using a Stuart SMP-300 melting point apparatus and capillary tubes. All the melting points determined were recorded uncorrected. ¹H and ¹³C NMR spectra were determined using a Bruker Avance III HD spectrometer at a frequency of 400 MHz and 100 MHz, respectively. Tetramethylsilane (TMS) was used as internal standard. All chemical shifts are reported in parts per million (ppm), relative to the internal standard. The following abbreviations are used to indicate the multiplicities of the respective signals: s – singlet; s – broad singlet; d – doublet; dd – doublet of doublets; t – triplet and m – multiplet.

4.2. General procedure for the synthesis of compounds 1–4

The appropriate amino- or hydroxylindole (1 mmol) was conjugated to carbomoyl chloride at position 5 or 6 in a 1:1 ratio, using acetonitrile (10 ml) and 1.2 mmol K₂CO₃. The mixture was stirred for 2 hours at room temperature. Once the reaction was complete the mixture was filtered to remove the K₂CO₃ and the solvent removed *in vacuo*. An extraction was

carried out using DCM (3 × 15 ml). The organic phase was further washed with brine and water. The DCM was removed *in vacuo* to render a blackish waxy product which was purified *via* column chromatography (1:2 hexane:ethylacetate) rendering the products as pure amorphous solids.

4.2.1. *N,N*-Diethyl-*N'*-1*H*-indol-5-ylurea (1). Yield: 74%; mp: 143 °C; Rf: 0.68; ¹H NMR: (400 MHz, CDCl₃) δ H: 7.89 (s, 1H, H - 4), 7.49–7.47 (d, 1H, *J* = 8.4 Hz, H - 7), 7.11–7.10 (t, 1H, *J* = 5.6; 2.8 Hz, H - 2), 6.77–6.75 (dd, 1H, *J* = 4.8; 2.4 Hz, H - 6), 6.45–6.34 (m, 1H, H - 3), 3.42–3.37 (m, 4H, H - 14; 16), 1.26–1.24 (t, 6H, *J* = 8.0; 4.0 Hz, H - 15, 17); ¹³C NMR (100 MHz, CD₃OD): 158.15, 137.74, 134.54, 126.26, 125.37, 120.74, 116.81, 106.73, 102.10, 42.43, 14.12; IR (FT-IR, cm⁻¹): 3247.42, 2978.13, 1220.22; MS (EI, 70 ev) *m/z*: 232.10 (M + H)⁺; HR-ESI [M + H]⁺: calcd. 232.1443, found. 232.1447.

4.2.2. *N,N*-Diethyl-*N'*-1*H*-indol-6-ylurea (2). Yield: 52%; mp: 141 °C; Rf: 0.71; ¹H NMR: (400 MHz, CDCl₃) δ H: 7.63 (s, 1H, H - 7), 7.28–7.26 (d, 1H, *J* = 8.0 Hz, H - 5), 7.16–7.15 (d, 1H, H - 4), 6.47–6.46 (t, 1H, *J* = 4.8; 2.4 Hz, H - 2), 6.26 (m, 1H, H - 3), 3.42–3.37 (m, 4H, H - 14; 16), 1.26–1.22 (t, 6H, *J* = 14.4; 7.2 Hz, H - 15, 17); ¹³C NMR (400 MHz, CD₃OD): 153.69, 126.35, 123.97, 121.48, 121.23, 114.60, 106.23, 97.42, 36.97, 13.41; IR (FT-IR, cm⁻¹): 3288.30, 2922.65, 1760.52, 1217.09; MS (EI, 70 ev) *m/z*: 232.15 (M + H)⁺; HR-ESI [M + H]⁺: calcd. 232.14443, found. 232.1442.

4.2.3. 1*H*-Indol-5-yl-diethylcarbamate (3). Yield: 81%; mp: 126 °C; Rf: 0.71; ¹H NMR: (400 MHz, CD₃OD) δ H: 7.19–7.8 (d, 1H, *J* = 8.8 Hz, H - 7), 7.14–7.13 (d, 1H, *J* = 2.8 Hz, H - 4), 6.92–6.91 (d, 1H, *J* = 4.0 Hz, H - 2), 6.60–6.40 (dd, 1H, *J* = 8.8; 2.4 Hz, H - 6), 6.27–6.25 (dd, 1H, *J* = 3.2; 0.8 Hz, H - 3), 3.21–3.16 (m, 4H, H - 14; 16), 1.13–1.09 (t, 6H, *J* = 14.4; 6.8 Hz, H - 15, 17); ¹³C NMR (400 MHz, CD₃OD): 158.38, 137.79, 134.52, 126.28, 125.43, 120.71, 116.83, 106.66, 102.06, 42.47, 14.13; IR (FT-IR, cm⁻¹): 3298.82, 2971.20, 1210.75; MS (EI, 70 ev) *m/z*: 233.11 (M + H)⁺; HR-ESI [M + H]⁺: calcd. 233.1285, found. 233.1289.

4.2.4. 1*H*-Indol-6-yl-diethylcarbamate (4). Yield: 80%; mp: 159 °C; Rf: 0.67; ¹H NMR: (400 MHz, CDCl₃) δ H: 7.63–7.26 (d, 1H, *J* = 1.6 Hz, H - 7), 6.95–6.94 (d, 1H, *J* = 2.0 Hz, H - 4), 6.68–6.65 (dd, 1H, *J* = 8.4; 2.0 Hz, H - 5), 6.48–6.46 (t, 1H, *J* = 6.4; 3.2 Hz, H - 2), 6.38–6.36 (m, 1H, H - 3), 3.43–3.37 (m, 4H, H - 14; 16), 1.26–1.22 (t, 6H, *J* = 14.4; 6.8 Hz, H - 15, 17); ¹³C NMR (400 MHz, CD₃OD): 158.52, 137.78, 134.55, 125.36, 122.75, 120.76, 116.79, 106.73, 101.26, 42.39, 14.08; IR (FT-IR, cm⁻¹): 3253.61, 1683.13, 1215.52; MS (EI, 70 ev) *m/z*: 233.13 (M + H)⁺; HR-ESI [M + H]⁺: calcd. 233.1285, found. 233.1287.

4.3. General procedure for the synthesis of compounds 5–8

Compounds 5–8 were synthesised by conjugating the propargyl bromide (1.2 mmol) to position *N*1 of the synthesised compounds, 1–4 (1 mmol). This mixture was stirred at room temperature in the presence of NaH (1 mmol) and dimethylformamide (10 ml) and irradiated under microwave conditions (95 °C, 150 W) for 2 to 4 hours. Once the reaction was complete water (30 ml) was added followed by at least five extractions with ethyl acetate (5 × 15 ml). Thereafter

the organic phase was washed with brine and water. The ethyl acetate was removed *in vacuo* to render the pure products as amorphous solids.

4.3.1. *N,N*-Diethyl-*N'*-[1-(prop-2-yn-1-yl)-1*H*-indol-5-yl]urea (5). Yield: 95%; mp: 158 °C; ¹H NMR: (400 MHz, CDCl₃) δ H: 7.64–7.63 (d, 1H, *J* = 1.6 Hz, H - 4), 7.29–7.27 (d, 1H, *J* = 8.8 Hz, H - 7), 7.15–7.14 (m, 2H, *J* = 10.4 Hz, H - 2, 6), 6.47–6.46 (t, 1H, *J* = 1.0, 2.0 Hz, H - 3), 4.12–4.11 (d, 2H, *J* = 4.0 Hz, H - 18), 3.42–3.37 (m, 4H, H - 14; 16), 2.22–2.20 (t, 1H, *J* = 4.8; 2.4 Hz, H - 20), 1.26–1.23 (t, 6H, *J* = 14.0; 6.8 Hz, H - 15, 17); ¹³C NMR (400 MHz, CD₃OD): 157.03, 153.96, 147.94, 139.35, 126.21, 120.56, 114.78, 110.24, 109.63, 105.20, 97.47, 43.55, 11.50; IR (FT-IR, cm⁻¹): 3293.69, 2922.34, 1761.13, 1217.23; MS (EI, 70 ev) *m/z*: 270.94, (M + H)⁺; HR-ESI [M + H]⁺: calcd. 271.1441, found. 271.1439.

4.3.2. *N,N*-Diethyl-*N'*-[1-(prop-2-yn-1-yl)-1*H*-indol-6-yl]urea (6). Yield: 87%; mp: 166 °C; ¹H NMR: (400 MHz, CD₃OD) δ H: 7.42–7.40 (m, 2H, H - 7, 4), 7.15–7.14 (d, 1H, *J* = 3.8 Hz, H - 3), 6.94–6.92 (d, 1H, *J* = 8.4 Hz, H - 5), 6.37–6.36 (d, 1H, *J* = 3.2 Hz, H - 2), 4.10–4.09 (d, 2H, *J* = 4.0, H - 18), 3.46–3.41 (m, 4H, H - 14, 16), 2.18–2.16 (t, 1H, *J* = 8.0; 4.0, H - 20); 1.23–1.19 (t, 6H, *J* = 14.4; 6.8 Hz, H - 15, 17); ¹³C NMR (400 MHz, CD₃OD): 157.13, 139.29, 127.18, 126.16, 120.56, 114.69, 110.23, 109.85, 105.26, 102.33, 97.49, 43.46, 11.61; IR (FT-IR, cm⁻¹): 3300.95, 3194.06, 2927.43, 1210.50; MS (EI, 70 ev) *m/z*: 271.15 (M + H)⁺; HR-ESI [M + H]⁺: calcd. 271.1441, found. 271.1443.

4.3.3. 1-(Prop-2-yn-1-yl)-1*H*-indol-5-yl-diethylcarbamate (7). Yield: 94%; mp: 187 °C; ¹H NMR: (400 MHz, CD₃OD) δ H: 7.50–7.46 (d, 1H, *J* = 16.0 Hz, H - 7), 7.24–7.19 (dd, 2H, *J* = 19.2; 3.2 Hz, H - 4, 6), 7.16–7.13 (dd, 1H, *J* = 10.4; 1.6 Hz, H - 2), 6.41–6.37 (dd, 1H, *J* = 15.6; 2.8 Hz, H - 3), 4.96–4.95 (d, 2H, *J* = 4.0, H - 18); 3.45–3.40 (m, 4H, H - 14; 16), 2.79–2.78 (t, 1H, *J* = 4.8; 2.4 Hz, H - 20), 1.23–1.19 (t, 6H, *J* = 14.4; 7.2 Hz, H - 15, 17); ¹³C NMR (400 MHz, CD₃OD): 158.52, 135.13, 132.06, 129.38, 125.73, 120.36, 116.34, 114.23, 111.79, 107.90, 102.34, 101.38, 42.32, 14.13; IR (FT-IR, cm⁻¹): 3299.52, 3195.74, 1210.83; MS (EI, 70 ev) *m/z*: 270.11 (M + H)⁺; HR-ESI [M + H]⁺: calcd. 270.1601, found. 270.1599.

4.3.4. 1-(Prop-2-yn-1-yl)-1*H*-indol-6-yl-diethylcarbamate (8). Yield: 95%; mp: 168 °C; ¹H NMR: (400 MHz, CD₃OD) δ H: 7.47–7.46 (dd, 1H, *J* = 4.0, 2.0 Hz, H - 7), 7.39–7.36 (d, 1H, *J* = 12.0 Hz, H - 4), 7.24–7.23 (d, 1H, *J* = 4.0 Hz, H - 5), 7.20–7.19 (d, 1H, *J* = 4.0 Hz, H - 2), 6.43–6.33 (m, 1H, H - 3), 4.96–4.95 (d, 2H, *J* = 4.0 Hz, H - 18); 3.46–3.40 (m, 4H, H - 14, 16), 2.79–2.70 (t, 1H, *J* = 8.0; 4.0 Hz, H - 20), 1.23–1.21 (t, 6H, *J* = 8.0; 4.0 Hz, H - 15, 17); ¹³C NMR (400 MHz, CD₃OD): 158.76, 135.15, 132.05, 129.32, 126.16, 126.08, 124.58, 119.97, 116.27, 111.79, 102.32, 101.29, 42.52, 14.14; IR (FT-IR, cm⁻¹): 3291.83, 2922.52, 1761.08, 1217.03; MS (EI, 70 ev) *m/z*: 270.59 (M + H)⁺; HR-ESI [M + H]⁺: calcd. 270.1601, found. 270.1609.

4.4. Recombinant human MAO-A and MAO-B inhibition studies

Stock solutions of the test compounds and controls (clorgiline for MAO-A and selegiline for MAO-B) were

prepared by dissolving the test compounds in DMSO and it was kept under refrigeration until use. The solutions were made at 10 mM concentrations and further serially diluted to achieve final concentrations of 100 μM , 10 μM , 1 μM , 0.1 μM and 0.01 μM . The potassium phosphate buffer was prepared in water at a concentration of 100 mM (adjusted to pH 7.4 by 2 N NaOH). It was also refrigerated until use. The stock solution of the substrate kynuramine was made at 750 μM (45 μM final assay concentration) for MAO-A and 500 μM (30 μM final assay concentration) for MAO-B. Using the phosphate buffer, MAO enzyme stock solution was prepared and stored in aliquots at $-80\text{ }^{\circ}\text{C}$ until use. To each vial 207.5 μL of phosphate buffer and 2.5 μL of corresponding test compound or control compound was added. This was followed by 25 μL of the enzyme at 10 second intervals. The mixture was then incubated for 10 minutes at $37\text{ }^{\circ}\text{C}$. Thereafter 15 μL of kynuramine was added also at 10 second intervals and it was then further incubated for 20 minutes. 150 μL of 2 N NaOH were used to stop the reaction. Finally, 200 μL of the mixture was added to each well of a black 96 well plate, placed in the fluorescent plate reader and read at excitation/emission wavelength of 310 nm/400 nm. Each assay was run in triplicate and the data was analysed with Graph Pad Prism 7 software (San Diego, CA, USA).

4.5. Time-dependent MAO inhibition studies

To investigate whether the observed enzyme inhibition is reversible or irreversible, time-dependant inhibition studies were carried out with the active inhibitors. Recombinant human MAO-A or human MAO-B (0.03 mg mL^{-1}) were preincubated for periods of 0, 15, 30, 60 min at $37\text{ }^{\circ}\text{C}$. The concentrations of inhibitor in these incubations were twofold the measured IC_{50} values for the inhibition of MAO. The incubations were carried out in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl). A final concentration of 45 μM kynuramine for MAO-A and 30 μM kynuramine for MAO-B were then added to the preincubated enzyme preparations and the resulting reactions were incubated at $37\text{ }^{\circ}\text{C}$ for 15 min. The final volumes of these incubations were 250 μL and the final concentration of the inhibitors were approximately equal to the IC_{50} values for the inhibition of the respective enzyme. The final enzyme concentrations of the MAO preparations were 0.015 mg mL^{-1} . The reactions were terminated with 100 μL NaOH (2 N) and a volume of 600 μL distilled water was added to each reaction. The rates of formation of 4-hydroxyquinoline were measured and quantified as described above. All measurements were carried out in triplicate and are expressed as mean $\pm$ SD.

4.6. Cholinesterase inhibition studies

AChE from electric eel, BuChE from equine serum, *S*-butyrylthiocholine iodide (BTCl), acetylthiocholine iodide (ATCl), 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB), donepezil and tacrine hydrochloride were purchased from Sigma-Aldrich. The inhibitory activities of the test com-

pounds were evaluated by Ellman's method.³⁶ The compounds were dissolved in DMSO and diluted with the buffer solution (50 mM Tris-HCl, pH = 8.0) to yield the corresponding test concentrations (DMSO less than 0.01%). In each well of the plate, 160 μL of 1.5 mM DTNB, 50 μL of AChE (0.22 U mL^{-1} eeAChE) or 50 μL of BuChE (0.12 U mL^{-1} eqBuChE) were incubated with 10 μL of different concentrations of test compounds (0.01–100 μM) at $37\text{ }^{\circ}\text{C}$ for 10 min. After this period, ATCl (15 mM) or BDCI (15 mM) as the substrate (30 μL) was added, incubated for a further 10 minutes, and thereafter the absorbance was measured at a wavelength of 405 nm at different time intervals (0, 60, 120, and 180 s). IC_{50} values were calculated as concentration of the compound that produces 50% enzyme activity inhibition, using the Graph Pad Prism 7 software (San Diego, CA, USA).

4.7. Molecular modelling studies

4.7.1. MAO docking studies. Computer-assisted docking was carried out using the CHARMM force field and hMAO-A co-crystallized with clorgiline (PDB ID: 2BXS)⁴⁰ and hMAO-B co-crystallized with safinamide (PDB ID: 2V5Z),⁴¹ which were recovered from the Brookhaven Protein Database (www.rcsb.org/pdb). Docking simulations were performed on the test compounds using Molecular Operating Environment (MOE)⁴³ with the following protocol. (1) Enzyme structures were checked for missing atoms, bonds and contacts. (2) Hydrogens and partial charges were added using the protonate 3D application in MOE. (3) The ligands were constructed using the builder module and were energy minimized. (4) Ligands were docked within the MAO-A or MAO-B active sites using MOEDock application, the poses were generated by the Triangle Matcher placement method. (5) The retained best poses were visually inspected and the interactions with binding pocket residues were analyzed. To determine the accuracy of this docking protocol, the co-crystallised ligand, clorgiline (PDB ID: 2Z5X), was re-docked into the MAO-A active site and the co-crystallised ligand, safinamide (PDB ID: 2V5Z), was re-docked into the MAO-B active site. This procedure was repeated three times and the best ranked solutions of clorgiline and safinamide exhibited RMSD values of less than 2 Å from the position of the co-crystallised ligand for MAO-A and MAO-B, respectively. In general, RMSD values smaller than 2.0 Å, indicate that the docking protocol is capable of accurately predicting the binding orientation of the co-crystallised ligand.^{61,62} These protocols were thus deemed to be suitable for the docking of inhibitors into the active site models of MAO-A and MAO-B.

4.7.2. Cholinesterase docking studies

4.7.2.1. Acetylcholinesterase. The dock function of MOE⁴³ was used to predict the interactions and binding modes of the synthesized inhibitors in the eeAChE active site. The crystal structure of the eeAChE protein co-crystallized with donepezil was acquired from the Protein Data Bank (PDB ID: 1EVE).⁴² The docking procedure followed the same protocol as described for the MAO docking. To determine the accuracy

of this docking protocol, the co-crystallised ligand, was re-docked into the AChE active site. This procedure was repeated three times and the best ranked solution exhibited an RMSD value of 1.15 Å from the position of the co-crystallised ligand. The RMSD value in this case is smaller than 2.0 Å indicating that the docking protocol is capable of accurately predicting the binding orientation of the co-crystallised ligand.^{61,62} This protocol was thus deemed to be suitable for the docking of inhibitors into the active site model of AChE.

4.7.2.2. Butyrylcholinesterase. Because no X-ray structure exists for eqBuChE, a homology model was used to rationalize the experimental data. The eqBuChE model was retrieved from the SWISS-MODEL Repository. This is a database of annotated three-dimensional comparative protein structure models generated by the fully automated homology-modeling pipeline SWISS-MODEL. A putative three-dimensional structure of eqBuChE has been created based on the crystal structure of hBuChE (PDB ID: 2 PM8), these two enzymes exhibited 89% sequence identity. Proper bonds, bond orders, hybridization and charges were assigned and CHARMM force field was applied using MOE.⁴³ The prepared protein was directly loaded into AutoDockTools (ADT; version 1.5.4), hydrogens were added and partial charges for proteins and ligands were calculated using Gasteiger charges. Flexible torsions in the ligands were assigned with the AutoTors module, and the acyclic dihedral angles were allowed to rotate freely. Docking calculations were performed with the program Autodock Vina.⁴⁶ Because VINA uses rectangular boxes for the binding site, the box center was defined and the docking box was displayed using ADT. All dockings were performed as blind dockings where a cube of 75 Å with grid points separated by 1 Å, was positioned in the middle of the protein ($x = 29.885$; $y = -54.992$; $z = 58.141$). Default parameters were used except num_modes, which was set to 40. The lowest docking-energy conformation was considered as the most stable orientation. Finally, the docking results generated were analysed using MOE.

4.8. SH-SY5Y cell viability assays

4.8.1. Cell line and culture conditions. The human neuroblastoma cell line SH-SY5Y was generously donated by our collaborator at the Division of Molecular Biology and Human Biology, Stellenbosch University, Tygerberg, Cape Town. Cells were cultured in monolayer using Dulbecco Modified Eagles Medium (DMEM, Gibco, Life Technologies Ltd) supplemented with 10% foetal bovine serum (FBS, Gibco, Life Technologies Ltd.) and 1% 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Lonza Group Ltd., Base Switzerland). Cells were grown at 37 °C, in a humidified atmosphere consisting of 5% CO₂ and 95% air. Media was replaced every two to three days and cells were sub-cultured by splitting with trypsin (Lonza Group Ltd., Basel Switzerland).

4.8.2. MTT cytotoxicity assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) is a yellow tetrazolium salt that is reduced to purple formazan in the pres-

ence of living cells. This assay which measures cell metabolic activity was used to determine cytotoxicity as described by Mosmann, (1983).⁵³ Briefly, SH-SY5Y cells were plated in flat bottom 96 well plates in growth medium as stated above at a density of 7500 cells per well. Cells were allowed to adhere to the plate surface for 24 hours and used media were replenished with fresh media containing test compounds at 10 µM, 50 µM and 100 µM. Vehicle control cells were treated with dimethyl sulfoxide (DMSO, solvent for dissolving test compounds) at a concentration similar to the amount contained in the highest concentration of test compounds. After 48 hours, 10 µL of MTT solution (5 mg mL⁻¹) was added to each well and incubated for 4 hours and the purple formazan formed was solubilized with 100 µL DMSO and plates were read spectrophotometrically to determine absorbance at 570 nm using a BMG Labtech Omega® POLARStar multimodal plate reader.

4.8.3. MPP⁺-induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were seeded onto a 96-well plate and treated with 1000 µM MPP⁺ for approximately 48 hours. Different concentrations (1 µM, 5 µM, 10 µM) of test compounds were administered two hours prior to MPP⁺ treatment. Afterwards, cell viability was measured by MTT colorimetric assay and performed as stated above in 4.8.2.

Conflicts of interest

The authors declare no competing interests.

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