CHAPTER 5

CONCLUSION

Parkinson’s disease is an age-related neurodegenerative disorder characterized pathologically by the loss of neurons in the SNpc. This loss in turn, leads to a striatal dopamine deficiency, which is responsible for the major symptoms of the disease. These symptoms include tremor at rest, postural instability, bradykinesia and in the later stages of the disease, non-motor symptoms such as psychosis. Current treatment only relieves the symptoms of the disease and do not have disease modifying effects, while also suffering from several side effects. MAO inhibitors have been shown to possess neuroprotective properties in addition to providing symptomatic relief in Parkinson’s disease. The potential of the chalcone scaffold to confer MAO inhibitory activity has furthermore been validated. The aim of this study was therefore to synthesize heterocyclic substituted chalcone analogues, as selective and reversible MAO-B inhibitors. The ideal chalcone inhibitor should be MAO-B selective with an IC\textsubscript{50} < 1 µM, reversible, have a competitive mode of binding, and be non-toxic.

Chemistry: Heterocyclic chalcone analogues were synthesized using the Claisen-Schmidt condensation reaction. Pyrrole, 5-methylthiophene, 5-chlorothiophene and 2-methoxypyridine groups were included as heteroaromatic substituents. Commercially available ketones were reacted with appropriate aldehydes in the presence of NaOH as base. All compounds were characterized with NMR and IR spectroscopy, as well as mass spectrometry. Purity, as determined by HPLC for all synthesized compounds ranged from 94-100%.

IC\textsubscript{50} determinations: The IC\textsubscript{50} values of all compounds were determined spectrofluorometrically using the recombinant human MAO-A and MAO-B enzyme sources, and kynuramine which is a mixed MAO-A/B substrate. The MAO-catalyzed oxidation of kynuramine, to yield 4-hydroxyquinoline, an excitation wavelength of 310 nm, and an emission wavelength of 400 nm was used to measure the catalytic activities of the MAO enzymes.

The results showed that all compounds were selective for the MAO-B isoform with SI values ranging from 4.6-240.7. Most compounds exhibited IC\textsubscript{50} values smaller than 1 µM, which indicates that these chalcones are potent MAO-B inhibitors. The most potent MAO-B inhibitor, compound 10i, exhibited an IC\textsubscript{50} value of 0.067 µM and also possessed the highest selectivity index of 240.7. The most potent MAO-A inhibitor, compound 10f, exhibited an IC\textsubscript{50} value of 3.805 µM and a SI value of 4.6. It was determined that compound 10i (IC\textsubscript{50} = 0.067 µM), was 1.3-fold more potent than the reversible MAO-B inhibitor lazabemide (IC\textsubscript{50} = 0.091 µM), which is currently in clinical use. Compound 10i was also 2.6-fold more potent than the most potent MAO-B inhibitor 9a (IC\textsubscript{50} = 0.174 µM), synthesized in a preceding study by Robinson et al. (2013).
Reversibility of binding: To determine whether binding of these heterocyclic chalcones to the MAO enzymes were reversible, the enzymatic activity after dilution of the enzyme-inhibitor complexes was determined. Compound **10i** and **10f**, the most potent MAO-B and MAO-A inhibitors, respectively, as well as compound **10e** was selected for this study. The irreversible inhibitors pargyline and \((R)\)-deprenyl were also included, for MAO-A and MAO-B, respectively, as positive controls.

The results of these studies indicated that compound **10f** had a reversible mode of binding to both the hMAO-B and hMAO-A isoforms, since enzyme activities were recovered after dilution of the reactions containing the enzymes and inhibitor. In contrast, for compound **10i**, hMAO-B enzyme activity was not fully recovered after dilution. These results indicated that **10i** possibly exhibited tight binding and that the inhibition caused by this compound was not readily terminated by dilution.

To determine whether the tight binding as exhibited by compound **10i** was due to the thiophene or 3-bromo-4-fluorophenyl moieties, the reversibility of binding of the pyrrole derivative **10e**, substituted with a similar phenyl group, was also examined. In this case, the results showed hMAO-B enzyme activity is recovered after dilution, which is consistent with a reversible interaction of **10e** with hMAO-B. This leads to the conclusion that the tight binding as exhibited by compound **10i** was due to the thiophene moiety.

To confirm that binding of **10i** was in fact not irreversible, the reversibility of binding of **10i** was also determined using dialysis. For this purpose MAO-B and **10i**, at a concentration of 4 × IC\(_{50}\), were preincubated for a period of 15 min and subsequently dialyzed for 24 h. The results of this study
confirmed that 10i had a reversible mode of binding, since the MAO-B activity was recovered by dialysis to a level of 83% of the control value after 24 h.

**Kinetic studies:** Since reversibility of binding was illustrated for compound 10f for both MAO isoforms, this compound was selected for kinetic studies. Lineweaver–Burk plots were constructed for the inhibition of hMAO-A and hMAO-B by 10f. For this purpose, the initial rates of kynuramine oxidation were measured at four different kynuramine concentrations (15, 30, 60 and 90 μM) in the absence and presence of four different test inhibitor concentrations. The lines of the constructed Lineweaver-Burk plots intersected on the y-axis, which is indicative of a competitive mode of binding. It may thus be concluded that compound 10f binds competitively to both the MAO-A and MAO-B isoforms.

**MTT cell viability:** The MTT cell viability assay was employed to obtain preliminary data regarding the cytotoxicity of selected compounds, 10e, 10f and 10i. The cytotoxicity of the test compounds were evaluated at concentrations of 1 and 10 μM, using HeLa cells. In the MTT assay, the MTT tetrazolium salt is metabolized by living cells to a purple coloured formazan product, which can be measured spectrophotometrically.

The most potent MAO-B inhibitor of the series examined, compound 10i, was non-toxic, with 100% and 96% cell viability obtained after exposure of the cultured cells to 1 and 10 μM, respectively, of the test compound. Interestingly, the pyrrole derivative 10f, the most potent MAO-A inhibitor of the series, exhibited significant toxicity at 10 μM, with only 5% viable cells remaining. To examine the possibility that the pyrrole moiety of 10f was responsible for its higher degree of toxicity, the cytotoxicity of another pyrrole derivative 10e, was also investigated. Interestingly, the results showed that 10e was non-toxic at 1 μM and 10 μM, with 99% and 98%, respectively, cell viability remaining. It thus appears that it is the substituent of the phenyl ring (a CF₃ group in this case) that is responsible for the observed cytotoxicity. Further investigation in this regard is required to determine the mode of cytotoxicity and to investigate the possibility that 10e, may be metabolized to reactive intermediates or toxic products.

**Molecular modeling:** The Windows based Accelrys® Discovery Studio 3.1 was used for the molecular docking studies. The crystal structure of hMAO-B co-crystallized with carboxaldehyde coumarin (a MAO-B inhibitor) was obtained from the Protein Data Bank (PDB code: 2V60). Molecular docking was carried out with the CDOCKER module in Discovery Studio and all compounds were successfully docked into the crystal structure model of the active site of hMAO-B. Visible inspection of the docked compounds further revealed that all compounds spanned both the substrate and entrance cavities of hMAO-B.
From the docking results it was also clear that the orientation of the docked ligands are dependent on the combination of substituents and that the orientation of the ligands cannot be predicted beforehand. It may be concluded from the results that a binding energy larger than -30.000 kcal/mol results in IC<sub>50</sub> values greater than 1 µM, thus higher binding energies result in poorer MAO-B inhibitory potency. Most compounds interacted with Tyr 326 or Tyr 398, while interactions with Cys 172, Gln 206, Ile 199 and Tyr 435 were also observed. The most potent MAO-B inhibitor of the series, compound 10i, bound to MAO-B with the phenyl ring facing the FAD cofactor. The phenyl ring of 10i forms a Pi-Pi interaction with Tyr 398 as well as a Pi-Sigma interaction between the thiophene moiety and Ile 199 (which is part of the gating switch of MAO-B). It is speculated that the tight binding component of hMOA-B inhibition by 10i may, at least in part, be attributed to the interaction of this compound with the gating switch amino acid, Ile 199. Although the docking studies yielded some insights, it is unlikely that molecular docking may be used to predict the MAO-B inhibitory potencies of chalcone-derived inhibitors.

In conclusion, this study resulted in the synthesis of potent, selective heterocyclic substituted chalcone MAO-B inhibitors. The compounds tested had a reversible, competitive mode of binding, but cytotoxicity of these chalcone derivatives should be further investigated as only two of the three compounds screened, were non-toxic to cultured cells. Analyses of the IC<sub>50</sub> values, reversibility of inhibition and results of the cytotoxicity results suggest that small variations in heterocyclic and aromatic substitution convey diverging biological properties. This study confirmed the validity of the chalcone scaffold in the design of potent, selective reversible MAO inhibitors and illustrated that heterocyclic substitution is a viable option.