CHAPTER 4

BIOLOGICAL EVALUATION

4.1 INTRODUCTION

In this study the human MAO-A and MAO-B inhibitory potencies of the synthesized chalcones were evaluated. This chapter will firstly give an overview of enzyme kinetics, which will be followed by the experimental methods and results of the MAO inhibition studies.

4.2 ENZYME KINETICS

4.2.1. Introduction

Enzyme kinetics may be used to examine the interaction of an inhibitor with an enzyme. For this purpose, the enzyme catalytic rate is measured in the absence and presence of the test inhibitor, and the rate data are fitted to the Michaelis-Menten equation.

4.2.2 Michaelis-Menten kinetics

The Michaelis-Menten equation (equation 1), describes the behavior of enzymes when substrate concentrations are varied (Rodwell, 1993).

\[ V_i = \frac{V_{\text{max}}[S]}{K_m + [S]} \]

**Equation 1**: The Michaelis-Menten equation.

In this equation, \( K_m \) is the substrate concentration that produces half-maximal velocity (also known as the Michaelis constant), \([S]\) is the substrate concentration and \( V_i \) is the measured initial velocity. \( V_{\text{max}} \) is the maximum velocity (Rodwell, 1993).

\( K_m \) can be determined by graphing \( V_i \) as a function of \([S]\). When \([S]\) is approximately equal to \( K_m \), it means \( V_i \) is very responsive to changes in \([S]\), and that the enzyme is working at half-maximal velocity (Rodwell, 1993).

In the next three points below, the Michaelis-Menten equation is evaluated to illustrate the dependence of the initial velocity of an enzyme-catalyzed reaction on \([S]\) and on \( K_m \):

1) When \([S]\) is much smaller than \( K_m \), \([S]\) may be removed from the denominator. Since \( V_{\text{max}} \) and \( K_m \) are constants, their ratio can be replaced with a new constant \( K \). Thus, when the substrate
concentration is much lower than that required to produce a half maximal velocity (the $K_m$ value), the initial velocity, $V_i$, is dependent on the substrate concentration, $[S]$ (equation 2) (Rodwell, 1993).

$$V_i = \frac{V_{\text{max}}[S]}{K_m + [S]} \sim \frac{V_{\text{max}}[S]}{K_m} \sim \frac{V_{\text{max}}[S]V_i}{K_m [S]}$$

Equation 2: When $[S]$ is less than $K_m$.

2) When $[S]$ is much greater than $K_m$ (equation 3), the addition of $K_m$ to $[S]$ results in a very small change in the denominator value. In this case, $K_m$ can be omitted from the denominator. When the substrate concentration $[S]$ is much larger than the $K_m$ value, the initial velocity, $V_i$, is thus equal to the maximal velocity, $V_{\text{max}}$ (Rodwell, 1993).

$$V_i = \frac{V_{\text{max}}[S]}{K_m + [S]} \sim \frac{V_{\text{max}}[S]}{[S]} \sim V_{\text{max}}$$

Equation 3: When $[S]$ is greater than $K_m$.

3) When $[S]$ is equal to $K_m$ (equation 4), the initial velocity, $V_i$, is equal to the half maximal reaction velocity. This analysis may be used to measure the $K_m$ value of a substrate for an enzyme since $K_m$ is equal to the substrate concentration where the initial velocity is half that of the maximal velocity (Rodwell, 1993).

$$V_i = \frac{V_{\text{max}}[S]}{K_m + [S]} = \frac{V_{\text{max}}[S]}{[S] + [S]} = \frac{V_{\text{max}}[S]}{2[S]} = \frac{V_{\text{max}}[S]}{2}$$

Equation 4: When $[S] = K_m$

4.2.2.1 $K_m$ and $V_{\text{max}}$ determinations

The linear form of the Michaelis-Menten equation is used to determine $K_m$ and $V_{\text{max}}$. The linear equation is obtained by plotting $1/V_i$ versus $1/[S]$ (equation 5) (Rodwell, 1993).

$$\frac{1}{V_i} = \frac{K_m}{V_{\text{max}}} \cdot \frac{1}{[S]} + \frac{1}{V_{\text{max}}}$$

Equation 5: The linear form of the Michaelis-Menten equation.

This is a linear equation: $y = a.x + b$, where $y = 1/V_i$ and $x = 1/[S]$. When $y (1/V_i)$ is plotted as a function of $x (1/[S])$, the $y$-intercept ($b$) is $1/V_{\text{max}}$, and the slope ($a$) is $K_m/V_{\text{max}}$ (figure 4.1).
To obtain the value of the negative x-intercept, \( y \) is set equal to 0 (Equation 6).

\[
\begin{align*}
\text{Equation 6: The value of the x intercept } (y = 0). \\
& \quad x = -\frac{b}{a} = -\frac{1}{K_m}
\end{align*}
\]

Such a plot is called a double reciprocal plot, in which the reciprocal of \( V_i \) (1/\( V_i \)) is plotted against the reciprocal of [S], (1/[S]). The double-reciprocal plot is also known as a Lineweaver-Burk plot (figure 4.2). By using this plot, \( K_m \) can be estimated from the slope and y-intercept, or the negative x intercept (Rodwell, 1993).

At a substrate concentration 100 times that of \( K_m \), the enzyme acts at a maximum rate, and the maximal velocity (\( V_{\text{max}} \)) will thus reflect the concentration of active enzyme present. In this case, the \( K_m \) value would indicate the concentration of substrate required for the measurement of \( V_{\text{max}} \) (Rodwell, 1993).
4.2.3 Competitive inhibition and \( K_i \) determination

\( K_i \) is the equilibrium constant used to indicate the reversibility of an enzyme-inhibitor complex.

When the double reciprocal plot is constructed in the presence of a fixed concentration of a competitive inhibitor (figure 4.3 A), the \( y \)-intercept \((1/V_{\text{max}})\) remains unchanged compared to when the value of the \( y \)-intercept of the double reciprocal plot is constructed in the absence of inhibitor. The two lines thus intercept on the \( y \)-axis, which is typically what is observed during competitive inhibition. On the other hand, the \( x \)-axis intercepts of the double reciprocal plot increases (becomes less negative) which means that the apparent \( K_m \) value increases.

![Figure 4.3: The Lineweaver-Burk plots demonstrating competitive inhibition (A) and non-competitive inhibition (B).](image)

If a reversible non-competitive inhibitor is evaluated, the maximum velocity \((V_{\text{max}})\) will be reduced, but the \( K_m \) values are not affected (figure 4.3B) (Rodwell, 1993). In this case, the two lines would intercept on the \( x \)-axis of the double reciprocal plot.

If \( 1/V_i \) was plotted versus inhibitor concentration [I], while the substrate concentration was kept constant, the lines constructed with two substrate concentrations would intersect to the left of the \( y \)-axis, which is also indicative of competitive inhibition. The value of \( x \) at the point where the lines intersect is equal to the \(-K_i\) value of the inhibitor (figure 4.4) (Rodwell, 1993).
Figure 4.4: The $K_i$ values for competitive inhibition (A) and for non-competitive inhibition (B) (Dixon, 1952).

For a competitive system, $K_i$ can be calculated by the following equation (equation 7).

\[
V_i = \frac{V \cdot S}{K_m (1 + [I]/K_i) + S}
\]

\[
K_i = \frac{K_m [I]}{(\frac{V \cdot S}{v_i}) - S - K_m}
\]

**Equation 7:** The Michaelis-Menten equation for a competitive system.

### 4.2.4 IC$_{50}$ value determination

The IC$_{50}$ value is the concentration of the inhibitor that reduces the enzyme activity by 50%. The IC$_{50}$ value gives an indication of the effectiveness of a compound in inhibiting a biological or biochemical function, in this case the concentration of inhibitor required to decrease enzyme activity by 50% (Novaroli et al., 2005, Burlingham & Widlanski, 2003). Lower IC$_{50}$ values is indicative of more potent inhibition (Burlingham & Widlanski, 2003).

The IC$_{50}$ value for competitive inhibitors can be calculated by equation 8.

\[
IC_{50} = K_i \left(1 + \frac{[S]}{K_m}\right)
\]

**Equation 8:** IC$_{50}$ determination

The IC$_{50}$ value of a competitive inhibitor is related to the $K_i$ value of the inhibitor as a function of the substrate concentration, [S], and the Michaelis-Menten constant, $K_m$, of the substrate (Burlingham & Widlanski, 2003). The IC$_{50}$ value can also be determined experimentally. In this dissertation, the potencies of the test inhibitors will be expressed as their IC$_{50}$ values for the
inhibition of the MAO enzymes. As discussed below, these IC50 values will be experimentally determined from sigmoidal concentration-inhibition curves.

4.3 THE IC50 VALUE DETERMINATION OF THE TEST INHIBITORS

To determine the MAO inhibitory potencies of the synthesized chalcones, the IC50 values were determined. For this study a compound with an IC50 value < 1 μM is considered to be a potent MAO inhibitor. To measure MAO activity this study employs kynuramine as enzyme substrate. Kynuramine is a MAO-A/B non-selective substrate and can thus be used to measure the activities of both MAO isoforms.

4.3.1 General background

\[
\begin{align*}
\text{Kynuramine} & \xrightarrow{\text{MAO}} \text{Intermediate aldehyde} \xrightarrow{} \text{4-Hydroxyquinoline}
\end{align*}
\]

*Figure 4.5:* Kynuramine is catalyzed to 4-hydroxyquinoline by MAO oxidation.

Kynuramine is oxidized by MAO-A and MAO-B to yield 4-hydroxyquinoline (figure 4.5). In the presence of a MAO inhibitor the oxidation of kynuramine to 4-hydroxyquinoline is inhibited. This reduction in the concentration of 4-hydroxyquinoline can be readily measured by fluorescence spectrophotometry since 4-hydroxyquinoline is fluorescent in alkaline media. The fluorescence of 4-hydroxyquinoline is measured at an excitation wavelength of 310 nm and an emission wavelength of 400 nm (Krajl, 1965).

4.3.2 Materials and instrumentation

Microsomes from baculovirus infected BTI insect cells containing recombinant human MAO-A and -B (5 mg/ml) served as the MAO enzyme sources. These enzymes as well as kynuramine were obtained from Sigma Aldrich. Dimethyl sulfoxide (DMSO) was obtained from Riedel-de Haën, while sodium hydroxide (NaOH), KH2PO4, K2HPO4 and KCl were obtained from Merck.
Fluorescence spectrophotometry was conducted with a Varian® Cary Eclipse® fluorescence spectrophotometer (Agilent® Technologies, Santa Clara, USA).

The MAO-A and MAO-B inhibitory properties of compounds 10a-j and 8 were determined as described by Strydom et al. (2011).

The enzymatic reactions were prepared in potassium phosphate buffer (KH$_2$PO$_4$) (100 mM, pH 7.4, made isotonic with KCl 20.2 mM). The reactions contained the substrate kynuramine (45 μM and 30 μM for MAO-A and MAO-B, respectively) as well as various concentrations of the test inhibitor (0, 0.003, 0.01, 0.1, 1, 10 and 100 μM). DMSO at a final concentration of 4% was added to all incubations as co-solvent, including those conducted in the absence of the inhibitor. MAO-A or MAO-B (0.0075 mg protein/ml) was added to initiate the reactions, and the reactions were subsequently incubated for 20 min at 37 °C. The reaction was terminated with the addition of 400 μl NaOH (2 N) and 1000 μl water (figure 4.6).

4.3.3 Experimental method for IC$_{50}$ determination

The concentrations of the MAO-generated 4-hydroxyquinoline in the supernatants of the reactions were spectrofluorometrically measured at an excitation wavelength of 310 nm and emission wavelength of 400 nm.

A linear calibration curve was constructed with known concentrations of 4-hydroxyquinoline (0.04690, 0.09375, 0.18750, 0.37500, 0.75000 and 1.50000 μM) dissolved in potassium phosphate buffer. The calibration standards were prepared to a volume of 500 μl and also contained NaOH (2N, 400 μl) and water (1000 μl). Sigmoidal dose–response curves were constructed by plotting the initial rates of kynuramine oxidation versus the logarithm of the inhibitor concentration. For this purpose the Prism® 5.0 software package (GraphPad® Software, La Jolla, USA) was used. The IC$_{50}$ values were determined in triplicate from the sigmoidal curves and are expressed as mean ± standard deviation (SD) (Petzer et al., 2012, Strydom et al., 2011).
Figure 4.6: Diagrammatic representation of the determination of MAO-A and MAO-B IC\textsubscript{50} values.
4.3.4 Results and discussion

Table 4.1 summarizes the results of the MAO inhibition studies. These results will be discussed in detail below.

From the results the following observations were made:
- All compounds were selective for the MAO-B isoform with SI values of 4.6-240.7. The most selective MAO-B inhibitor was compound 10i with a SI value of 240.7.
- Most compounds exhibited IC\(_{50}\) 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\(_{50}\) value of 0.067 μM and also possesses the highest selectivity index (SI) of 240.7. The most potent MAO-A inhibitor, compound 10f, exhibited an IC\(_{50}\) value of 3.805 μM and a SI value of 4.6.

Table 4.1: IC\(_{50}\) and SI values obtained for the inhibition of hMAO-A and hMAO-B by the synthesized chalcones.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chalcone structure</th>
<th>MAO-B IC(_{50}) ± SD (μM)</th>
<th>MAO-A IC(_{50}) ± SD (μM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td><img src="image" alt="Structure 10a" /></td>
<td>0.133 ± 0.048</td>
<td>7.561 ± 1.360</td>
<td>56.8</td>
</tr>
<tr>
<td>10b</td>
<td><img src="image" alt="Structure 10b" /></td>
<td>3.274 ± 0.419</td>
<td>143.133 ± 24.058</td>
<td>43.7</td>
</tr>
<tr>
<td>10c</td>
<td><img src="image" alt="Structure 10c" /></td>
<td>0.330 ± 0.053</td>
<td>15.327 ± 6.158</td>
<td>46.4</td>
</tr>
</tbody>
</table>
The selectivity index (SI) is the selectivity of the MAO-B isoform. This ratio is calculated as $SI = \frac{IC_{50} (MAO-A)}{IC_{50} (MAO-B)}$. 

<table>
<thead>
<tr>
<th>10d</th>
<th><img src="https://example.com/10d.png" alt="Chemical Structure" /></th>
<th>$0.185 \pm 0.050$</th>
<th>$9.992 \pm 2.638$</th>
<th>54.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>10e</td>
<td><img src="https://example.com/10e.png" alt="Chemical Structure" /></td>
<td>$0.803 \pm 0.037$</td>
<td>$8.981 \pm 1.515$</td>
<td>11.2</td>
</tr>
<tr>
<td>10f</td>
<td><img src="https://example.com/10f.png" alt="Chemical Structure" /></td>
<td>$0.830 \pm 0.100$</td>
<td>$3.805 \pm 0.387$</td>
<td>4.6</td>
</tr>
<tr>
<td>10g</td>
<td><img src="https://example.com/10g.png" alt="Chemical Structure" /></td>
<td>$1.396 \pm 0.140$</td>
<td>$267.233 \pm 80.516$</td>
<td>191.4</td>
</tr>
<tr>
<td>10h</td>
<td><img src="https://example.com/10h.png" alt="Chemical Structure" /></td>
<td>$0.116 \pm 0.030$</td>
<td>$10.493 \pm 1.198$</td>
<td>90.5</td>
</tr>
<tr>
<td>10i</td>
<td><img src="https://example.com/10i.png" alt="Chemical Structure" /></td>
<td>$0.067 \pm 0.016$</td>
<td>$16.130 \pm 2.138$</td>
<td>240.7</td>
</tr>
<tr>
<td>8</td>
<td><img src="https://example.com/8.png" alt="Chemical Structure" /></td>
<td>$0.093 \pm 0.022$</td>
<td>$8.591 \pm 0.666$</td>
<td>92.4</td>
</tr>
</tbody>
</table>
When comparing the MAO-A and MAO-B inhibitory potencies of the pyrrole derivatives, 10f (IC50 = 0.830 µM) and 10g (IC50 = 1.396 µM), it is clear that trifluoromethyl substitution in position 4 of the phenyl ring (10f) is preferable over substitution in position 3 (10g). Interestingly, the SI value of the m-substituted derivative, however, is significantly higher. The position of substitution therefore seems to play a role in both potency and selectivity of these derivatives.

On the other hand, comparison of the MAO-B inhibitory potencies of the 5-chlorothiophene derivatives 10a (IC50 = 0.330 µM) and 10h (IC50 = 0.116 µM) indicate that a 3-bromo-4-fluorophenyl substituent yields more potent MAO-B inhibition than a p-trifluoromethylphenyl substituent.

Compound 8 was previously investigated (Chimenti et al., 2009) and an IC50 value of 0.004 µM reported for the inhibition of hMAO-B. This was the most active derivative identified in a series of 1,3-diphenyl-2-propen-1-ones and it was resynthesized in this study for comparative purposes. When the IC50 value of this compound was determined in this study, an IC50 value of 0.093 µM was obtained. Compound 10i, the most potent derivative identified in this study, has an IC50 value 0.067 µM, which is more potent than compound 8, and indicates that heterocyclic substitution of the chalcone scaffold (with a methylthiophene ring in particular) is a viable design strategy.

Furthermore, comparison of the MAO-B inhibitory potencies of 3-bromo-4-fluorophenyl derivatives 10i (IC50 = 0.067 µM) and 10h (IC50 = 0.116 µM), reveals that an electron donating methyl substituent in the thiophene ring (10i), results in improved MAO-B inhibitory activity compared to substitution with an electron withdrawing chlorine substituent (10h).

In addition, when the MAO-B inhibitory potencies of the 3-bromo-4-fluorophenyl derivatives 10h (IC50 = 0.116 µM) and 10e (IC50 = 0.803 µM), and the MAO-B inhibition potencies of the 4-trifluorophenyl derivatives 10a (IC50 = 0.133 µM) and 10f (IC50 = 0.830 µM) are compared, the results indicate that 5-chlorothiophene substitution is preferable over pyrrole substitution.

Similarly, when the MAO-B inhibitory potencies of the 3-chlorophenyl derivatives, 10c (IC50 = 0.330 µM) and 10b (IC50 = 3.274 µM) are compared, it is evident that 2-methoxypyridine substitution is also preferable over pyrrole substitution.
The results of this study were also compared to the results obtained for a series of furanochalcones (table 4.2) synthesized by Robinson et al. (2013). This direct comparison was possible, since these derivatives were screened using the same conditions.

In order to identify the heterocyclic substituent that conferred the most potent MAO-B inhibitory activity, the MAO-B inhibitory potencies of the following derivatives were compared:

The 3-chlorophenyl derivatives 9b-9d (IC$_{50}$ = 0.529, 2.10, 0.490 µM, respectively), 10b (IC$_{50}$ = 3.274 µM) and 10c (IC$_{50}$ = 0.330 µM). An analysis of the MAO-B inhibitory activities of these compounds indicate that the effect of the heteroaromatic/aromatic substituent on activity, in decreasing order is: 2-methoxypyridine > 5-methylfuran > phenyl > furan > pyrrole.

The 3-bromo-4-fluorophenyl derivatives, namely 9f (IC$_{50}$ = 0.200 µM), 10e (IC$_{50}$ = 0.803 µM), 10h (IC$_{50}$ = 0.116 µM) and 10i (IC$_{50}$ = 0.067 µM). An analysis of the MAO-B inhibitory activities indicates that the effect of the heteroaromatic substituent for these compounds, on activity, in decreasing order is: 5-methylthiophene > 5-chlorothiophene > 5-methylfuran > pyrrole.

The 4-trifluoromethylphenyl derivatives, 9g (IC$_{50}$ = 0.275 µM), 10a (IC$_{50}$ = 0.133 µM) and 10f (IC$_{50}$ = 0.830 µM). An analysis of the MAO-B inhibitory activities indicate that the effect of the heteroaromatic substituent for these compounds, on activity, in decreasing order is: 5-chlorothiophene > 5-methylfuran > pyrrole.

These results show that substitution with a 5-methylthiophene group is an improvement on furan or methylfuran substitution and results in optimal MAO-B inhibitory activity, while pyrrole substitution is associated with decreased MAO-B inhibitory activity.

In conclusion, compound 10i (IC$_{50}$ = 0.067 µM), the most potent MAO-B inhibitor of the present series, is 1.3-fold more potent than the reversible MAO-B inhibitor lazabemide (IC$_{50}$ = 0.091 µM), which is currently in clinical use. Compound 10i is also 2.6-fold more potent than the most potent furanochalcone MAO-B inhibitor (9a) (IC$_{50}$ = 0.174 µM) investigated by Robinson et al. (2013) and has similar activity to 8, previously investigated by Chimenti and co-workers (2009).

It should be kept in mind that this is a preliminary study and future work will include the expansion of this series, to investigate the effect of further variations in the substitution of the phenyl ring in particular, and also to investigate the effect of dihetero-aryl substitution.
### Table 4.2: IC$_{50}$ values for the inhibition of human MAO-B of a series of furanochalcones (Robinson et al., 2013).

<table>
<thead>
<tr>
<th>Structure</th>
<th>MAO-B IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Structure 9a" /></td>
<td>0.174</td>
</tr>
<tr>
<td><img src="image" alt="Structure 9b" /></td>
<td>0.529</td>
</tr>
<tr>
<td><img src="image" alt="Structure 9c" /></td>
<td>2.10</td>
</tr>
<tr>
<td><img src="image" alt="Structure 9d" /></td>
<td>0.490</td>
</tr>
<tr>
<td><img src="image" alt="Structure 9e" /></td>
<td>0.616</td>
</tr>
<tr>
<td><img src="image" alt="Structure 9f" /></td>
<td>0.200</td>
</tr>
<tr>
<td><img src="image" alt="Structure 9g" /></td>
<td>0.275</td>
</tr>
</tbody>
</table>
4.4 THE REVERSIBILITY OF MAO INHIBITION (DILUTION METHOD)

In this section the reversibility of MAO inhibition by the most potent MAO-B and MAO-A inhibitors, 10i, and 10f, respectively, were examined. This study also investigated the reversibility of MAO-B inhibition by compound 10e.

4.4.1 General background

To examine the reversibility of MAO-A and MAO-B inhibition by the test compounds, the recoveries of the enzymatic activities after dilution of the enzyme-inhibitor mixtures were evaluated. MAO-A and MAO-B were preincubated with the test compounds at concentrations of 10 × IC$_{50}$ and 100 × IC$_{50}$ for the inhibition of the respective enzymes. After a 30 min preincubation, the incubations were diluted 100-fold to yield inhibitor concentrations of 0.1 × IC$_{50}$ and 1 × IC$_{50}$. For reversible inhibition, dilution of the enzyme-inhibitor mixtures to an inhibitor concentration of 0.1 × IC$_{50}$ is expected to result in approximately 90% recovery in enzyme activity, while dilution to 1 × IC$_{50}$ is expected to result in approximately 50% recovery in enzyme activity. In contrast, after similar treatment of MAO-A and MAO-B with the irreversible inhibitors pargyline and (R)-deprenyl, respectively, the MAO-A and MAO-B activities were not expected to recover.

4.4.2 Materials and instrumentation

Materials and instrumentation were the same as in section 4.3.2. Pargyline and (R)-deprenyl were obtained from Sigma-Aldrich (Petzer et al., 2012).
4.4.3 Experimental method for reversibility determination (dilution method)

**REVERSIBILITY DILUTION METHOD**

**Prepare KH₂PO₄ buffer: 100 mM, pH 7.4, made isotonic with KCl 20.2 mM.**

**Prepare incubations: Add KH₂PO₄ buffer, DMSO, and test inhibitors at concentrations of 0, 10, and 100 x IC₅₀.**

**Enzyme concentrations: Add MAO-A [0.0075 mg/ml] or MAO-B [0.0075 mg/ml].**

**Incubate for 30 min at 37 °C.**

**Dilution: Incubations diluted 100-fold yielding concentrations of 0, 0.1 and 1 x IC₅₀ of the test inhibitors. Kynuramine substrate (45 µM for MAO-A and 30 µM for MAO-B) was added to all incubations.**

**Incubated further for 20 min at 37 °C.**

**Terminate the reaction with the addition of 400 µl NaOH and 1000 µl distilled water.**

**Measure 4-hydroxyquinoline concentrations spectrofluorometrically at an excitation wavelength of 310 nm and emission wavelength of 400 nm.**

**Reactions were done in triplicate and the residual enzyme catalytic rates expressed as mean ± SD. Pargyline or (R)-deprenyl, were included as positive controls.**

**Figure 4.7:** Diagrammatic representation of the determination of reversibility of MAO-A and MAO-B using the dilution method.
The literature protocol of Strydom et al. (2011), were used to carry out these studies.

**Preincubation**: The test inhibitors, at concentrations of $10 \times IC_{50}$ and $100 \times IC_{50}$ were pre-incubated with recombinant human MAO-A or MAO-B (0.75 mg protein/ml) at 37 °C for of 30 min in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl). Control incubations were conducted in the absence of inhibitor, and DMSO (4%) was added as co-solvent to all preincubations (figure 4.7).

**Dilution**: After these preincubations, the reactions were diluted 100—fold with the addition of kynuramine (45 μM and 30 μM for MAO-A and -B, respectively) to yield inhibitor concentrations equal to $0.1 \times IC_{50}$ and $1 \times IC_{50}$ value, respectively. The final concentration of MAO-A and –B in these diluted mixtures was 0.0075 mg/ml.

**MAO activity measurement**: The reactions were incubated for a further 20 min at 37 °C, terminated and the residual rates of 4-hydroxyquinoline formation were measured as described previously. For this purpose the concentrations of the MAO-generated 4-hydroxyquinoline in the reactions were spectrofluorometrically measured at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. These reactions were carried out in triplicate and the residual enzyme catalytic rates were expressed as mean ± SD.

**Positive controls**: For comparison, similar studies were carried out with the irreversible inhibitors pargyline and (R)-deprenyl. For this purpose pargyline and (R)-deprenyl were preincubated with MAO-A and MAO-B, respectively, at concentrations equal to $10 \times IC_{50}$ and then diluted 100-fold to yield final inhibitor concentrations of $0.1 \times IC_{50}$ (Petzer et al., 2012, Strydom et al., 2011).

### 4.4.4 Results and discussion

The pyrrole derivative, 10f, which was the most potent MAO-A inhibitor, was selected and reversibility of binding for both MAO-A and –B was determined. The results, given in Fig. 4.8, show that after dilution of compound 10f, to concentrations equal to $0.1 \times IC_{50}$ and $1 \times IC_{50}$, the MAO-B activities were recovered to levels of 85% and 32%, respectively, of the control value. This behavior is consistent with a reversible interaction of 10f with MAO-B. As mentioned above, for reversible inhibition, dilution of the enzyme-inhibitor mixtures to an inhibitor concentration of $0.1 \times IC_{50}$ is expected to result in approximately 90% recovery in enzyme activity, while dilution to $1 \times IC_{50}$ is expected to result in approximately 50% recovery in enzyme activity. In contrast, after similar treatment of MAO-B with the irreversible inhibitor (R)-deprenyl, the MAO-B activity was not recovered as dilution of (R)-deprenyl to a concentration of $0.1 \times IC_{50}$, resulted in the recovery of only 3% enzyme activity, of the control value.
**Figure 4.8:** Reversibility of inhibition of MAO-B by 10f. MAO-B was preincubated with 10f at concentrations equal to 10 × IC\(_{50}\) and 100 × IC\(_{50}\) for 30 min and then diluted to 0.1 × IC\(_{50}\) and 1 × IC\(_{50}\). The residual enzyme activities were subsequently measured. For comparison, MAO-B was similarly preincubated with (R)-deprenyl at concentrations equal to 10 × IC\(_{50}\) and then diluted to 0.1 × IC\(_{50}\).

Furthermore, as illustrated in Fig. 4.9, dilution of compound 10f, to concentrations equal to 0.1 × IC\(_{50}\) and 1 × IC\(_{50}\), resulted in the recovery of the MAO-A activities to levels of 90% and 67%, respectively, of the control value. This behavior is also consistent with a reversible interaction of 10f with MAO-A. In contrast, after similar treatment of MAO-A with the irreversible inhibitor pargyline, the MAO-A activity was not recovered since dilution of the enzyme-pargyline complex, to a concentration equal to 0.1 × IC\(_{50}\), resulted in the recovery of the enzyme activities to only 1% of the control value. These results were as expected, since a reversible mode of binding was also illustrated for a related series of furanochalcones (Robinson *et al*., 2013).
Figure 4.9: Reversibility of inhibition of MAO-A by 10f. MAO-A was preincubated with 10f at concentrations equal to $10 \times IC_{50}$ and $100 \times IC_{50}$ for 30 min and then diluted to $0.1 \times IC_{50}$ and $1 \times IC_{50}$. The residual enzyme activities were subsequently measured. For comparison, MAO-A was similarly preincubated with pargyline at concentrations equal to $10 \times IC_{50}$ and then diluted to $0.1 \times IC_{50}$.

Contrastingly, the results obtained when the reversibility of binding of the most potent MAO-B inhibitor, 10i, was determined, was unexpected. These results, given in Fig. 4.10, show that after diluting mixtures containing MAO-B and compound 10i, to concentrations equal to $0.1 \times IC_{50}$ and $1 \times IC_{50}$, the MAO-B activities were recovered to levels of only 26% and 5%, respectively, of the control value. This behavior was not fully consistent with a reversible interaction of 10i with MAO-B. As mentioned above, for reversible inhibition, dilution of the enzyme-inhibitor mixtures to an inhibitor concentration of $0.1 \times IC_{50}$ is expected to result in approximately 90% recovery in enzyme activity, while dilution to $1 \times IC_{50}$ is expected to result in approximately 50% recovery in enzyme activity. A possible explanation for this finding was that 10i could exhibit tight binding to the MAO-B enzyme, and the inhibition caused by this compound was not readily terminated by dilution. This finding was further examined in the following section where the reversibility of MAO-B inhibition by 10i was further examined by dialysis. The graph (Fig. 4.10) also shows after treatment of MAO-B with the irreversible inhibitor (R)-deprenyl and subsequent dilution to a concentration of $0.1 \times IC_{50}$, the recovered enzyme activity was only 5% of the control value. In this case, the results obtained for 10i, especially at $1 \times$ the $IC_{50}$ value, thus appears very similar to those obtained for the irreversible inhibitor.
Figure 4.10: Reversibility of inhibition of MAO-B by 10i. MAO-B was preincubated with 10i at concentrations equal to 10 × IC₅₀ and 100 × IC₅₀ for 30 min and then diluted to 0.1 × IC₅₀ and 1 × IC₅₀. The residual enzyme activities were subsequently measured. For comparison, MAO-B was similarly preincubated with (R)-deprenyl at concentrations equal to 10 × IC₅₀ and then diluted to 0.1 × IC₅₀.

To investigate whether the tight binding of 10i was due to the presence of the phenyl or thiophene moieties, the reversibility of MAO-B inhibition of 10e was also examined. These results, given in Fig. 4.11, show that after diluting mixtures containing MAO-B and compound 10e, to concentrations equal to 0.1 × IC₅₀ and 1 × IC₅₀, the MAO-B activities were recovered to levels of 81% and 60%, respectively, of the control value. This behavior is consistent with a reversible interaction of 10e with MAO-B. For (R)-deprenyl, as in the preceding dilution studies, enzyme activity was only recovered to 3% of the control value, after dilution to a concentration of 0.1 x the IC₅₀. From these results it could thus be derived that the tight binding observed for 10i was due to the presence of the thiophene moiety, as the pyrrole derivative 10e, with a similar phenyl substituent, bound reversibly to MAO-B.
Figure 4.11: Reversibility of inhibition of MAO-B by 10e. MAO-B was preincubated with 10e at concentrations equal to 10 × IC₅₀ and 100 × IC₅₀ for 30 min and then diluted to 0.1 × IC₅₀ and 1 × IC₅₀. The residual enzyme activities were subsequently measured. For comparison, MAO-B was similarly preincubated with (R)-deprenyl at concentrations equal to 10 × IC₅₀ and then diluted to 0.1 × IC₅₀.

4.5 THE REVERSIBILITY DETERMINATION ASSAY (DIALYSIS METHOD)

4.5.1 General background

In the section above, it was shown that the inhibition of MAO-B by compound 10i appeared not to be completely reversible, and 10i therefore could exhibit tight binding to the MAO-B active site. To further explore the results obtained in the dilution assay, the reversibility of MAO-B inhibition by 10i was examined by dialysis. For this purpose MAO-B and 10i, at a concentration of 4 × IC₅₀, were preincubated for a period of 15 min and subsequently dialyzed for 24 h.
4.5.2 Materials and instrumentation

Materials and instrumentation were the same as in section 4.3.2. (R)-Deprenyl as well as the chemicals required to prepare the dialysis buffer were obtained from Sigma Aldrich. Slide-A-Lyzer® dialysis cassettes were obtained from Thermo Scientific and had a molecular weight cut-off of 10 000 and a sample volume capacity of 0.5–3 ml.

4.5.3 Experimental method for reversibility determination (dialysis method)

The literature protocol of Petzer et al. (2013), as set out below, was used:

**Preincubation:** MAO-B (0.03 mg/ml) and 10i, at a concentration equal to 4 x IC50 were preincubated for 15 min at 37°C. These incubations were buffered with potassium phosphate buffer (100 mM, pH 7.4) containing 5% sucrose. The final volume of the incubations were 0.8 ml and DMSO (4%) was added as co-solvent to all preincubations. As control, MAO-B were similarly preincubated in the absence of inhibitor and presence of the irreversible inhibitor, (R)-deprenyl [IC50 (MAO-B) = 0.079 µM] at a concentration of 4 x IC50 (figure 4.12).

**Dialysis:** The reactions (0.8 ml) were subsequently dialyzed at 4 °C in 80 ml of outer buffer (100 mM potassium phosphate, pH 7.4, 5% sucrose). The outer buffer was replaced with fresh buffer at 3 h and 7 h after the start of dialysis.

**Residual MAO-B activity:** At 24 h after dialysis was started the reactions were diluted twofold with the addition of kynuramine (dissolved in potassium phosphate buffer, 100 mM, pH 7.4, made isotonic with KCl) and the residual MAO activities were measured as described previously. For this purpose the concentrations of the MAO-generated 4-hydroxyquinoline in the reactions were spectrofluorometrically measured at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. The final concentration of kynuramine in these reactions was 50 µM, while the final inhibitor concentrations were equal to 2 x IC50. These reactions were carried out in triplicate and the residual enzyme catalytic rates were expressed as mean ± SD.

**Undialyzed reactions:** For comparison, undialyzed mixtures of MAO-B with 10i were maintained at 4 °C over the same time period (Petzer et al., 2013, Strydom et al., 2011).
Prepare KH$_2$PO$_4$ buffer: 100 mM, pH 7.4, containing 5% sucrose

Prepare incubations: Add KH$_2$PO$_4$ buffer, DMSO, and test inhibitor at concentrations of 4 x IC$_{50}$

Enzyme concentrations: Add MAO-B [0.03 mg/ml]

Incubate for 15 min at 37 °C

Dialysis: Reactions were dialyzed at 4 °C in 80 ml outer buffer. The outer buffer was replaced at 3 h and 7 h after start of the dialysis.

After 24 h, the reactions were diluted 2-fold yielding test inhibitor concentrations of 2 x IC$_{50}$

Kynuramine substrate was added

Incubated further for 20 min at 37 °C

Terminate the reaction with the addition of 400 µl NaOH and 1000 µl distilled water

Measure 4-hydroxyquinoline concentrations spectrofluorometrically at an excitation wavelength of 310 nm and emission wavelength of 400 nm.

Reactions were done in triplicate and the residual enzyme catalytic rates expressed as mean ± SD. (R)-deprenyl, was included as a positive control.

**Figure 4.12**: Diagrammatic representation of the determination of reversibility of MAO-B, using the dialysis method.
4.5.4 Results and discussion

The results of the dialysis study showed that the inhibition of MAO-B by 10i was almost completely reversed after 24 h of dialysis, with the MAO-B activity recovering to levels of 83% of the control value (which is the MAO-B activity recorded in the absence of inhibitor). In contrast, MAO-B activity in undialyzed mixtures of the enzyme with 10i was only recovered to 18% of the control value. Pleasingly, this behaviour was consistent with reversible inhibition of MAO-B by 10i. For comparison, after similar preincubation and dialysis of mixtures of MAO-B with the irreversible inhibitor (R)-deprenyl the enzyme activity was not recovered with a residual enzyme activity of only 5% of the control values (figure 4.13).

**Figure 4.13:** Reversibility of inhibition of MAO-B by 10i. MAO-B and 10i were preincubated for a period of 15 min, dialyzed for 24 h and the residual enzyme activities were subsequently measured ([I] 2 x IC50 (Dialyzed)). For comparison, the MAOs were similarly preincubated in the absence ([I] 0 x IC50 (Dialyzed)) and presence of the irreversible inhibitor, (R)-deprenyl ([Depr] 2 x IC50 (Dialyzed)), respectively, and dialyzed. For comparison, the MAO-B activity of undialyzed mixtures ([I] 2 x IC50 (Not dialyzed)) of 10i and MAO-B is given.
4.6 MODE OF MAO INHIBITION

4.6.1 General background

In the previous sections, it was shown that inhibitor 10f acts as a reversible inhibitor of MAO-A and MAO-B. To determine whether this compound exhibited a competitive or non-competitive mode of inhibition (for this study a competitive mode of binding is preferred), Lineweaver-Burk plots for the inhibition of MAO-A and MAO-B by 10f was constructed. For a competitive inhibitor the lines of the Lineweaver-Burk plots should intersect on the y-axis. If the lines intersect on the x-axis, the test inhibitor would have a non-competitive mode of binding.

4.6.2 Materials and instrumentation

Materials and instrumentation were the same as in section 4.3.2.

4.6.3 Experimental method for construction of Lineweaver-Burk plots

Lineweaver–Burk plots were constructed for the inhibition of MAO-A and MAO-B. 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 concentrations of the test inhibitor that was selected for the studies with MAO-A and MAO-B were: 0, 0.95, 1.90 and 3.81 μM for MAO-A; and 0, 0.21, 0.42 and 0.83 μM for MAO-B. The enzyme concentration in these incubations was 0.015 mg protein/ml. The rates of formation of the MAO generated 4-hydroxyquinoline were measured by fluorescence spectrophotometry as described above. The Prism® version 5.0 software package was used to perform the linear and non-linear regression analyses (Petzer et al., 2012, Strydom et al., 2011).

Figures 4.14 and 4.15 illustrates the Lineweaver-Burk plots that were constructed in the absence and presence of various concentrations of the test inhibitor, compound 10f. The lines intersected on the y-axis, illustrating that the test inhibitor (10f) exhibited a competitive mode of binding to both MAO-A and MAO-B isoforms.
### 4.6.4 Results and discussion

**Figure 4.14:** A graph illustrating the Lineweaver-Burk plots of kynuramine oxidation by recombinant human MAO-B. The Lineweaver-Burk plots are constructed in the absence (blue diamonds) and presence of various concentrations of test inhibitor (10f). The red squares, green triangles, and purple dots represent 0.21 μM, 0.42 μM and 0.83 μM of the test inhibitor, respectively.

**Figure 4.15:** A graph illustrating the Lineweaver-Burk plots of kynuramine oxidation by recombinant human MAO-A. The Lineweaver-Burk plots are constructed in the absence (blue diamonds) and presence of various concentrations of test inhibitor (10f). The red squares, green triangles, and purple dots represent 0.95 μM, 1.90 μM and 3.81 μM of the test inhibitor, respectively.
4.7 TOXICOLOGY

4.7.1 General background

In this section, the toxicity of selected inhibitors, 10e, 10f and 10i, towards cultured cells was determined. For this purpose, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was employed. The cytotoxicity of the test compounds were evaluated at concentrations of 1 and 10 μM, and HeLa cells were used. In the MTT assay, a tetrazolium salt is metabolized by living cells to a formazan product, which has a purple colour. The concentration of the formazan product may subsequently be measured spectrophotometrically.

4.7.2 Materials and instrumentation

MTT and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich. The cell culture media (Dulbecco's Modified Eagle Medium; DMEM), fetal bovine serum (FBS), penicillin (10 000 units/ml)/streptomycin (10 mg/ml), fungizone (250 µg/ml) and trypsin/EDTA (0.25%/0.02%) were from Gibco, while the 24-well culture plates and 96-well spectrophotometric plates were from Corning. Syringe filters were obtained from Pallman.

A Multiscan RC UV/Vis platereader (from Labsystems) were used to measure the absorbances in 96-well microplates.

4.7.3 MTT cell viability assay

The literature protocol of Mosmann (1983) was used, for the cytotoxicity assay.

For the cell viability assay, both a negative (100% cell viability with no treatment, DMSO only) and a positive control (100% cell death, induced by 0.03% formic acid) were included. All compounds were screened in triplicate. Stock solutions of test compounds were prepared in DMSO and sterilized by filtration (using a 0.22 μm syringe filter).

Preparation of HeLa cells: 1 liter flasks were used to maintain the HeLa cells. The flasks also contained 30 ml DMEM, 10% fetal bovine serum, 1% penicillin (10 000 units/ml), streptomycin (10 mg/ml) and 0.1% fungizone (250 µg/ml). The cells were incubated at 37 °C in a 10% CO₂ atmosphere. Once the cells reached confluency, as determined by counting with a haemocytometer, they were detached using trypsin / EDTA (0.25%/0.02%, 3 ml). Cells were then seeded at 500 000 cells/well in 24-well plates, followed by a 24 hour incubation period. After incubation, the wells were rinsed with 0.5 ml DMEM containing no fetal bovine serum.
**MTT assay:** A volume of 0.99 ml DMEM (containing no fetal bovine serum) was subsequently added to each well, followed by the addition of 10 µl of the test compound. DMSO was used to prepare the stock solutions of the test drugs, and were prepared by sterilizing it through a 0.22 µm syringe filter (Pallman). For each 24-well plate, selected wells were reserved for the positive controls (100% cell death via lyses with 0.33% formic acid) and negative controls (0% cell death as a result of no treatment). The well-plates were incubated for another 24 h, where after the culture medium was aspirated. After washing each well with PBS (5ml) 200 µl of 0.5% MTT reagent, dissolved in PBS was added to each well. The well-plates were then incubated at 37 °C for 2 hours in the dark, followed by aspiration of the MTT-reagent. Isopropanol (250 µl) was added to each well to stop the reaction; and the well-plates incubated at room temperature for another 5 minutes to dissolve the purple formazan crystals. The isopropanol phase of each well (100 µl) was transferred to a 96-well plate. The absorbance of each 96-well was measured spectrophotometrically at 560 nm.

Equation 9 was used to determine the metabolic activity. The answer is expressed as a percentage of the negative control (100% cell viability).

\[
\frac{\% \text{ Viable Cells}}{100} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{positive control}}}{\text{Abs}_{\text{negative control}} - \text{Abs}_{\text{positive control}}}
\]

Where Abs = absorbance as read by the spectrophotometer, \(\text{Abs}_{\text{sample}}\) = absorbance from spectrophotometer of the sample, \(\text{Abs}_{\text{positive control}}\) = absorbance of the cells treated with 0.3% formic acid (100% cell death), \(\text{Abs}_{\text{negative control}}\) = absorbance of cells without treatment.

### 4.7.4 Results and discussion

The results of the MTT cell viability study are shown in table 4.3. The percentages given represent the percentage viable cells remaining after exposing the HeLa cells to the test compounds for 24 h. Thus, a high percentage indicates that a compound is relatively non-toxic at the tested concentration, and is preferred. As shown, the most potent MAO-B inhibitor of the series examined, compound 10i, was non-toxic at 1 and 10 µM, with 100% and 96% viable cells, respectively, remaining. Compound 10f, the most potent MAO-A inhibitor of the series unfortunately exhibited significant toxicity at 10 µM, with only 5% viable cells remaining. As expected, at the lower 1 µM concentration, a smaller degree of toxicity (77% cell viability) was observed. Compared to 10i, compound 10f is thus toxic to cultured HeLa cells.
Table 4.3: MTT assay results.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chalcone</th>
<th>% Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 µM</td>
</tr>
<tr>
<td>10i</td>
<td><img src="image1" alt="Structure" /></td>
<td>100%</td>
</tr>
<tr>
<td>10f</td>
<td><img src="image2" alt="Structure" /></td>
<td>77%</td>
</tr>
<tr>
<td>10e</td>
<td><img src="image3" alt="Structure" /></td>
<td>99%</td>
</tr>
</tbody>
</table>

To determine whether the pyrrole of phenyl substituents were responsible for the observed toxicity of 10f, 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% cell viability, obtained respectively. Since 10e was non-toxic, this result suggested that it was not the presence of the pyrrole substituent of 10f that was responsible for its higher degree of cytotoxicity, but the trifluoromethyl substituted phenyl ring. Further investigation in this regard is required to determine the mode of cytotoxicity and to investigate the possibility that 10f is metabolized to reactive intermediates or toxic products.

In the next section, the molecular docking of all compounds in the active site model of the hMAO-B enzyme will be discussed, in an attempt to rationalise the observed MAO-B inhibitory activities of these compounds.
4.8 MOLECULAR MODELING

4.8.1 Introduction

Molecular modeling can be used for the design of novel compounds for a specific receptor or enzyme target. Molecular modeling may also be used to determine important interactions between the ligand and active site and to determine possible binding modes of inhibitors to an active site.

4.8.2 Method

The Windows based Accelrys® Discovery Studio was used for the molecular docking studies and molecular docking was carried out with the CDOCKER module. The crystal structure of human MAO-B co-crystallized with carboxaldehyde coumarin (a MAO-B inhibitor) was obtained from the Protein Data Bank (PDB code: 2V60) and was used for the molecular modeling studies. The enzyme model was prepared by using the clean protein function, which corrects the structures and valences of amino acids, the carboxaldehyde coumarin and FAD. In addition, the protonation states at pH 7.4 were calculated. The enzyme model was typed with the CHARMM force field. A fixed atom constraint was applied to the backbone of the model and the model was minimized (Maximum steps 50000). For this purpose the generalized Born solvation model with molecular volume was used. The dielectric constant was set to 4. A binding sphere with a 10 Å radius was defined by using the existing ligand, the carboxaldehyde coumarin. The ligand was subsequently removed from the receptor. The selected inhibitors were drawn in Discovery Studio and prepared for docking. All waters of crystallization were removed, and the inhibitors were typed with the CHARMM Momany-Rone force field before docking. The reference ligand, the carboxaldehyde coumarin and the selected inhibitors were subsequently docked, allowing for a maximum of 10 conformers. The CDOCKER interaction energies were recorded and orientation of the 10 conformers of each ligand was evaluated. Afterwards in situ ligand minimization was performed on the selected conformers.

4.8.3 Results and discussion

All compounds were successfully docked into the crystal structure model of human MAO-B using Discovery Studio 3.1. The docking results are given in table 4.4, and all figures are included in the addendum.
From the docking results the following observation were made:

- As discussed previously, the active site of MAO-B consists of substrate and entrance cavities, which are separated by the gating switch, residues Ile 199 and Tyr 326. The substrate cavity contains Tyr 435, Tyr 398 and Gln 206, while the entrance cavity contains Pro 102. On the left-hand side of figure 4.16, selected amino acids present in these cavities are illustrated, and it is shown that all compounds are docked into both the substrate as well as entrance cavities.

- From the docking results it is 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.

- The binding energies were determined by Discovery Studio 3.1. In this case, the binding energy was only a crude predictor of the enzyme inhibitor activity. It may be concluded from the results (table 4.4) that a binding energy higher than -30.000 kcal/mol results in IC₅₀ values greater than 1 µM, thus higher binding energies result in poorer MAO-B inhibitory potency. For example, compound 10b, which had the highest binding energy also possessed the lowest MAO-B inhibitory potency. Similarly, the binding energy of compound 10g, was also higher than -30.000 kcal/mol and also was a relatively weak MAO-B inhibitor (table 4.4).

- Among the compounds evaluated was the previously synthesized compound 8. The docking results indicated that this compound is orientated with the hydroxyl-methoxyphenyl ring facing the FAD cofactor. This result correlates with the docking results of Chimenti et al. (2009). Our results, however, indicated a difference in interactions from Chimenti et al. (2009), with compound 8 undergoing a Pi-Pi interaction as well as two hydrogen bond interactions. The orientation of compound 8 correlated with the structurally similar compound 10d whose hydroxyl-methoxyphenyl ring also faces the FAD cofactor. Interestingly both compound 8 and 10h formed hydrogen bonds with Cys 172.

- The results also indicated that all the compounds, with the exception of 10b and 10e, formed a Pi-Pi interaction with Tyr 398. This interaction is most likely important for inhibitor binding to MAO-B.
Table 4.4: The results of the docking experiments and the IC$_{50}$ values of the selected inhibitors for the inhibition of MAO-B.

<table>
<thead>
<tr>
<th>Name</th>
<th>Orientation of compound: Which moiety faces the FAD</th>
<th>Interactions</th>
<th>Binding energy (kcal/mol)</th>
<th>MAO-B IC$_{50}$(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hydrogen bonds</td>
<td>Pi-Sigma interactions</td>
<td>Pi-Pi interactions</td>
</tr>
<tr>
<td>10a</td>
<td>Phenyl moiety</td>
<td>Tyr 398 and Phenyl moiety</td>
<td>-47.7386</td>
<td>0.133</td>
</tr>
<tr>
<td>10b</td>
<td>Phenyl moiety</td>
<td>Tyr 326 and Pyrrole moiety</td>
<td>-22.7799</td>
<td>3.274</td>
</tr>
<tr>
<td>10c</td>
<td>Methoxypyridine moiety</td>
<td>Tyr 398 and Pyridine moiety</td>
<td>-40.3116</td>
<td>0.330</td>
</tr>
<tr>
<td>10d</td>
<td>Hydroxy-methoxyphenyl moiety</td>
<td>Tyr 398 and Hydroxy-methoxyphenyl moiety</td>
<td>-40.6882</td>
<td>0.185</td>
</tr>
<tr>
<td>10e</td>
<td>Pyrrole moiety</td>
<td>Gln 206 and Pyrrole moiety</td>
<td>-31.052</td>
<td>0.803</td>
</tr>
<tr>
<td>10f</td>
<td>Phenyl moiety</td>
<td>Tyr 326 and Pyrrole moiety</td>
<td>-38.2236</td>
<td>0.830</td>
</tr>
<tr>
<td>10g</td>
<td>Pyrrole moiety</td>
<td>Ile 199 and phenyl moiety</td>
<td>-26.8939</td>
<td>1.396</td>
</tr>
<tr>
<td>10h</td>
<td>Thiophene moiety</td>
<td>Cys 172 and Carbonyl</td>
<td>-46.3103</td>
<td>0.116</td>
</tr>
<tr>
<td>10i</td>
<td>Phenyl moiety</td>
<td>Ile 199 and pyrrole moiety</td>
<td>-38.694</td>
<td>0.067</td>
</tr>
<tr>
<td>8</td>
<td>Hydroxy-methoxyphenyl moiety</td>
<td>Cys 172 and Carbonyl; Tyr 435 and Hydroxy-methoxyphenyl moiety</td>
<td>-46.4928</td>
<td>0.093</td>
</tr>
</tbody>
</table>
Figure 4.16: A figure illustrating compounds 10e, 10f, 10i and 10g docked into the crystal structure of human MAO-B. The figures on the left illustrate selected residues surrounding the ligands, while the figures on the right illustrate only the residues with whom the ligands interacted. The hydrogen atoms are hidden for clarity. The FAD and ligands are displayed in stick, while the interacting residues are displayed in wire frame.

The docking results of selected compounds 10i, 10e and 10f are illustrated in figure 4.16. Since the reversibility of MAO inhibition by these compounds were evaluated, their docking results will be discussed in more detail. The docking results may provide some insight into the MAO inhibitory...
activities of these compounds. Compound 10i was the most potent MAO-B inhibitor and was shown to be a reversible MAO-B inhibitor, although it exhibited tight binding to MAO-B. Compound 10f was the most potent MAO-A inhibitor and was shown to be a reversible inhibitor of both MAO isoforms, while compound 10e was shown to be a reversible MAO-B inhibitor. The results of the docking study for compound 10g are similar to those of compound 10i, and thus is included in this discussion.

- Compound 10f had a binding energy of -38.2236 kcal/mol and an IC$_{50}$ value of 0.830 µM. It was orientated with the phenyl moiety facing the FAD cofactor. A Pi-Pi interaction between the phenyl ring and Tyr 398 as well as a hydrogen bond interaction between the pyrrole moiety and Tyr 326 were observed for this compound.

- The structurally similar compound 10e, in contrast was orientated with the pyrrole moiety facing the FAD co-factor. The phenyl ring of 10e forms a Pi-Pi interaction with Tyr 326. A hydrogen bond interaction is further present between the heterocyclic pyrrole ring and Gln 206. This compound had a binding energy of -31.052 kcal/mol and an IC$_{50}$ value of 0.803 µM.

- The most potent MAO-B inhibitor of the series, compound 10i, docked into the MAO-B active site with the phenyl ring facing the FAD cofactor. The phenyl ring of 10i forms a Pi-Pi interaction with Tyr 398 and a Pi-Sigma interaction between the thiophene ring and Ile 199 (which is part of the gating switch of MAO-B) is further observed. It is speculated that the tight binding component of MOA-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. This compound had a binding energy of -38.694 kcal/mol and an IC$_{50}$ value of 0.067 µM. Although 10i has the most potent MAO-B inhibitory activity, it does not have the lowest binding energy of the series. Compound 10a displayed the lowest binding energy of -47.7386 kcal/mol although exhibiting a lower potency IC$_{50}$ value of 0.133 µM. It may thus be concluded that the potency of an MAO-B inhibitor is only partly dependent on the binding energy of the inhibitor enzyme complex.

- The only other compound that exhibited a Pi-Sigma bond interaction with the gating switch Ile 199, was compound 10g. This compound is orientated differently than 10i, with the pyrrole moiety now facing the FAD cofactor. A Pi-Sigma interaction is present between the phenyl moiety and Ile 199. The pyrrole ring of 10g also forms a Pi-Pi interaction with Tyr 398. Although this compound showed the same type of interactions as compound 10i, it was considerably less potent than 10i as a MAO-B inhibitor (IC$_{50}$ 1.396 µM) and has a
much larger binding energy (-26.8939 kcal/mol). Unfortunately, the reversibility of binding of this compound was not assessed.

In conclusion, 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.

4.9 SUMMARY

In this chapter the *in vitro* biological evaluation of the novel chalcones were discussed. The MAO inhibitory potential of all compounds were determined with a fluorometric assay using kynuramine as substrate. Reversibility of binding was determined for compounds 10i, 10e and 10f. To determine the mode of binding, kinetic analysis of the MAO inhibition by 10f were done for both the MAO isoforms. A MTT cell viability assay was used to examine the cytotoxicity of compounds 10i, 10e and 10f, at concentrations of 1 and 10 µM. All compounds were successfully docked into a crystal structure model of human MAO-B using Discovery Studio 3.1.

In general the chalcone derivatives examined in this dissertation are potent, selective, reversible and competitive inhibitors of the MAO-B isoform and mostly non-toxic.