Identification of novel scaffolds for Monoamine oxidase B inhibitors

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By
Hasanain Odhar
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Thesis written by

Hasanain Odhar

B.S., University of Baghdad, 2007

M.S., Kent State University, 2014

Approved by

__________________________, Chair, Master’s Thesis Committee

__________________________, Member, Master’s Thesis Committee

__________________________, Member, Master’s Thesis Committee

__________________________, Director, School of Biomedical Sciences

__________________________, Dean, College of Arts and Sciences
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Chapter one: Introduction

1.1- Parkinson's disease (PD)

It is an age related neurodegenerative disease that affects about one million persons in the United States [1]. The prevalence of Parkinson's disease usually increases from 1-2% at age 50 to about 5% at age 85 [2, 3]. Pathologically the disease is characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta, this depletion of dopaminergic neurons will result in four major motor related hallmarks including tremor, rigidity, bradykinesia and postural imbalance [4]. These manifestations usually appear when about 60-70% of dopaminergic neurons are lost [5]. Non-motor symptoms (like depression and dementia) may also appear as the disease progress over time [6]. Another characteristic pathological feature of Parkinson's disease is the presence of proteinaceous inclusions called Lewy bodies within the surviving dopaminergic neurons, these inclusions consist mainly of α-synuclein which is highly ubiquitinated [7]. Although the exact cause of Parkinson's disease remains unclear, several factors may play a role in increasing susceptibility to develop the disease. These factors include: age, environmental factors (ex: Paraquat PQ) [8, 9] and genetic factors (autosomal dominant and recessive genes like α-synuclein, parkin, DJ-1, LRRK2) [10].

Current therapeutic approach focuses on symptoms attenuation only [11] through dopamine replacement therapy by using either L-dopa (the immediate
metabolic precursor of dopamine) or dopamine receptor agonist [12, 13]. Dopamine replacement therapy does not halt or slow down the pathological degeneration process and as the disease is advancing, the efficiency of such therapeutic approach is falling down [14]. In this situation, higher medication dose is required to maintain adequate therapeutic benefit. Such decision is usually restricted by the fact that the tolerated levodopa dose is limited by several side effects associated with long term therapy such as dyskinesia and behavioral disturbances [15, 16]. Drugs that can lower L-dopa dose and/or delay the need for L-dopa are considered essential for Parkinson's disease therapy approach [17, 18]. These adjunct drugs involve the following categories: Monoamine oxidase B (MAO-B) inhibitors, Catechol-O-methyltransferase inhibitors and amantadine [11]. No drug with neuroprotective activity has been approved by FDA for Parkinson's disease treatment till this point [19, 20].

Parkinson's disease is a complex neurologic condition with multiple underlying etiological pathways and treating such disorder with a molecule that target a single pathway is inefficient, a drug molecule that target different pathways seems to be more convenient [21-28]. A possible candidate for such multi-targeted drug design is MAO-B inhibitors, by inhibiting MAO-B enzyme; these compounds can elevate the level of both endogenous and exogenous dopamine [29, 30]. By blocking the activity of MAO-B enzyme, these compounds can also lower the production of hydrogen peroxide (a byproduct of oxidative deamination reaction) and by this way oxidative stress can be minimized [31]. The rationale for using MAO-B inhibitors as a basic model for the development of a new antiparkinsonian therapy is encouraged by several facts. The first
one is that the activity level of MAO-B enzyme inside the brain increases with age [32-34] while MAO-A level is not affected by age [87]. Also in patients with Parkinson's disease, the level of MAO-B is elevated as a result of gliosis. Finally, the activity level of MAO-B in human basal ganglia is higher than that of MAO-A and since both isozymes metabolize dopamine with the same level of efficiency, then it is more reasonable to target MAO-B and not MAO-A [36].

1.2- **Monoamine oxidase enzyme (MAO)**

MAO is an outer mitochondrial membrane bound flavin containing enzyme with a molecular weight of about 60 kDa, it plays an important role in the metabolism of endogenous and exogenous amines [35, 45]. Intraneuronal MAO terminates the action of endogenous amine neurotransmitters, regulates intraneuronal amine stores and protects cells from dietary amines (false transmitters) [36].

This enzyme exists in two isoforms (MAO-A) and (MAO-B), these isoforms shares about 70% sequence identity and represent separate X chromosome related gene products [37]. These isoforms have different substrate specificities in that MAO-A catalyzes the oxidation of serotonin (5-HT) while MAO-B catalyzes the oxidation of both benzylamine and 2-phenylethylamine. Other substrates like dopamine, epinephrine, norepinephrine, tyramine and tryptamine are oxidized by both isoforms [38]. During embryonic development, MAO-A is expressed first but the expression level of MAO-B inside the brain is increased dramatically after birth [32, 39, 40]. MAO enzyme level of activity is different among various regions of human brain, where the hypothalamus and
basal ganglia show the highest activity while cerebellum and neocortex show the lowest activity [41]. Radio labeled inhibitors were used to identify the distribution of MAO isozymes throughout the brain, it was found that basal ganglia contains mainly MAO-B [42].

Immunohistochemical analysis reveals that serotonergic neurons contain predominantly MAO-B isoform while catecholaminergic neurons contain MAO-A mainly [43]. These immunohistochemical analysis data would suggest two conclusions: first, the role of MAO-B in serotonergic neurons is to eliminate any foreign amines and prevent them from reaching the synaptic cleft while the second conclusion is that MAO-A in glial cells should be responsible for degradation of serotonin in brain regions rich in serotonergic neurons. Noradrenergic neurons contain MAO-A and MAO-B, both isoforms can catalyze the oxidative deamination of norepinephrine efficiently but because the uptake of norepinephrine into synaptic vesicle is strongly favorable over binding to MAO therefore the substrate can escape metabolic degradation to some extent [44].

1.3- Structure of MAO-B

MAO-B enzyme consists mainly from protein (520 amino acids) and FAD (redox cofactor), it crystallizes as a dimer and each monomer involves the following subunits: membrane binding domain, substrate binding domain and flavin binding domain [46, 47]. Figure 1.1 shows a three dimensional representation for human MAO-B monomer. MAO-B undergoes post translational modification (acetylation) where the amino terminal methionine is cleaved and the resultant N-terminal serine is acetylated [48, 49].
Figure 1.1- Three dimensional structure of human MAO-B enzyme (monomer A). The membrane binding domain is in green color (residues 489–500), the substrate binding domain is in red color (residues 80–210, 286–390 and 454–488) and the flavin binding domain is in blue color (residues 4–79, 211–285 and 391–453). This figure was generated by using PyMOL v.1.3 (www.schroedingar.com) [154] and human MAO-B crystal with the code 2BK3; the sequences of amino acids for the designated domains were obtained from reference [55].
1.3.1- **membrane binding domain**

C-terminal truncation experiments together with sequence analysis had suggested that the C-terminal region can function as the membrane binding domain (C-terminal α helix), this 27 residue transmembrane helix has apolar surface which would encourage its insertion into mitochondrial membrane [50, 51]. Truncation experiments showed that C-terminal truncated MAO-B can retain some affinity towards membranes but not as strong or as specific as intact enzyme and this would suggest the presence of other membrane binding regions. The electrostatic surface maps show positively charged domains that can form electrostatic interactions with the anionic membrane surface [54].

1.3.2- **Substrate binding domain**

In order to access covalent FAD and undergo metabolism, substrates must cross a specific path with a distance of about 20 Å [52]. The journey starts by negotiation with a flexible loop (residue 99-112) which function as a gating switch at the surface of outer mitochondrial membrane, this loop must be moved so that the substrate can reach active site cavities [53]. The substrate then must pass through two hydrophobic cavities; the first one is called the entrance cavity with a volume of ~290 Å³, the second larger cavity is called the substrate cavity with a volume of ~400 Å³ [47, 54]. These two cavities are separated by an Isoleucine 199 side chain which can function as a gate; depending on the nature of the substrate, the Isoleucine (Ile) 199 side chain can exhibit either an open conformation (fuse both cavities) or closed conformation (separate both cavities) [53].
Tyr326 residue also plays a role in separating the active site cavities of MAO-B; the side chain of Tyr326 undergoes conformational changes upon substrate binding [72].

The outer mitochondrial membrane is covered with negative charges and these charges will attract amine substrates (positively charged) into MAO-B enzyme [52]. FAD cofactor is located at the end of substrate cavity where it is covalently bound to Cysteine 397 through thioether linkage [46].

1.3.3- **Flavin binding domain**

Flavin adenine dinucleotide (FAD) is considered as an essential entity for MAO enzyme catalytic activity (redox cofactor); it is bound covalently to MAO-B apoenzyme through a thioether linkage which is formed between cysteine 397 residue and the 8α-methylene of isoalloxazine ring [46, 37]. This binding is located near the C-terminus of MAO enzyme [55].

FAD coenzyme is incorporated in an extended conformation into a hydrophobic environment within MAO-B, specific interactions are found between FAD and MAO-B apoenzyme [55]. These interactions are mainly hydrogen bonds between FAD and either amino acids side chains or amide linkages in the protein. Mutagenesis experiments had shown that if Arg42 was replaced by Ala or Lys then this would adversely influence FAD incorporation into MAO-B, These results showed the requirement of electrostatic interaction between anionic pyrophosphate group of FAD and the positively charged guanidino group of Arg42 [56]. The amino acid residues that interact with FAD in MAO-
B enzyme are conserved in MAO-A enzyme and this suggests that FAD binding site is identical in both enzymes [55]. The only difference between these two enzymes is that Ser59 is replaced with Ala in MAO-A and since the peptide bond (not amino acid side chain) at this position is involved in hydrogen bonding with the flavin C-4 carbonyl oxygen, then such amino acid substitution is not thought to cause a significant difference in FAD binding domain between the these two isozymes. Flavin coenzyme analogs analysis had shown that both MAO-A and MAO-B have a similar FAD incorporation mechanism and a similar FAD binding sit [59, 60]. Analogs analysis results also provide additional support for covalent flavinylation mechanism (quinone methide); it is believed that both MAO-A and MAO-B follow this mechanism for FAD incorporation [61].

Structural data, obtained through diffraction analysis, had shown that the amide bond between Cys397 and Tyr398 is in cis rather than the favorable trans configuration [53]. This energetically unfavorable configuration (cis configuration) of the peptide bond is necessary to make the phenolic side chain of Tyr398 in a perpendicular orientation to the flavin ring, this orientation is essential for catalytic activity (aromatic cage) [58]. Two main steric constraints that affect flavin reactivity had been recognized, first one is the bending about (N5)-(N10) axis of isoalloxazine ring so that it will deviate 30° from normal planar conformation while the second one is being puckered about the pyrimidine portion of the ring. These steric deformations will make the reactive sites of flavin ring (N-5 and C-4) assuming a more sp3 rather than a planar sp2 configuration and this will enhance MAO enzyme reactivity [53].
To determine the effect of covalent binding of FAD coenzyme on the activity and
general stability of MAO enzyme, two mutagenesis experiments were performed. First
experiment had shown that replacement of Cys397 with Ser would result in an inactive
enzyme, it is clear that the hydroxyl group of serine does not have enough nucleophilicity
to form covalent bond with FAD [62]. The second experiment involved the expression of
Cys406Ala mutant MAO-A in riboflavin dependent strain of S. cerevisiae. Mutant MAO-
A was expressed in the absence of riboflavin and it did not show any activity until FAD
was added to the medium. The level of MAO-A activity was dependent on the
concentration of FAD added. This experiment suggested that enzyme activity is not
absolutely dependent on covalent FAD linkage and it also showed the importance of this
covalent linkage in structural stabilization of the enzyme against detergents solubilization
[63].

1.3.4- Structural comparison between human MAO-B and human MAO-A

The most important structural difference between these two isozymes is that
human MAO-B crystallizes as a dimer while human MAO-A crystallizes as a monomer
[64, 47, 58]. It was suggested that in human MAO-A, a Glu-151 is replaced by a Lys
residue. This selective mutation at the dimeric interface may be responsible for
destabilization of the dimeric form in human MAO-A [65]. This shift in the oligomeric
state of human MAO-A mainly affects the temperature stability of the enzyme; it was
found that rat MAO-A (dimer) is more thermally stable than human MAO-A (monomer)
[66]. Dipole moment calculations show a significant change in the direction of
monomeric MAO-B dipole moment upon dimerization. In dimeric MAO-B, the orientation of positively charged end is directed towards the anionic membrane surface which will provide addition interaction. Such alteration in dipole direction is less pronounced in MAO-A [54]. New results of distance measurements by pulsed EPR-DEER (electron paramagnetic resonance-double electron-electron resonance) experiments showed that human MAO-A is present in dimeric form in the mitochondrial membrane, it is believed that solubilization of human MAO-A by using detergent will result in a mixture of both monomeric and dimeric forms of human MAO-A and the monomeric form will be crystallized more readily [71]

Other significant differences are also present between human MAO-B and human MAO-A regarding active site shape and volume. The active site in human MAO-B is bipartite with a total volume of ~700Å³ (entrance cavity: 290Å³ and substrate cavity: ~400Å³) while in human MAO-A, the active site is monopartite with a volume of ~550Å³ [67, 64]. The shape of active site in human MAO-B is longer and narrower than that of human MAO-A. This difference in active site shape and volume may be in part as a result of variation in the conformation of cavity shaping loop, this six residue loop is present in a more compact conformation in human MAO-B as compared with human MAO-A [64, 68]. Active site in both isozymes also show important amino acid replacements for example human MAO-A contains Phe-208 (Ile-199 in human MAO-B) and Ile-335 (Tyr-326 in human MAO-B) [64]. A combination of both conformational changes and residue replacements affect the shape and size of the active site and this in turn will modify the substrate specificity of the isozyme.
Rat MAO-A is about 92% identical with human MAO-A in terms of sequence identity [64]. Rat MAO-A crystallizes as a dimer with active site cavity volume of about 450 Å³, its cavity shaping loop conformation is very similar to that of human MAO-B [64, 70]. These structural data about rat and human MAO-A would suggest a possible relationship between dimer formation and the conformation of cavity shaping loop through a long range effect [64]. Such long range effect hypothesis had been observed in a number of other enzymes like dihydrofolate reductase [69]. Kinetic studies showed that rat MAO-A metabolizes human MAO-B substrates (like benzylamine) more efficiently than does human MAO-A [66]. Based on the facts mentioned above, the approach of using rat MAO-A as a laboratory model for the development of human MAO-A specific inhibitor should be re-evaluated [64].

1.4- Reaction mechanism of MAO enzyme

The enzyme catalyzes the oxidative deamination of primary, secondary and some tertiary amines, this reaction will result in the formation of hydrogen peroxide, an aldehyde and either ammonia (if the substrate is primary amine) or substituted amine (if the substrate is secondary amine) [36, 52]. For MAO-B, the reaction pathway depends on the type of substrate. In this regard the oxidation of benzylamine follows ternary complex mechanism where the imine product dissociates from the oxidized enzyme. On the other hand the binary complex mechanism is usually followed by phenethylamine and in this case the imine product will dissociate from reduced enzyme before oxidation of the reduced enzyme-imine complex takes place [73, 74]. Figure 1.2 shows the catalytic
reaction pathway for MAO enzyme. The existence of these two pathways (binary and ternary) depends on the relationship between the rate of imine dissociation from reduced enzyme and the rate of reduced enzyme-imine complex oxidation [75]. For ternary complex mechanism (benzylamine), the oxidation rate of reduced enzyme-imine complex is faster than the rate of imine dissociation from reduced enzyme. In binary complex mechanism (phenethylamine), the rate of imine dissociation is faster than the rate of reduced enzyme-imine complex oxidation [73, 75]. Catalysis pathway for MAO-A had been found to follow ternary complex mechanism only [76].
Figure 1.2 - A schematic representation for catalytic reaction pathway followed by MAO enzyme. This figure was adapted from reference [52].
MAO enzyme catalytic mechanism involves the cleavage of Cα-H bond in amine substrate with the transfer of two reducing equivalents to the enzyme (flavin ring) and this will result in formation of imine product and flavin hydroquinone [68]. It is believed that the amine substrate should be in the deprotonated form so that it can enter the active site and undergo metabolism, however the exact mechanism of substrate deprotonation at physiological condition is not known yet [68, 77, 86]. Kinetic isotope studies had demonstrated that Cα-H bond cleavage is the rate limiting step in oxidative deamination reaction [77, 78]. Stereochemical analysis showed that only pro-R hydrogen can be transferred in this catalytic reaction (undergoes abstraction) [73, 79, 80]. The abstraction of pro-R H can happen in one of three possibilities: as a proton, a hydride ion or a hydrogen atom. To identify hydrogen form that undergo abstraction, the effect of electron withdrawing group in para substituted substrate on reaction rate was tested. It was found that increasing electron withdrawing power will increase reaction rate and this fact supports the possibility of proton abstraction [77, 78].

Two hypotheses had been suggested for the transfer of reducing equivalents in MAO catalyzed reaction. The first and more acceptable hypothesis is called polar nucleophilic mechanism. In this mechanism, substrate amine will attack flavin C4a and this will form flavin-substrate adduct. Once this adduct is formed, N5 position of flavin ring becomes a strong base that can abstract proton from Cα [77]. Figure 1.3 shows a schematic representation for polar nucleophilic mechanism of MAO catalysis. NMR studies had observed that the pKa value of N5 proton for flavin hydroquinone to be ~24 and this support the idea of N5 sufficient basicity to perform abstraction [81].
mentioned previously in FAD binding domain structure, the isoalloxazine ring is in a bent conformation about (N5-N10) axis which deviates about 30° from normal planarity [53]. This bending will make N5 atom closer to sp3 than sp2 conformation and this will lead to a higher electron density at N5 position (better nucleophile) and a lower electron density at C4a position (better electrophile). This will facilitate the nucleophilic attack by a suitable substrate at C4a position [68].

The other and less acceptable hypothesis is called single electron transfer (SET) mechanism; this mechanism involves one electron oxidation of substrate amine nitrogen leading to the formation of aminium cationic radical and flavin anionic radical. The production of aminium radical will lower pKa value of Cα-H and this will facilitate proton abstraction by a base in the active site of MAO enzyme [82-84]. Figure 1.4 shows a schematic picture for single electron transfer hypothesis. Different structural data showed that no amino acid within MAO active site can function as an active base or acid [52]. Experimental monitoring failed to identify intermediate radical and thus it is difficult to provide any support for SET hypothesis [85]. The formation of aminium cationic radical is expected to interact with tyrosine residues in aromatic cage (π-cationic interaction) and this can halt any further reaction [52, 75].
Figure 1.3- A schematic representation for the proposed polar nucleophilic mechanism of MAO enzyme catalysis. This figure was adapted from reference [52].
Figure 1.4- A schematic representation for the proposed single electron transfer (SET) mechanism of MAO catalysis. This schematic figure was adapted from reference [52].
1.5- Functional role of aromatic cage

Previous structural analyses had observed that the active site of MAO enzyme contains two tyrosine residues (Tyr398 and Tyr435 in MAO-B), these residues are situated in front of the re face of flavin ring and they exhibit an approximately perpendicular conformation relative to the flavin ring. The phenolic side chains of these two residues are separated from each other by about 7.8Å [47]. A number of mutagenesis analyses targeting Tyr435 in MAO-B were performed to identify possible functional roles of the aromatic cage. Targeting Tyr398 in these analyses was excluded because this residue is involved in a cis bonding with Cys397 which binds covalently through thioether linkage with flavin. It is believed that any change in Tyr398 may adversely affect covalent flavin binding [68, 88].

The aromatic cage does not seem to perform any important structural role within the active site of MAO enzyme but mutant enzymes show lower stability upon solubilization by detergents as compared to wild enzyme [88]. Other flavin dependent amine oxidase enzymes contain aromatic amino acids in a conformation similar to that observed in the aromatic cage of MAO enzyme and this observation suggests a possible functional role for aromatic cage in catalytic activity [89, 90]. One of the expected roles of aromatic cage is to polarize the electron single pair of substrate amine and this will enhance substrate nucleophilicity. As mentioned above, the distance between phenolic side chains of aromatic cage was calculated to be 7.8Å which is believed to be optimal for substrate amine polarization [88]. Aromatic cage may also play a steric role through
dipole moment effect of Tyrosine side chain. These dipolar effects can simulate the presence of electron withdrawing group at para position of substrate leading to a reduction in the distance between amine nitrogen and flavin C4a, this will facilitate the transfer of charge from amine to flavin and encourage the formation of flavin C4a adduct [91]. Figure 1.5 shows us a schematic representation for the role of aromatic cage in substrate oxidation.

The dipole effect of aromatic cage may also contribute to substrate specificity of MAO-B, it is suggested that using a substrate having a dipole moment in the same direction as do the aromatic cage dipoles will result in repulsive interaction and a change in substrate orientation within active site cavity [88]. All these facts about aromatic cage structure and function strengthen polar nucleophilic mechanism of MAO-B catalysis [88].
Figure 1.5- A schematic representation for the effect of dipole moments of Tyr398 and Tyr435 residues in MAO-B on the electron lone pair of the substrate amine before the nucleophilic attack takes place. This figure was adapted from reference [88].

1.6- **MAO inhibitors with a possible or approved clinical application**

A summary about these chemicals is shown in the below table (table 1.1) which was adapted from reference [134].
Table 1.1- A brief summary about MAO-inhibitors with a possible or approved clinical application.

<table>
<thead>
<tr>
<th>MAO inhibitor</th>
<th>Selectivity</th>
<th>Binding</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenelzine</td>
<td>MAO-A/MAO-B</td>
<td>Irreversible, covalent.</td>
<td>Antidepressant and anxiolytic.</td>
</tr>
<tr>
<td>Tranylcypromine</td>
<td>MAO-A/MAO-B</td>
<td>Irreversible, covalent.</td>
<td>Antidepressant and anxiolytic.</td>
</tr>
<tr>
<td>Selegiline (L-deprenyl)</td>
<td>MAO-B</td>
<td>Irreversible, covalent.</td>
<td>Antiparkinsonian</td>
</tr>
<tr>
<td>Rasagiline</td>
<td>MAO-B</td>
<td>Irreversible, covalent.</td>
<td>Antiparkinsonian</td>
</tr>
<tr>
<td>Lazabemide</td>
<td>MAO-B</td>
<td>Reversible, covalent.</td>
<td>Development as antiparkinsonian was discontinued due to toxicity issues.</td>
</tr>
<tr>
<td>Safinamid</td>
<td>MAO-B</td>
<td>Reversible, non-covalent.</td>
<td>Currently undergo phase III clinical trials as antiparkinsonian.</td>
</tr>
<tr>
<td>Zonisamide</td>
<td>MAO-B</td>
<td>Reversible, non-covalent.</td>
<td>Antiepileptic and it is approved in Japan as antiparkinsonian.</td>
</tr>
<tr>
<td>Moclobemide</td>
<td>MAO-A</td>
<td>Reversible, non-covalent.</td>
<td>Antidepressant and anxiolytic. It is not approved in USA.</td>
</tr>
</tbody>
</table>
1.7- MAO-B inhibitors with a possible disease modifying effect

Traditional drug design approach focuses on a single target within disease pathologic pathway; this discovery tactic is based on the idea of minimizing adverse effects by designing a highly selective ligand that will avoid interaction with other unwanted biological targets (cross reactivity) [22, 23]. Such method for drug design and discovery proved to be incompetent when dealing with diseases that involve complex pathological picture; a good example for such conditions is the neurodegenerative diseases. New attempts, to address an effective therapeutic approach for neurodegenerative diseases, concentrate on designing a ligand that targets multiple abnormalities within disease pathway. This novel pharmaceutical instrument shows some successful results regarding disease process modification with no significant elevation in the incidence of side effects [21, 92]. Below we will mention some compounds that were designed using designed multiple ligand approach.

A- Rasagiline:

It is an irreversible and selective inhibitor of MAO-B [93]. It was approved by FDA for Parkinson's disease treatment in 2006 either as an early monotherapy or as an adjunct treatment to levodopa [11]. Rasagiline exhibits a disease modifying capability when given in a dose of 1 mg/ day [94]. It seems that the propargylamine moiety is responsible for the neuroprotective feature of rasagiline; it was observed that the S-enantiomer is more than 1000 times weaker than rasagiline (R-enantiomer) as a MAO inhibitor however the S-enantiomer is still able to show neuroprotective activity [95-98].
The main metabolite of rasagiline (aminoindan) also shows neuroprotective effect in vitro studies [98, 99].

The neuroprotective mechanism of propargylamine moiety depends on defending the mitochondria through several means like activation of protein kinase C (PKC), Bcl-2, phosphoinositide kinase 3 and mitogen activated protein kinase (MAPK). It also down regulates pro-apoptotic proteins like Bax, Bad and Fas [93]. Rasagiline also stimulates the liberation of the soluble form of amyloid precursor protein α (sAPPα) through PKC-MAP mediated activation of α-secretase [27, 28]. Rasagiline seems to possess neurorescuing ability through induction of brain derived neurotrophic factors (BDNF) and glial derived neurotrophic factors (GDNF) [100, 155].

B- Ladostigil:

This drug was designed by combining the propargylamine moiety of rasagiline with the carbamate cholinesterase inhibitory moiety of rivastigmine. The resultant drug can inhibit acetylcholine esterase, butyrylcholine esterase, MAO-A and MAO-B [101]. The drug shows a neuroprotective effect similar to that of rasagiline [101, 102]. Ladostigil also shows antidepressant activity through inhibition of MAO-A inside the brain leading to an elevation in the level of dopamine, norepinephrine and serotonin [108]. This compound is believed to be effective in the treatment of Alzheimer's disease and depression.
C- HLA20:

This lead compound was prepared by combining the antioxidant chelator moiety of 8-hydroxyquinoline derivative of the iron chelator VK-28 with the propargylamine moiety of rasagiline or selegiline [103-105]. This compound exhibits a moderate selectivity for MAO-B enzyme and also acts as a free radical scavenger. The rationale behind combining iron chelation effect with MAO-B inhibition emerges from the fact that brain level of iron and MAO-B increases with age and it also increases in neurodegenerative diseases as a result of gliosis. The rise in MAO-B level will elevate the level of oxidative deamination leading to an increase in hydrogen peroxide production as a byproduct. The resultant hydrogen peroxide will undergo Fenton reaction in the presence of elevated iron content leading to exaggerated hydroxyl radical level and inevitable oxidative stress [95, 106].

Based on this scenario M30 compound was introduced, this compound is a potent inhibitor of MAO-A and MAO-B (inhibits both of them equally) and it has iron chelation characteristics similar to desferoxamine. The neuroprotection features of M30 are like those of rasagiline and ladostigil [103, 107].

D- Istradefylline (KW-6002):

It is MAO-B enzyme inhibitor and Adenosine A2a receptor blocker [92]. Adenosine A2a receptor is a G-protein coupled receptor which will stimulate adenylate cyclase upon activation leading to an increase in cAMP level [109]. This receptor subtype
is found mainly in the striatum (GABAergic striatopallidal neurons) where it is co-localized with dopamine D2 receptor [110-112]. It is believed that activation of adenosine A2a receptor can produce a functional antagonistic effect on dopamine D2 receptor through reduction in G-protein coupling activity of dopamine D2 receptor and lowering the binding affinity of dopamine D2 agonist [113, 114]. Because both dopamine D2 receptor and adenosine A2a receptor are coupled to adenylate cyclase and have some similarities in signaling system, it is possible that the functional antagonistic effect can also be mediated at the downstream of signaling pathway (second messenger level) [115].

Based on the localization of adenosine A2a receptor and their functional relationship with dopamine D2 receptor, it is obvious that the blockade of adenosine A2a receptor can be used as a non-dopaminergic approach to treat Parkinson's disease. Blockade of adenosine A2a receptor can improve motor activity in animal models of Parkinson's disease through strengthening dopamine D2 neurotransmission [116-118]. Some studies showed that adenosine A2a receptor antagonism can also produce a beneficial motor effect in dopamine D2 receptor knockout mice suggesting that adenosine A2a receptor antagonism can produce such effect through dopamine D2 receptor independent pathway [119].

Many epidemiological studies had shown that blockade of adenosine A2a receptors can provide a protective effect against dopaminergic neurons degeneration observed in Parkinson's disease and MPTP induced neurotoxicity [115, 120, 121]. Caffeine ingestion (non-selective adenosine A1/ A2a receptor blocker) is shown to be
associated with a lower tendency to develop Parkinson's disease in men [122-125]. The neuroprotective effect produced by adenosine A2a receptor antagonists is not fully understood, a possible explanation for such effect involves the reduction of glutamate release through adenosine A2a receptor blockade and this can lower neurodegeneration induced excitotoxicity [115, 120].

E- Pioglitazone and rosiglitazone:

These drugs are used for the treatment of type II diabetes (rosiglitazone is subjected to usage restrictions due to possible cardiotoxic effects) [126, 127]. These drugs (Thiazolidinedione TZD) reduce insulin resistance through targeting peroxisome proliferator activated receptor gamma (PPARγ) [128]. Recently these compounds receive a lot of attention for a possible use as neuroprotective agents in stroke and Parkinson's disease [129]. The neuroprotective effect is attributed to three main effects: modulation of inflammation, MAO-B inhibition and activation of mitoNEET.

These compounds can manipulate inflammatory pathways leading to a reduction in reactive oxygen species (ROS) production through inhibition of matrix metallopeptidase 9 and Inhibition of iNOS [92]. They can also increase the level of CuZn-superoxide dismutase and this can enhance the neuroprotective effect [130]. It had been shown that pioglitazone can lower the liberation of cytokines in cultured cells upon lipopolysaccharide treatment [131]. Both rosiglitazone and pioglitazone can inhibit the deposition of beta amyloid in the brain and lower the release of inflammatory mediator induced by beta amyloid [132, 156, 157].
The neuroprotective effect of pioglitazone and rosiglitazone can be explained partially through the inhibition of MAO-B enzyme and this will prevent the conversion of MPTP to the neurotoxic form MPP+ in MPTP Parkinsonian mouse model [133].

Finally pioglitazone can function as a ligand for a mitochondrial protein named mitoNEET and through this pathway pioglitazone can stabilize mitoNEET and change the functional state of mitochondria leading to a neuroprotective output. Binding of ligands to mitoNEET protein can result in depolarization of mitochondrial membrane, alteration in mitochondrial oxidative condition and attenuation of rotenone toxic effect [92].
Chapter two: Materials and Methods

2.1- Prospective candidates

Twenty four chemical compounds were purchased from Chembridge online chemical store (www.hit2lead.com) as possible candidates for MAO-B inhibition. The chemical structures for these compounds were predicted using similarity analysis approach which depends on molecular shape comparison using Gaussian function for volume matching [135]. Some specifications about these candidates are shown in table 2.1. Each compound was dissolved in an appropriate volume of dimethyl sulfoxide (DMSO) so that a stock solution with 10 mM concentration was produced.

Initially, virtual approaches (docking studies) were used to build an early impression about the binding tendency of these compounds to both MAO-B and bovine serum albumin. Based on docking results, these compounds were screened in the laboratory for both inhibition potential and selectivity against MAO-A and MAO-B. In order to obtain a better understanding of these compounds' pharmacokinetic profile, bovine serum albumin (BSA) binding assay and parallel artificial membrane permeability assay (PAMPA) were performed. Finally in our hope to discover a possible neuroprotective activity, these compounds were tested for antioxidant effect using oxygen radical absorbance capacity (ORAC) assay. All these experimental procedures can facilitate drawing the complete picture of possible scaffolds for potent, selective and

Table 2.1- Prospective candidates' specifications.

<table>
<thead>
<tr>
<th>ID code</th>
<th>Chemical structure</th>
<th>Chemical name</th>
<th>Mol. Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>7909619</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>4-[3-(6-chloro-2-hydroxy-4-phenyl-3-quinolinyl)-5-(2-pyridinyl)-4, 5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid.</td>
<td>500.9</td>
</tr>
<tr>
<td>5476423</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>7, 7'-[1,4-piperazinediylbis (methylene)] di (8-quinolinol).</td>
<td>400.5</td>
</tr>
<tr>
<td>7747412</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>4-[(2, 4-dimethyl-1, 3-thiazol-5-yl) carbonyl]-3-hydroxy-5-[4-(methylthio) phenyl]-1-(3-pyridinyl methyl)-1, 5-dihydro-2H-pyrrol-2-one.</td>
<td>451.6</td>
</tr>
</tbody>
</table>
Table 2.1- (Continued) Prospective candidates' specifications.

<table>
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<tr>
<th>ID code</th>
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<th>Chemical name</th>
<th>Mol. Weight g/ mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>7655826</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>2-methyl-5-(3-methyl-4-oxo-3, 4-dihydro-1-phthalazinyl)-N-[2-(4-morpholinyl) ethyl] benzene sulfonamide.</td>
<td>442.5</td>
</tr>
<tr>
<td>6656215</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>1-[3-(dimethyl amino) propyl]-4-(2-furoyl)-3-hydroxy-5-(3-nitrophenyl)-1, 5-dihydro-2H-pyrrol-2-one.</td>
<td>399.4</td>
</tr>
<tr>
<td>6405277</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>6-methoxy-N-[3-(4-morpholinyl) propyl]-2-naphthalene sulfonamide.</td>
<td>364.5</td>
</tr>
<tr>
<td>7917584</td>
<td><img src="image" alt="Chemical structure" /></td>
<td>1-(4, 5-dimethyl-1, 3-thiazol-2-yl)-5-(2-furyl)-3-hydroxy-4-[(7-methoxy-1-benzofuran-2-yl) carbonyl]-1, 5-dihydro-2H-pyrrol-2-one.</td>
<td>450.5</td>
</tr>
<tr>
<td>ID code</td>
<td>Chemical structure</td>
<td>Chemical name</td>
<td>Mol. Weight g/mole</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>7730595</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>5-(2-fluorophenyl)-3-hydroxy-4-[(4-methyl-2-phenyl-1, 3-thiazol-5-yl)carbonyl]-1-[2-(4-morpholiny)ethyl]-1, 5-dihydro-2H-pyrrol-2-one.</td>
<td>507.6</td>
</tr>
<tr>
<td>7839510</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>1-(3, 4-dimethoxybenzyl)-3-hydroxy-3-[2-(2-naphthyl)-2-oxoethyl]-1, 3-dihydro-2H-indol-2-one.</td>
<td>467.5</td>
</tr>
<tr>
<td>7917586</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>3-hydroxy-4-[(7-methoxy-1-benzofuran-2-yl)carbonyl]-1-(5-methyl-3-isoxazolyl)-5-phenyl-1, 5-dihydro-2H-pyrrol-2-one.</td>
<td>430.4</td>
</tr>
</tbody>
</table>

Table 2.1- (Continued) Prospective candidates' specifications.
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<table>
<thead>
<tr>
<th>ID code</th>
<th>Chemical structure</th>
<th>Chemical name</th>
<th>Mol. Weight g/ mole</th>
</tr>
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<tbody>
<tr>
<td>5140311</td>
<td><img src="image1.png" alt="Chemical structure" /></td>
<td>3-{[4-(dimethylamino)benzylidene]-4-oxo-2-thioxo-1,3-thiazolidin-3-yl}propanoic acid.</td>
<td>336.4</td>
</tr>
<tr>
<td>5108394</td>
<td><img src="image2.png" alt="Chemical structure" /></td>
<td>5-(2,4-dimethoxybenzylidene)-2-(phenylimino)-1,3-thiazolidin-4-one.</td>
<td>340.4</td>
</tr>
<tr>
<td>5108400</td>
<td><img src="image3.png" alt="Chemical structure" /></td>
<td>5-(4-chlorobenzylidene)-2-(phenylimino)-1,3-thiazolidin-4-one.</td>
<td>314.8</td>
</tr>
<tr>
<td>7919469</td>
<td><img src="image4.png" alt="Chemical structure" /></td>
<td>1-(3,4-dihydroxy phenyl)-2-[[1-(1-naphthyl)-1H-tetrazol-5-yl]thio]ethanone.</td>
<td>378.4</td>
</tr>
</tbody>
</table>
Table 2.1- (Continued) Prospective candidates' specifications.

<table>
<thead>
<tr>
<th>ID code</th>
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<th>Mol. Weight g/ mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>5143886</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>5-[(5-phenyl-2-furyl)methylene]-2-thioxo-1, 3-thiazolidin-4-one.</td>
<td>287.4</td>
</tr>
<tr>
<td>5140321</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>[5-(4-methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]acetic acid.</td>
<td>309.4</td>
</tr>
<tr>
<td>5144779</td>
<td><img src="image3" alt="Chemical structure" /></td>
<td>5-(1, 3-benzodioxol-5-ylmethylene)-3-(4-methylphenyl)-2-thioxo-1, 3-thiazolidin-4-one.</td>
<td>355.4</td>
</tr>
</tbody>
</table>
Table 2.1- (Continued) Prospective candidates' specifications.

<table>
<thead>
<tr>
<th>ID code</th>
<th>Chemical structure</th>
<th>Chemical name</th>
<th>Mol. Weight g/ mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>7542216</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>Ethyl N-(1, 1-dioxido-1, 2-benzisothiazol-3-yl)-N-methylglycinate.</td>
<td>282</td>
</tr>
<tr>
<td>7738249</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>5, 6-dimethyl-4-{{(2-methyl-1, 3-thiazol-4-yl)methyl}thio}thieno[2,3-d] pyrimidine.</td>
<td>307</td>
</tr>
<tr>
<td>7929249</td>
<td><img src="image3" alt="Chemical structure" /></td>
<td>{{(1-methyl-1H-pyrrol-2-yl)methyl}(3-pyridinylmethyl) amine hydrochloride.</td>
<td>238</td>
</tr>
<tr>
<td>7954103</td>
<td><img src="image4" alt="Chemical structure" /></td>
<td>3-methyl-2-{{[2-(4-methyl-1, 3-thiazol-5-yl) ethyl]thio}-4(3H)-quinazolinone.</td>
<td>317</td>
</tr>
</tbody>
</table>
Table 2.1- (Continued) Prospective candidates' specifications.

<table>
<thead>
<tr>
<th>ID code</th>
<th>Chemical structure</th>
<th>Chemical name</th>
<th>Mol. Weight g/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>7935085</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>N-[(2-hydroxy-8-methyl-3-quinolinyl) methyl]-N-(4-methoxyphenyl) methane sulfonamide.</td>
<td>372.4</td>
</tr>
<tr>
<td>5124176</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>[5-(anilinomethylene)-4-oxo-2-thioxo-1, 3-thiazolidin-3-yl] acetic acid.</td>
<td>294.4</td>
</tr>
<tr>
<td>7697413</td>
<td><img src="image3" alt="Chemical structure" /></td>
<td>Methyl [(4-methyl-5, 6, 7, 8-tetrahydro-2-quinazolinyl)thio] acetate.</td>
<td>252</td>
</tr>
</tbody>
</table>
2.2- Docking studies

The 2D and 3D structures of the tested compounds were prepared using MarvinSketch 6.0.3 (www.chemaxon.com). Human MAO-B crystal (2BK3) and bovine serum albumin (BSA) crystal (4F5S) were obtained from RCSB Protein Data Bank (www.rcsb.org). Both MAO-B and BSA crystals were prepared for docking using UCSF Chimera 1.8 (www.cgl.uctsf.edu/chimera) [150] and AutoDockTools (ADT) 1.5.6 (http://mgltools.scripps.edu) [151, 152]. ADT 1.5.6 was also used to prepare the tested compounds (ligands) for docking operation. Docking of the ligands into these targets (crystals) was done using AutoDock 4.2 (http://autodock.scripps.edu) [151]. The final results were analyzed using AutoDockTools (ADT) 1.5.6, LigPlot+ v.1.4.5 [153], UCSF Chimera 1.8 and PyMOL v.1.3 (www.schrodinger.com) [154].

2.3- Monoamine oxidase inhibition assay

This assay measure the compound's inhibition strength and degree of selectivity toward MAO-A and MAO-B. The basic concept of this assay involves the ability of both MAO-A and MAO-B to convert the non-fluorescent and non-selective substrate (kynuramine) into the intermediate 3-(2-aminophenyl)-3-oxo-propionaldehyde which will undergo spontaneous intramolecular condensation leading to the formation of the fluorescent product 4-Hydroxyquinoline (4-HQ) [136]. Based on this concept, the level of MAO activity can be determined through measuring the fluorescence level of 4-Hydroxyquinoline (4-HQ). In the presence of the tested compound, any reduction in
fluorescence level will reflect the inhibition capacity of that compound. Figure 2.1 shows the basic principle of this assay.

In this test, KPO4 solution (0.1 M, pH 7.4) was used as a buffer which was warmed to 37°C prior to performing the test. Falcon black 96 well plates (Cat#353241) were also used in this test. Kynuramine was purchased from Sigma-Aldrich (Cat#: k-3250) and a stock solution with a concentration of 25 mM was prepared by dissolving kynuramine in the appropriate volume of distilled water. This stock solution was further diluted with KPO4 buffer to yield a final concentration that is close to the Km value for the reaction catalyzed by either MAO-A or MAO-B (the final concentration of kynuramine solution after dilution was 40 µM for MAO-A and 20 µM for MAO-B). Human MAO-A and human MAO-B were purchased from Fisher Scientific (www.fishersci.com). These enzymes were used in this assay as 5 mg/ ml stock solutions and in order to minimize thermal induced inactivation of MAO, both enzymes were stored at -80°C and they even kept on ice till their employment in the assay. The stock solutions of MAO-A and MAO-B were further diluted with KPO4 buffer to obtain final solutions with concentrations of 0.006 mg/ ml for MAO-A and 0.015 mg/ ml for MAO-B. 2 N NaOH was used as a stopping reagent by causing protein denaturation.

In this assay, the tested compounds were serially diluted with KPO4 buffer (100 µl/ well in duplicate) across the plate to produce eight points dose inhibition curve. As mentioned previously, the tested compounds were dissolved in DMSO and the final concentration of DMSO in the assay was less than 0.5%. It is reported that DMSO shows
the least MAO inhibitory effect. The enzyme activity (fluorescence level) in the absence of any inhibitor (only DMSO) was considered as a control (100% MAO activity). After the compounds were serially diluted, the plate was covered and incubated at 37°C for 10 minutes so that both the compounds and the plate can reach a temperature of 37°C. Then the substrate (kynuramine) was added as 50 µl/well after that the enzyme (either MAO-A or MAO-B) was added as 50 µl/well in order to start the reaction. The plate was then covered and incubated at 37°C for 20 minutes in order to obtain the ultimate level of activity for the enzyme. The final assay volume was 200 µl per well. After incubation period, 75 µl of 2 N NaOH solution was added per well to stop the reaction. The fluorescence level of 4-Hydroxyquinoline was measured at 310/380 nm wavelength pair by using a Flourometer (top reader, BMG-FlouStar).
Figure 2.1- Conversion of kynuramine into 4-Hydroxyquinoline as catalyzed by both MAO-A and MAO-B, this figure was adapted from reference [137].
2.4- *Bovine serum albumin (BSA) binding assay*

This assay measures the binding affinity of the tested compounds toward serum bovine albumin. Fluorescence quenching represents the main principle of this assay and it involves the reduction in fluorescence level (quantum yield) of a fluorophore upon interaction with a quencher molecule [138, 139]. Bovine serum albumin (BSA) shows intrinsic fluorescence activity (fluorophore) due to the presence of two types of aromatic amino acids (tryptophan and tyrosine). When BSA is excited at 280 nm, it will show a strong fluorescence emission at 342 nm and this emission is mainly attributed to tryptophan [140-142]. This test is based on the idea that higher binding of the tested compound to BSA will result in greater quenching of the fluorescence activity of BSA and this can be measured experimentally.

In this assay, KPO4 solution (0.1 M, pH 7.4) was used as a buffer. Bovine serum albumin was obtained from Boston BioProducts (Ashland, MA, USA). A stock solution of BSA (1 mg/ ml) was prepared by dissolving BSA in KPO4 buffer just before starting the test. Falcon black 96 well plates (Cat# 353241) were used for this test. The final assay volume was 200 µl/ well. The tested compounds were serially diluted (100 µl/ well in duplicate) with KPO4 buffer across the plate so that the desired range of concentrations (an eight points data curve) was obtained. As mentioned before, dimethyl sulfoxide (DMSO) was used to dissolve the tested compounds and the fluorescence level of BSA in the absence of any tested compound (only DMSO) was considered as a control (no BSA binding). The stock solution of BSA was added as 100 µl/ well (the final concentration of
BSA in each well was 7.5 µM) after that the plate was covered and incubated at room temperature for 30 minutes. After incubation, the plate was read at 280/340 nm wavelength pair using a Fluorometer (BMG-FlouStar).

2.5- Parallel Artificial Membrane Permeability Assay (PAMPA)

This assay represents a high throughput approach to predict passive, transcellular permeability of the tested compounds in early stages of drug development process. This assay is cheap, highly successful and reproducible [143]. It uses a synthetic lipid layer immobilized on filter support to simulate the passive permeability of the compound across different biological barriers (blood brain barrier, skin and gastrointestinal tract) [143-146]. This assay can't determine active transport of the compound across biological membranes [144].

The Hexadecane Method of PAMPA (HDM-PAMPA) measures the passive diffusion of the tested compound from the donor compartment (which contains the desired concentration of the compound dissolved in buffer medium) to an acceptor compartment (compound free compartment, contains only buffer medium). These two compartments are separated by a hexadecane liquid layer on a polycarbonate membrane support. The concentration of the tested compound can be determined in these two compartments by measuring the fluorescence level for the compound.

For this assay, 5% DMSO in phosphate buffered saline pH 7.4 was used as a buffer system. Hexadecane and hexane were obtained from commercial sources and a 5%
(v/v) hexadecane in hexane mixture was prepared. Compound under investigation (5140311) was diluted with buffer system (5% DMSO in BPS) to yield a final solution with a concentration of 500 µM. A 96 well MultiScreen filter plate for permeability (donor plate) was ordered from Millipore Corporation (Billerica, MA, USA, Cat# MAPBMN310). The 96 well acceptor plate was also obtained from Millipore (Cat# MSSACCEPTOR).

The assay was initiated by the addition of 15 µl/ well of 5% (v/v) hexadecane in hexane solution into the donor plate; the plate was then placed uncovered in a fume hood for one hour in order to ensure that hexane was completely evaporated and a fine layer of hexadecane was generated on polycarbonate film. This was followed by the addition of buffer solution (5% DMSO in PBS) into the acceptor plate as 300 µl per each well. The donor plate was then placed on the acceptor plate so that the underside of the membrane for donor plate is in contact with the buffer solution in each well of the acceptor plate. The compound (5140311) solution was added to the donor plate as 150 µl/ well in triplicate. After that, the donor plate was covered with its lid and incubated at room temperature for a period of four hours. During incubation period, equilibrium solution for compound (5140311) was prepared by simply mixing donor compartment volume (150 µl of compound solution) with acceptor compartment volume (300 µl of buffer solution). After incubation period, 250 µl was transferred from each well of the acceptor plate into Falcon black 96 well plate (Cat# 353241). A similar volume of equilibrium solution for compound (5140311) was transferred into the same 96 well black plate. Also compound (5140311) solution was serially diluted with assay buffer across the black plate to yield a
suitable eight points fluorescence-concentration curve. The generation of such curve will help us to predict the concentration of the compound in the acceptor compartment based on the observed fluorescence (interpolation). The fluorescence level of the compound (5140311) was measured at 540/620 nm wavelength pair by using FLX 800 microplate fluorescence reader (Biotek instruments).

2.6- Oxygen Radical Absorbance Capacity (ORAC) assay

This assay measures the ability of the tested compounds to scavenge peroxyl radicals that are generated by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), thereby it can screen these compounds for any possible antioxidant activity [147, 148]. The assay principle is based on the idea that the presence of peroxyl radicals can lower the fluorescence level of a fluorophore (fluorescein) as a result of oxidative degradation. A compound with antioxidant power can preserve the fluorescence activity by reducing the oxidative degradation of fluorescein [149].

For this assay, KPO4 solution (0.1 M, pH 7.4) was used as a buffer, Falcon black 96 well plates (Cat# 353241) were also used and the final volume for this assay was 200 µl per each well. AAPH was obtained from Sigma-Aldrich (St. Louis, MO, USA) and it was stored in a dry environment. Before starting the assay, AAPH was dissolved in an appropriate volume of KPO4 buffer to yield a stock solution with a concentration of 72 mM. This stock solution was kept on ice until assay initiation. Fluorescein was also purchased from Sigma-Aldrich (St. Louis, MO, USA) and prior to assay initiation, it was also dissolved in KPO4 buffer to prepare a stock solution with a concentration of 112
nM. Vitamin C (L-ascorbic acid) was also ordered from Sigma-Aldrich (St. Louis, MO, USA); before starting the test, it was dissolved in KPO4 buffer to obtain a solution with a concentration of 10 mM. Vitamin C was used in this assay as a reference substance (positive control). As mentioned previously, DMSO was used as a vehicle for the tested compounds, the fluorescence level of fluorescein in the absence of any tested compound (only DMSO) was considered as a negative control.

The compounds under investigation were serially diluted with KPO4 buffer across the assay plate (20 µl/ well in duplicate) to yield the required range of concentrations. Fluorescein solution was then added as 80 µl per each well. This was followed by the addition of peroxyl radical generator (AAPH) as 100 µl per each well. The plate was then covered and incubated at room temperature for one hour. After that, the fluorescence level was read at 485 nm excitation and 528 nm emission wavelengths through using FLX 800 microplate fluorescence reader (Biotek instruments).

2.7- Data analysis

Most of the results for the previously mentioned experiments were displayed as (Mean ± SEM). Prism 5 (www.graphpad.com) was used for columns generation, curves fitting (nonlinear regression) and lines fitting (linear regression).
Chapter three: Results

3.1- Selection of the most efficient candidates

Based on the results of our experiments, a scatter plot was generated by plotting MAO-B enzyme inhibition potential (capacity) against antioxidant potential (capacity) of the prospective candidates. The scatter plot is shown in figure 3.1 and it was generated by using 100µM concentration of each compound in MAO-B inhibition assay and 20µM concentration of each compound in antioxidant (ORAC) assay.

By careful examination of this plot, we can easily choose the most efficient candidates that display a perfect combination of both MAO-B inhibition capacity and antioxidant effect. According to the scatter graph, six compounds show such efficient combination and the identification codes for these chemicals are: 5143886, 5140311, 7730595, 5140321, 7919469 and 7747412. In this section we will focus only on the results of these compounds since they represent encouraging candidates for possible antiparkinsonian agents with disease modifying capability. We will also mention the results for additional two chemicals and these are: 5476423 and 7954103. According to the plot, compound 5476423 shows excellent antioxidant behavior with minor MAO-B inhibition ability. On the other hand, compound 7954103 shows a potent MAO-B inhibition effect with minor antioxidant activity.
Figure 3.1- A scatter plot of MAO-B inhibition potential (percentage) against antioxidant potential (percentage) for our chemicals. For antioxidant potential, vitamin C was used as a reference (100% potential).
3.2- Docking studies results

In order to obtain preliminary expectations about the pharmacodynamics and pharmacokinetics characteristics of our candidates, docking analyses were performed by targeting human MAO-B crystal and bovine serum albumin crystal respectively. Docking studies can also give us a virtual insight into compound's orientation within the active site of our target.

Based on our results of docking studies, it is obvious that compounds: 5140311, 5140321, 5143886, 7919469 and 7954103 exhibit an extended conformation that will occupy both the entrance cavity and the substrate cavity within the active site of MAO-B crystal. Such orientation will force Ile 199 residue to gain an open configuration leading to the fusion of both entrance cavity and substrate cavity. The results of docking studies for these five compounds are shown in figure 3.2, 3.3, 3.4, 3.5 and 3.6 respectively. According to these figures, we can easily notice that these five candidates are situated in the active site with a position that directly facing the FAD moiety. In figure 3.2, we can recognize that there is a hydrogen bond between the oxygen atom attached to position four of the 2-thioxo-1, 3-thiazolidine moiety of compound 5140311 and the hydroxyl group of phenyl moiety in Tyr 435 (residue of aromatic cage). Another important finding to mention is the presence of two hydrogen bonds in the docking results of compound 7919469 as can be seen in figure 3.5. The first hydrogen bond is formed between the nitrogen atom at position three of tetrazole moiety of compound 7919469 and the hydroxyl group in carboxylic acid moiety of Phe 168. The second and most important
hydrogen bond is formed between the p-hydroxyl group of dihydroxy phenyl moiety in compound 7919469 and N (5) in the isoalloxazine ring system of FAD moiety. For these five compounds, hydrophobic interactions with residues of active site contribute significantly to the binding energy.

The results for the three remaining compounds: 5476423, 7730595 and 7747412 are shown in figure 3.7, 3.8 and 3.9 respectively. From these results, we can see clearly that these compounds do not enter to the active site of MAO-B crystal. These three compounds occupy a position close to the gating switch (loop 99-112) and through hydrophobic interactions with the amino acid residues in this loop (mainly Phe 103 and Pro 102); these compounds can hinder the entrance of substrates into the entrance cavity of MAO-B enzyme. The values for energy of binding and dissociation constant (K_I) for the last three compounds are higher than those for the first five compounds and this may be attributed to the difference in the location of binding.
Figure 3.2(A)- Two dimensional representation for the docking of compound 5140311 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen, nitrogen and sulfur atoms are represented by black, red, blue and yellow colors respectively. Hydrogen bonds are represented by green dashed line while hydrophobic interactions are drawn as small multiple red lines.
Binding energy: -7.75 kcal/mol
Ki: 2.08 µM

Figure 3.2(B)- Three dimensional representation for the docking of compound 5140311 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red and the hydrogen bond is shown as a green line.
Figure 3.3(A)- Two dimensional representation for the docking of compound 5140321 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen, nitrogen and sulfur atoms are represented by black, red, blue and yellow colors respectively. Hydrophobic interactions are drawn as small multiple red lines.
**Binding Energy:** -7.39 kcal/mol

**kl : 3.85uM**

Figure 3.3(B)- Three dimensional representation for the docking of compound 5140321 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red.
Figure 3.4(A)- Two dimensional representation for the docking of compound 5143886 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen, nitrogen and sulfur atoms are represented by black, red, blue and yellow colors respectively. Hydrophobic interactions are drawn as small multiple red lines.
Figure 3.4(B)- Three dimensional representation for the docking of compound 5143886 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red.
Figure 3.5(A)- Two dimensional representation for the docking of compound 7919469 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen, nitrogen and sulfur atoms are represented by black, red, blue and yellow colors respectively. Hydrogen bonds are represented by green dashed line while hydrophobic interactions are drawn as small multiple red lines.
Figure 3.5(B)- Three dimensional representation for the docking of compound 7919469 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red and the hydrogen bond is shown as a green line.
Figure 3.6(A)- Two dimensional representation for the docking of compound 7954103 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen, nitrogen and sulfur atoms are represented by black, red, blue and yellow colors respectively. Hydrophobic interactions are drawn as small multiple red lines.
Figure 3.6(B)- Three dimensional representation for the docking of compound 7954103 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red.
Figure 3.7(A)- Two dimensional representation for the docking of compound 5476423 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen and nitrogen atoms are represented by black, red and blue colors respectively. Hydrophobic interactions are drawn as small multiple red lines.
Figure 3.7(B)- Three dimensional representation for the docking of compound 5476423 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red.
Figure 3.7(C)- This figure shows the location of binding for compound 5476423 (red color) according to docking studies in MAO-B crystal. This compound occupies a location close to the gating loop and may interfere with substrates accessibility to the active site. This figure was generated by using UCSF Chimera 1.8 (www.cgl.ucsf.edu/chimera) [150].
Figure 3.7(D)- A close view for the previous figure, Here we can see how compound 5476423 (red color) is located near the gating loop and may interact with residues located in that loop like Pro 102 and Phe 103. This figure was generated by using UCSF Chimera 1.8 (www.cgl.ucsf.edu/chimera) [150].
Figure 3.8(A)- Two dimensional representation for the docking of compound 7730595 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen, nitrogen, fluoride and sulfur atoms are represented by black, red, blue, green and yellow colors respectively. Hydrophobic interactions are drawn as small multiple red lines.
**Binding Energy:** -3.46 kcal/mol

kl : 2.92mM

Figure 3.8(B)- Three dimensional representation for the docking of compound 7730595 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red.
Figure 3.8(C)- A close view for the binding position of compound 7730595 (red color) as indicated by docking studies using MAO-B crystal as a target. As shown here the compound is located close to the gating loop and may be involved in interactions with residues located within the gating loop like Pro 102 and Phe 103. This figure was generated by using UCSF Chimera 1.8 (www.cgl.ucsf.edu/chimera) [150].
Figure 3.9(A)- Two dimensional representation for the docking of compound 7747412 into MAO-B crystal. This image was generated by LigPlot+ v.1.4.5 [153]. Carbon, oxygen, nitrogen, fluoride and sulfur atoms are represented by black, red, blue, green and yellow colors respectively. Hydrophobic interactions are drawn as small multiple red lines. The green dashed line represents hydrogen bond between the amine group at position two of Threonine 478 residue and the hydroxyl group attached to position three at the 1, 5- dihydro-pyrrol-2-one moiety of compound 7747412.
Figure 3.9(B)- Three dimensional representation for the docking of compound 7747412 into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red and the hydrogen bond is shown as a green line.
Figure 3.9(C)- A close view for the binding position of compound 7747412 (red color) as indicated by docking studies using MAO-B crystal as a target. As shown here the compound is located close to the gating loop and may be involved in hydrophobic interactions with residues located within the gating loop like Pro 102 and Phe 103. The green line represents hydrogen bond between Thr 478 residue and our compound. This figure was generated by using UCSF Chimera 1.8 (www.cgl.ucsf.edu/chimera) [150].
Because the main binding sites in serum albumin for aromatic and heterocyclic compounds are subdomain IIA (site I) and subdomain IIIA (site II) [158,159], docking studies in BSA crystal were targeted toward these two binding sites only. By taking the virtual binding parameters (energy of binding and dissociation constant) into our consideration, it is clear that our candidates bind efficiently with nearly equal affinity to site I and site II. The only compound that deviates from this established principle is 7919469, which shows some preference for binding to site I over site II. Table 3.1 shows virtual binding parameters for our selected compounds in BSA docking studies.
Table 3.1- Docking results for BSA crystal.

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Subdomain IIA</th>
<th>Subdomain IIIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Energy of binding</td>
<td>Energy of binding</td>
</tr>
<tr>
<td></td>
<td>(kcal/mol)</td>
<td>KI (µM)</td>
</tr>
<tr>
<td>5140311</td>
<td>-5.53</td>
<td>89.11</td>
</tr>
<tr>
<td>5140321</td>
<td>-5.78</td>
<td>58.01</td>
</tr>
<tr>
<td>5143886</td>
<td>-6.35</td>
<td>22.03</td>
</tr>
<tr>
<td>7919469</td>
<td>-7.52</td>
<td>3.1</td>
</tr>
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<td>7954103</td>
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<td>5476423</td>
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<td>7747412</td>
<td>-4.96</td>
<td>232.32</td>
</tr>
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</table>
3.3- Monoamine oxidase inhibition assay results

The results of this assay provide a clear insight into the potential and selectivity of inhibition produced by our candidates against MAO enzyme isoforms. Our results for this test are summarized in figure 3.10, figure 3.11 and table 3.2. Figure 3.10 describes the strength of MAO-B inhibition (potency of inhibition) produced by using 100µM concentration of each selected compound. According to this figure, the highest inhibition is produced by compound 5143886 where MAO-B activity falls down to a percentage value of (2.148 ± 0.2697) and statistically this value represents (Mean ± SEM). The lowest inhibition is produced by compound 5476423 where the activity of MAO-B enzyme only falls down to a percentage value of (78.99 ± 0.1677). The remaining compounds produce a strong inhibitory effect which generally lowers the activity of MAO-B to below 50% level.

On the other hand, figure 3.11 shows the potency of the inhibitory effect against MAO-A for only four compounds (5140311, 5140321, 5143886 and 5476423). This potency of effect was generated by using a concentration of 100µM for each tested compound. In this figure, the strongest inhibitory effect is produced by compound 5143886 with a reduction in MAO-A level of activity into a percentage value of (42.37 ± 1.737) while the weakest effect is produced again by compound 5476423 with a reduction in MAO-A activity into a percentage value of (78.81 ± 0.4983). The other two compounds appear to be able to lower enzyme activity to around 60%. By comparing data from figure 3.10 with those in figure 3.11, we can obtain some information about the
selectivity in action of the tested compounds against MAO enzyme isoforms. It is very clear that compound 5476423 shows no preference in its inhibitory effect against MAO enzyme isoforms. Compounds 5140311 and 5143886 appear to be highly selective for MAO-B inhibition while compound 5140321 shows a slight preference for the inhibition of MAO-B over MAO-A.

Table 3.2 gives us information about the degree of inhibition selectivity toward MAO-B and MAO-A isozymes. This table compares the IC50 value for MAO-B inhibitory effect against that value for MAO-A inhibitory effect. Unfortunately, we were only able to determine MAO-A IC50 for compounds 5140311 and 5476423 due to technical restrictions. Compound 5140311 seems to be highly selective for inhibiting MAO-B enzyme since the MAO-B IC50 value is about 16 times lower than IC50 value for MAO-A.
Figure 3.10- MAO-B inhibition potential as produced by our compounds. Values are presented as (Mean ± SEM). The concentration used for each compound was 100µM.
Figure 3.11- The potency of the inhibitory effect against MAO-A as produced by the tested compounds. Results are presented as (Mean ± SEM); the concentration used to generate such effect is 100µM.
Table 3.2- Inhibition selectivity toward MAO isozymes.

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>MAO-B IC50 µM</th>
<th>MAO-A IC50 µM</th>
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</thead>
<tbody>
<tr>
<td>5140311</td>
<td>9.71</td>
<td>155</td>
</tr>
<tr>
<td>5140321</td>
<td>105</td>
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<td>5143886</td>
<td>2.64</td>
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<tr>
<td>7919469</td>
<td>1.19</td>
<td>N/A</td>
</tr>
<tr>
<td>7730595</td>
<td>62.8</td>
<td>N/A</td>
</tr>
<tr>
<td>7747412</td>
<td>94.3</td>
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</tr>
<tr>
<td>5476423</td>
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<td>242</td>
</tr>
<tr>
<td>7954103</td>
<td>6.27</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.4- Bovine serum albumin (BSA) binding assay results

This assay provides a general view about compounds' degree of binding to serum albumin and the influence of such interaction on the pharmacokinetics features of these selected compounds. The binding potentials for these chemicals are shown in figure 3.12. The results in this figure were generated by using 100µM concentration for each tested compound; the outcomes in this figure were reported as (Mean ± SEM).

Examination of this figure reveals that these compounds, in general, show high tendency for BSA binding. The lowest binding percentage is produced by compound 5476423 with a binding value of (44.16 ± 5.205) while the highest binding percentage is produced by compound 5143886 with a value of (97.57 ± 0.1944). With the exception of compound 5140311, all the remaining compounds show high degree of BSA binding that is greater than 70%.

By using different concentrations for each compound (serial dilution), we were able to generate a relative BSA binding curve (nonlinear regression). This curve was utilized to obtain an estimation of EC50 value. Figure 3.13 shows the BSA binding curve for each compound together with its calculated EC50. With some exceptions these EC50 values can be correlated with those results presented in figure 3.12. For instance, the smallest EC50 values (highest binding) were calculated for compounds 5143886 and 5140321 while the largest value (least binding) was reported for compound 5476423.
Figure 3.12- BSA binding potential of the tested chemicals. These results were generated by using a concentration of 100µM for each compound and the outcomes were presented as (Mean ± SEM).
Figure 3.13- Relative BSA binding curves for the selected compounds. The calculated EC50 values are also presented with these curves.
Figure 3.13- (Continued) Relative BSA binding curves for the selected compounds. The calculated EC50 values are also presented with these curves.
Figure 3.13- (Continued) Relative BSA binding curves for the selected compounds. The calculated EC50 values are also presented with these curves.
Figure 3.13- (Continued) Relative BSA binding curves for the selected compounds. The calculated EC50 values are also presented with these curves.
3.5- Parallel artificial membrane permeability assay (PAMPA) results

This assay gives us an early prediction about the ability of the compound (under investigation) to pass the blood brain barrier and reach CNS. This assay was only applied to compound 5140311 because it was the only one that fluoresced at the excitation wavelength range provided by the fluorescence reader. By the end of this assay, the fluorescence level was determined for both the acceptor compartment and equilibrium solution as this level of fluorescence reflects the concentration of compound 5140311 in these solutions. The results of fluorescence reader were then applied to the following equation (provided by the manufacturer of assay apparatus) in order to calculate the rate of permeation or effective permeability (Pe):

$$\log Pe = \log \{ C \times -\ln (1 - \frac{[drug]_{acceptor}}{[drug]_{equilibrium}}) \}$$

Where

$$C = \left( \frac{V_D \times V_A}{(V_D + V_A) \times \text{Area} \times \text{time}} \right)$$

$$V_D = \text{Volume of donor compartment} = 0.15 \text{ cm}^3.$$  

$$V_A = \text{Volume of acceptor compartment} = 0.3 \text{ cm}^3.$$  

$$\text{Area} = \text{Active surface area of membrane} = 0.048 \text{ cm}^2.$$  

$$\text{Time} = \text{Incubation time for the assay and in our case it was four hours} = 14400 \text{ seconds.}$$
By applying all of the previously mentioned variables into our equation we get the following:

\[ C = \left( \frac{0.15 \times 0.3}{0.15 + 0.3} \right) \frac{0.048 \times 14400}{0.048 \times 14400} \]

\[ C = 1.446 \times 10^{-4} \text{ cm s}^{-1} \]

\[ \log Pe = \log \left\{ (1.446 \times 10^{-4}) \times -\ln \left(1 - \frac{611}{17013}\right) \right\} \]

\[ \log Pe = -5.276 \text{ cm s}^{-1} \]

\[ Pe = 5.2966 \times 10^{-6} \text{ cm s}^{-1} \]

According to the classification of PAMPA-BBB results (based on Pe ranges) [160], it is very clear that our compound 5140311 is highly permeable through blood brain barrier since our calculated Pe value is greater than \( 4.0 \times 10^{-6} \text{ cm s}^{-1} \). As we mentioned previously in the materials and methods chapter (chapter two), compound 5140311 was serially diluted to generate a standard curve (eight points fluorescence-concentration curve). We can estimate the concentration of our compound in the acceptor compartment based on the measured level of fluorescence (interpolation using the standard curve). In our case the concentration of compound 5140311 in the acceptor compartment was 2.098125\( \mu \text{M} \).
3.6- Oxygen radical absorbance capacity (ORAC) assay results

This assay predicts the antioxidant capacity of compound under investigation through screening the ability of that compound to scavenge peroxyl radicals that are generated by AAPH (radical initiator) [147, 148]. By scavenging these radicals, our compound will be able to prevent the degradation of fluorescein thereby preserving the fluorescence level of this fluorophore [149]. According to this principle, the fluorescence level will reflect the antioxidant capacity of the tested compound.

The results for this assay are summarized in figure 3.14; these results were obtained by using 20µM concentration of each tested compound. The outcomes are presented as (Mean ± SEM); vitamin C (Ascorbic acid) was used as a positive control (100% activity). In this figure, all the tested compounds show excellent antioxidant potentials that are either equivalent or higher than that reported for Ascorbic acid (with the exception of compound 7954103). The highest radical scavenging capacity was reported for compound 7730595 with a percentage value of (205.2 ± 1.952) while the least scavenging capacity was observed in compound 7954103 with a percentage value of (31.24 ± 0.2169).

In this assay, serial dilution was used for each compound in order to generate eight points fluorescence – concentration curve (nonlinear regression). The generation of such curve helped us to estimate the value of EC50 for each compound. Unfortunately, we were unable to correlate the calculated EC50 values (shown in table 3.3) with results
in figure 3.14. This may be due to technical restrictions within curve fitting (nonlinear regression).

Figure 3.14- Percentage of antioxidant power for the selected chemicals. This figure was generated by using 20µM concentration for each compound. The outcomes are presented as (Mean ± SEM); Ascorbic acid was used as a positive control (100% activity).
Table 3.3- EC50 values for (antioxidant capacity – concentration) curve of each tested compound.

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>EC50 µM</th>
</tr>
</thead>
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<td>Ascorbic acid</td>
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</tr>
<tr>
<td>5140311</td>
<td>0.5748</td>
</tr>
<tr>
<td>5140321</td>
<td>3.698</td>
</tr>
<tr>
<td>5143886</td>
<td>N/A</td>
</tr>
<tr>
<td>7919469</td>
<td>12.56</td>
</tr>
<tr>
<td>7730595</td>
<td>0.9124</td>
</tr>
<tr>
<td>7747412</td>
<td>0.6416</td>
</tr>
<tr>
<td>5476423</td>
<td>3.256</td>
</tr>
<tr>
<td>7954103</td>
<td>3.491</td>
</tr>
</tbody>
</table>
3.7- Structure activity relationship (SAR) study results

We established a basic structure activity relationship (SAR) study as an attempt to build a hypothetical scaffold for the development of potent, selective and centrally active MAO-B inhibitors with a possible neuroprotective effect that can be applied for the treatment of Parkinson's disease. Through comparing the structure and activity for compounds with related building blocks, we tried to identify the structural units that are essential for MAO-B inhibition and those that are critical for peroxyl radical quenching.

For simplicity in presentation, the data of the scatter plot in figure 3.1 are re-introduced here in the form of columns (only for compounds under investigation in SAR study). Figures 3.15, 3.16 and 3.17 show the relative activity for the concerned compounds (MAO-B inhibition activity and antioxidant potential) together with the structural formula of these compounds.

Based on figure 3.15, it is very easy to notice that both compounds 5140311 and 5140321 have almost the same antioxidant potential, compound 5140311 appears to have a stronger MAO-B inhibition effect. This variation in MAO-B inhibition potential can be attributed in part to the difference in the substitution type at position four of the benzylidene moiety in these compounds. The dimethyl amino group in compound 5140311 has more electron donating effect than does the methoxy moiety in compound 5140321; this may provide a more favorable position and electron density for compound 5140311 within MAO-B active site so that a hydrogen bond can be formed between oxygen atom (attached to position four of the 2-thioxo-1, 3-thiazolidine ring) and the
hydroxyl group of phenyl moiety in Tyr 435 residue. The presence of such hydrogen bonding was confirmed for compound 5140311 by using docking analysis. Compounds 5140311 and 5140321 also show difference in substitution length at position three of the 2-thioxo-1, 3-thiazolidin-4-one ring; longer substitution (propanoic acid in 5140311 versus acetic acid in 5140321) may have a positive impact on the potential and selectivity of MAO-B inhibition effect by providing a more extended conformation.
Figure 3.15(A)- The potency of the inhibitory effect against MAO-B as produced by compounds 5140311 and 5140321; the data are displayed as (Mean ± SEM).
Figure 3.15(B)- Percentage of peroxyl radical quenching potential (antioxidant) for compounds 5140311 and 5140321, values are presented as (Mean ± SEM) and Ascorbic acid was used as positive control (100% activity).
Figure 3.15(C)- Chemical structural formula for compounds 5140311 and 5140321.
Figure 3.15(D)- Two dimensional representation for the docking of compound 5140311 into human MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153].
Figure 3.15(E)- Two dimensional representation for the docking of compound 5140321 into human MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153].
In figure 3.16(A), only compounds 5143886 and 5108394 show high MAO-B inhibition potential. The other two compounds 5144779 and 5108400 have a minor MAO-B inhibitory effect. Compounds 5108394 and 5108400 are very similar in terms of structural formula but they show significant difference regarding MAO-B inhibition effect (in vitro). Structural comparison for these two compounds show that the only difference between them is the presence of two electron donating groups (methoxy group) attached to positions two and four of the benzylidene moiety in compound 5108394 while compound 5108400 has an electron withdrawing group (chlorine atom) attached to position four of the benzylidene moiety. This variation in substituent type will affect the orientation and the electron density of these compounds within MAO-B active site. Docking studies showed that compound 5108394 occupies a position opposite in direction to that occupied by compound 5108400 within MAO-B crystal; such orientation will favor the formation of hydrogen bond between oxygen atom attached to position four of 1, 3-thiazolidine ring in compound 5108394 and the sulfhydryl group attached to position three of Cysteine 172 residue within MAO-B active site. The reversed orientation in compound 5108400 will facilitate the formation of hydrogen bond between the sulfur atom at position one of 1, 3-thiazolidin-4-one ring and the sulfhydryl group in Cysteine 172 residue, this hydrogen bond is weaker than that formed in case of compound 5108394 and this may account for the difference in MAO-B inhibition potential.

Previous studies showed that nitrogen atom in position three and oxygen atom attached to position four of the thiazolidinedione ring within pioglitazone molecule are
involved in hydrogen bonding with conserved active site water molecules within the aromatic cage of Tyr398 and Tyr435 [128]. We believe that such hydrogen bonding can also exist between the conserved active site water molecules and (nitrogen atom in position three and oxygen atom attached to position four of the 1, 3-thiazolidine ring). We expect that attachment of 4-methylphenyl group to the nitrogen atom at position three of the 2-thioxo-1, 3-thiazolidin-4-one ring in compound 5144779 can hinder the formation of hydrogen bond with conserved active site water molecule and this may adversely impact MAO-B inhibitory effect.

In figure 3.16(B), we can clearly notice that only compound 5143886 has peroxyl radical quenching activity, the other three compounds (5144779, 5108400 and 5108394) have very low or almost no antioxidant activity. Chain breaking antioxidants produce their radicals scavenging activity through donating hydrogen atoms (radicals reduction) and formation of stable radicals that are unable to continue chain reaction [149, 182]. We believe that nitrogen atom at position three of 2-thioxo-1, 3-thiazolidin-4-one ring represents a good hydrogen donor, and we also expect that the presence of thioxo group at position two of 1, 3-thiazolidin-4-one ring is considered essential for antioxidant activity. Such criteria are readily available in compound 5143886. Similar structural criteria for antioxidant activity can be also observed in compounds 5140311 and 5140321 by taking into consideration that the nitrogen atom at position three of 2-thioxo-1, 3-thiazolidin-4-one is attached to carboxylic acid moiety that can still donate hydrogen atom.
Figure 3.16(A)- The potency of the inhibitory effect against MAO-B as produced by compounds 5143886, 5144779, 5108394 and 5108400; the data are displayed as (Mean ± SEM).
Figure 3.16(B)- Percentage of peroxyl radical quenching potential (antioxidant) for compounds 5143886, 5144779, 5108394 and 5108400, values are presented as (Mean ± SEM) and Ascorbic acid was used as positive control (100% activity).
Figure 3.16(C)- Chemical structural formula for compounds 5108394, 5108400, 5144779 and 5143886.
Figure 3.16(D)- Two dimensional representation for the docking of compound 5108394 into human MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153].
Figure 3.16(E)- Two dimensional representation for the docking of compound 5108400 into human MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153].
Figure 3.17 clearly shows that compounds 7935085 and 5476423 have a very weak MAO-B inhibition activity. Compound 5476423 appears to be a potent radical quencher; on the other hand compound 7935085 shows a weaker potency for radical reduction. This variation in antioxidant power can be attributed in part to the difference in the substitution type and position within quinoline moiety. Previous researches showed that attachment of hydroxyl group to position eight in quinoline moiety can greatly enhance the radical scavenging activity [182]. Such structural requirement for antioxidant potential is clearly evident in compound 5476423 with the presence of two 8-hydroxyquinoline moieties; the weaker scavenging activity of compound 7935085 can be partially explained by the presence of methyl group attached to position eight of quinoline moiety and the attachment of hydroxyl group to position two of quinoline system.
Figure 3.17(A)- The potency of the inhibitory effect against MAO-B as produced by compounds 7935085 and 5476423; the data are displayed as (Mean ± SEM).
Figure 3.17(B)- Percentage of peroxyl radical quenching potential (antioxidant) for compounds 5476423 and 7935085, values are presented as (Mean ± SEM) and Ascorbic acid was used as positive control (100% activity).
Figure 3.17(C)- Chemical structural formula for both compounds 5476423 and 7935085.
Chapter four: Discussion

Parkinson's disease is a progressive neurodegenerative disorder that mainly affects elderly people; it holds the second rank after Alzheimer's disease in terms of neurodegenerative disorders prevalence among this segment of population [161]. Parkinson's disease is characterized by a progressive loss (degeneration) of dopaminergic neurons in the substantia nigra pars compacta region of the midbrain, this gradual damage will negatively impact nigrostriatal pathway leading to the emergence of classical motor related symptoms. As the disease progresses in course, non-motor symptoms (like depression and dementia) may also appear due to sporadic degeneration within cholinergic, noradrenergic and serotonergic pathways in the brain [162].

Parkinson's disease is a complex neurologic condition with multiple etiological factors being involved in guiding the pathophysiological course of this disorder. Current therapeutic approaches for this disorder focus only on symptomatic mitigation through dopamine replacement therapy [11-13]. No treatment is currently available that can hinder or slow down the progression of the neurodegenerative process [19, 20]. The application of designed multiple ligands (DMLs) approach seems to be the most convenient tool to produce a molecule capable of targeting more than one pathway within the pathological process with a possible disease course modifying effect [21-28].
Monoamine oxidase B (MAO-B) enzyme appears to be a promising target for such therapeutic approach. MAO-B enzyme catalyzes the oxidative deamination of dopamine and several other amines [38] and by blocking the effect of this enzyme we can elevate the level of both endogenous and exogenous dopamine [29, 30]. The inhibition of MAO-B enzyme can also lower the production of hydrogen peroxide as a byproduct of oxidative deamination reaction [31]. It is believed that the produced hydrogen peroxide can undergo Fenton reaction in the presence of ferrous iron to yield hydroxyl radicals [163]. By lowering the level of hydrogen peroxide, we can minimize the activity of Fenton reaction and protect neurons from detrimental oxidative stress status.

\[
\text{Fe}^{2+} \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{OH}^- \quad \text{(Fenton reaction)}
\]

It is important to mention that the expression level of antioxidant enzymes decreases with age leading to a reduction in the antioxidant capacity of the brain [164, 165]. The cellular iron level inside the brain is elevated with age and with neurodegenerative conditions [92]. MAO-B but not MAO-A expression level inside the brain is increased with age [32, 33, 34, 87]. MAO-B is expressed in glial cells; the level of MAO-B inside the brain is elevated in patients with Parkinson's disease as a direct result of gliosis. Human basal ganglia shows high level of activity for MAO-B as compared with MAO-A and since both isozymes catalyze the oxidative deamination (termination) of dopamine neurotransmitter with equal efficiency then it is very logical from pharmacological perspective to target MAO-B selectively [36].
Based on monoamine oxidase inhibition assay results, it is very obvious that compounds 5143886 and 5140311 are highly potent and selective in terms of inhibition effect against MAO-B enzyme. This potency and selectivity in action can be explained through docking studies results; it appears that these two compounds can exhibit an extended conformation that traverses both the entrance cavity and substrate cavity of the active site in MAO-B enzyme. Such conformation will force Ile 199 residue to acquire an open configuration that lead to the fusion of these two cavities. It is believed that the configuration of Ile 199 residue plays a critical role in determining substrate and inhibitor binding specificity for MAO-B [167]. When small molecule (substrate or inhibitor) enters the active site of MAO-B, Ile 199 residue will obtain a closed conformation and this will produce the bipartite configuration of the active site. On the other hand, Isoleucine 199 residue will obtain an open conformation when long, planar molecule (extended conformation) enters the active site and this will result in the fusion of both entrance cavity and substrate cavity.

Another interesting finding in MAO-B inhibition assay results is that both compounds 7730595 and 7747412 seem to be potent inhibitors, however docking studies predict that these compounds don't enter the active site of MAO-B. The images generated by docking programs reveal that these two compounds occupy a position that is very close to the gating switch (loop 99-112), it is very clear to notice that these compounds are involved in hydrophobic interactions with amino acid residues in the gating loop (mainly Phe 103 and Pro 102). Based on these images, we can build an assumption that these compounds produce their inhibitory effect through retarding the accessibility of
substrates to the active site of MAO-B enzyme. This difference in docking position between compounds (5140311, 5143886) and compounds (7747412, 7730595) can be attributed to the difference in structural geometry, the structures of compounds 7747412 and 7730595 are bulkier (four to five rings system) than those for compounds 5140311 and 5143886 (two to three rings system). Although the chemical structure of compound 7919469 is composed of four rings, this compound appears to be capable of entering the active site of MAO-B enzyme as shown in docking images. According to MAO-B inhibition assay results, compound 7919469 seems to be a potent inhibitor. We expect that the four rings in this compound are arranged in a way that can provide a planar and an extended conformation and this will eventually allow compound 7919469 to access MAO-B active site and produce a potent inhibitory effect.

A recent literature proposed the presence of a conformational relationship between Ile 199 residue and Phe 103 residue [166]. When Ile 199 residue exhibits an open conformation, the side chain of Phe 103 residue will be forced to acquire a conformation that makes the gating switch (loop) closes the entrance into the active site of MAO-B. On the other hand, when Ile 199 residue is in closed conformation then the side chain of Phe 103 will obtain a conformation that favors opening of the gating loop. In the ligand free condition, these two conformations are rapidly interchangeable and this will provide accessibility to the active site of MAO-B enzyme.
Albumin is the most abundant form of plasma protein in human beings; it acts as a vehicle for the transport of many endogenous and exogenous compounds through reversible binding [142, 168]. Exploring the profile of drug binding to serum albumin is considered an important step in drug development process; such binding can influence pharmacokinetics and pharmacodynamics parameters of drug under investigation [169-173]. Binding of drug molecules to plasma albumin can change the volume of distribution; lower the rate of clearance and increase plasma half-life for that drug [174, 175]. There are two main binding regions in albumin molecule for aromatic and heterocyclic compounds, these binding regions are hydrophobic in nature and are located in subdomain IIA (site I) and subdomain IIIA (site II) [158,159]. Drug molecules usually bind to plasma albumin in a dynamic and reversible style with a very fast dissociation rate [176].

We have used bovine serum albumin (BSA) as an alternative for human serum albumin (HSA) for the binding assay because BSA is readily available and because of its structural homology with HSA [177]. Our results for BSA binding assay clearly show that most of the tested compounds have a high tendency for BSA binding; this may negatively affects the distribution of these compounds throughout body tissues and limits the accessibility to site of action. The only exceptions are compounds 5476423 and 5140311 which show moderate tendency to bind BSA.
One of the main challenges in producing a successful neurotherapeutic agent is to design a molecule capable of passing through the blood brain barrier (BBB) and reaching the required site of action [178]. Blood brain barrier (BBB) is the main site for molecular exchange between the brain and blood stream; it functions to maintain a stable microenvironment for the brain. This barrier is composed mainly from endothelial cells that line cerebral microvessels; it represents a combination of both physical and metabolic barriers. The physical barrier involves the presence of intercellular tight junctions between the endothelial cells with a very limited vesicular transport. The blood brain barrier also contains active transporters; some of these transporters transfer nutrients and other materials from blood to brain (influx) while the other transporters mediate the clearance of waste products and some drugs from brain to blood (efflux). The metabolic barrier involves phase I and phase II metabolic enzymes that can limit the ability of lipophilic compounds to penetrate BBB by converting them into a more hydrophilic products [179-181]. Most CNS active drugs penetrate BBB through transcellular passive diffusion process [143].

Parallel artificial membrane permeability assay (PAMPA) is used to predict BBB permeability potential for compound under investigation. We performed this assay only for compound 5140311; this compound was the only one that fluoresced at the excitation wave length range produced by the fluorescence reader. We calculated the value of effective permeability (Pe) as mentioned previously and then we used the following table (classification of PAMPA-BBB results based on Pe ranges provided by reference [160]) to interpret our results.
Table 4.1-Classification of PAMPA-BBB results.

<table>
<thead>
<tr>
<th>BBB permeability</th>
<th>$\text{Pe} \left(10^{-6}\text{ cm s}^{-1}\right)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (CNS+)</td>
<td>$&gt;4.0$</td>
</tr>
<tr>
<td>Low (CNS-)</td>
<td>$&lt;2.0$</td>
</tr>
<tr>
<td>Uncertain (CNS+/−)</td>
<td>4.0-2.0</td>
</tr>
</tbody>
</table>

The calculated $\text{Pe}$ value for compound 5140311 was equal to $5.2966 \times 10^{-6}$ cm s$^{-1}$ and according to this table, our compound is highly permeable.

Oxidation reaction can produce free radicals; these radicals can start a chain reaction that involves several steps (initiation, propagation, branching and termination of these radicals). Such chain of oxidative events can eventually damage cellular contents like lipid membranes, DNA and proteins. In general, antioxidants can block such reaction through two major mechanisms: They can either inhibit the formation of free radicals from their unstable precursors (preventive antioxidants) or they can break the reaction chain through inhibiting the propagation and branching steps (chain breaking antioxidants). Most antioxidants are classified as chain breaking agents; they exert their effect through scavenging free radicals. The chain breaking antioxidants should be able to act as good hydrogen donors in order to reduce free radicals. Once they donate hydrogen atom, they should form stable radicals that are unable to continue the chain reaction or they continue it but with very low efficiency [149, 182].
Based on our results for oxygen radical absorbance capacity (ORAC) assay, it is very clear that all of the tested compounds show high antioxidant capacity that is either equivalent or higher than that reported for Ascorbic acid (positive control). The only exception is compound 7954103 which shows minor antioxidant effect.

It is evident from all these virtual and in vitro assays that candidate 5140311 represents a potential lead compound for the development of new antiparkinsonian agents. This compound is a potent and selective inhibitor for MAO-B, it is highly permeable through BBB and shows moderate tendency for BSA binding. Finally this compound shows excellent potential for peroxyl radicals quenching.

As a conclusion for our basic structure activity relationship (SAR) study, it is very clear that 2-thioxo-1, 3-thiazolidin-4-one ring plays an essential role as a structural requirement for both MAO-B inhibition activity and radicals quenching potential. For MAO-B inhibitory effect, we believe that the oxygen atom attached to position four of 2-thioxo-1, 3-thiazolidine ring can be involved in hydrogen bonding with a certain amino acid residue or a conserved water molecule within the active site. The nitrogen atom in position three of the 2-thioxo-1, 3-thiazolidin-4-one ring can be also involved in the formation of hydrogen bond with a conserved active site water molecule. For antioxidant potential, we assume that amine group at position three of the 2-thioxo-1, 3-thiazolidin-4-one ring can act as a good hydrogen donor. The presence of thioxo group at position two of the 1, 3-thiazolidin-4-one ring appears to be essential for antioxidant activity.
8-hydroxyquinoline has been shown to exhibit a strong iron chelating activity and an antioxidant potential [183, 184]. This molecule has been also shown to be capable of passing through blood brain barrier [57]. 8-hydroxyquinoline has been successfully used as a building block for the design and synthesis of several novel bi-functional neuroactive compounds like HLA20 and M30 [103, 104, 105, 107]. Based on these facts and our results, it is very clear that 8-hydroxyquinoline represents a promising building block for iron chelating activity and radical scavenging potential.

By using 2-thioxo-1, 3-thiazolidin-4-one ring and 8-hydroxyquinoline as building blocks, we were able to design two possible hypothetical scaffolds for MAO-B inhibitors. We hope that the combination of these two building blocks can help us in producing potent, selective and centrally acting MAO-B inhibitors with a promising iron chelating effect and a possible antioxidant potential. Figure 4.1 and figure 4.2 show the chemical formula for our scaffolds (scaffolds A and B) together with initial results of docking analysis. Docking results for scaffold A in figure 4.1 confirm the presence of two hydrogen bonds; the first bond is between the oxygen atom attached to position four of 2-thioxo-1, 3-thiazolidine ring and the hydroxyl group of phenyl moiety in Tyr 435 (residue of aromatic cage) while the second hydrogen bond is between the hydroxyl group attached to position eight of the quinoline moiety and the sulfhydryl group of Cysteine 172 residue. In figure 4.2, docking images for scaffold B confirm the presence of one hydrogen bond between the oxygen atom attached to position four of 2-thioxo-1, 3-thiazolidine ring and the sulfhydryl group of Cysteine 172 residue.
5-[(8-hydroxyquinolin-7-yl) methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one

Figure 4.1(A)- Chemical structure together with IUPAC name for our first prospective scaffold (scaffold A).
Figure 4.1(B)- Two dimensional representation for the docking of scaffold A into MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153]. Hydrogen bonds are represented by green dashed lines.
Figure 4.1(C)- Three dimensional representation for the docking of scaffold A into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red and the hydrogen bonds are shown as cyan lines.
Figure 4.2(A)- Chemical structure together with IUPAC name for our second prospective scaffold (scaffold B).

5-[(8-hydroxyquinolin-5-yl) methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one

Scaffold B

Molecular weight = 288.34 g/mole
Figure 4.2(B)- Two dimensional representation for the docking of scaffold B into MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153]. Hydrogen bonds are represented by green dashed lines.
Figure 4.2(C)- Three dimensional representation for the docking of scaffold B into MAO-B crystal. This image was generated by PyMOL v.1.3 (www.schrodinger.com) [154]. Our ligand is colored by red and the hydrogen bonds are shown as cyan lines.
As part of our attempts to refine the overall structure of these scaffolds and to optimize their expected pharmacokinetic profile, virtual evaluation of oral bioavailability and CNS permeability was performed for both scaffolds. For this purpose, a number of physicochemical parameters were initially calculated by using ChemAxon’s calculators and calculator plugins (www.chemaxon.com). These parameters were then assessed for their degree of obedience to Lipinski’s rule of five for oral bioavailability [185] and Norinder’s two rules (AstraZeneca’s rules) for CNS permeability [186]. For simplicity in presentation, we have listed the standards for the above rules together with our calculated parameters in tables 4.2 and 4.3.

Based on data in table 4.2, we can clearly notice that both scaffolds A and B will probably show a good oral activity profile. These scaffolds are in complete agreement with Lipinski’s rule of five. In table 4.3, on the other hand, these two scaffolds deviate from the last two standards for CNS permeability (parameters with red color). Such deviation may cause a future problem in CNS accessibility during lead optimization. From our point of view, we expect that such probable obstacle can be defeated by reducing the size of polar mass in these scaffolds. We believe that removal of nitrogen atom from 8-hydroxyquinoline part can enhance the value of \( \{ \log P - (N+O) \} \) and this will eventually increase the value of log BB. Such modification in the structure of these scaffolds will also lower the value of PSA (Polar surface area) and by this way the modified scaffolds will obey all CNS permeability rules. Based on this assumption, we have modified the structure of scaffolds A and B by using hydroxynaphthalene moiety instead of 8-hydroxyquinoline and this gave us two new scaffolds, namely scaffold C and
scaffold D. To avoid redundancy, we have listed the physicochemical parameters for these modified scaffolds in table 4.2 and 4.3 also. Based on these parameters, we can easily observe that these modified scaffolds (C and D) will probably have a good oral absorption profile and a satisfactory CNS permeation capacity.

Docking studies in figure 4.3 show that scaffold C exhibits a very similar configuration to that of scaffold A within MAO-B crystal. In figure 4.4, MAO-B docking studies for scaffold D show that the thiazolidine ring occupies a reversed orientation as compared to that of scaffold B. This reversed direction can be attributed to rotation of the single bond between 4-hydroxynaphthalene moiety and methyldiene group (the only rotatable bond in our design); this rotation will lead to the loss of hydrogen bond between the oxygen atom attached to position four of 2-thioxo-1, 3-thiazolidine ring and the sulphydryl group of Cysteine 172 residue.

Such proposed structural modification (removal of nitrogen atom from 8-hydroxyquinoline) may not adversely impact MAO-B inhibition capacity of our design. It is believed that hydroxynaphthalene moiety can quench radical species through single electron transfer process. This will convert hydroxynaphthalene molecule into a stabilized radical form which in turn may undergo dimerization (coupling) reaction [189]. In addition some scientific literatures have shown that phenolic compounds can form complex with iron [190]. Based on these facts, we hope that both scaffolds C and D can still meet our goals to produce a potent, selective and centrally acting MAO-B inhibitor with a possible antioxidant activity and a promising iron chelating ability.
Table 4.2- The obedience degree to Lipinski’s rule of five. It is important to mention that the number of rotatable bonds condition in this table was obtained from Veber’s rules for oral bioavailability of drug candidates [187].

<table>
<thead>
<tr>
<th>Physicochemical parameter</th>
<th>Standard value</th>
<th>Scaffolds A&amp;B</th>
<th>Scaffolds C&amp;D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular Weight</strong></td>
<td>≤ 500 g/mol</td>
<td>288.34 g/mol</td>
<td>287.36 g/mol</td>
</tr>
<tr>
<td><strong>Log P</strong></td>
<td>≤ 5</td>
<td>2.59</td>
<td>3.43</td>
</tr>
<tr>
<td><strong>H-bond donors (OH + NH)</strong></td>
<td>≤ 5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>H-bond acceptors (O + N)</strong></td>
<td>≤ 10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Number of rotatable bonds</strong></td>
<td>≤ 10</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4.3- The obedience degree to Norinder’s rules (AstraZeneca’s rules) for CNS permeability. It is important to notice that the third condition for PSA value in this table was obtained from reference [188].

<table>
<thead>
<tr>
<th>Physicochemical parameter</th>
<th>Standard value</th>
<th>Scaffolds A&amp;B</th>
<th>Scaffolds C&amp;D</th>
</tr>
</thead>
<tbody>
<tr>
<td>N + O atoms count</td>
<td>≤ 5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Log P – (N + O) value</td>
<td>&gt; 0 (positive)</td>
<td>-1.41</td>
<td>+0.43</td>
</tr>
<tr>
<td>Polar surface area (PSA)</td>
<td>&lt; 60 – 70 Å²</td>
<td>62.22 Å²</td>
<td>49.33 Å²</td>
</tr>
</tbody>
</table>
5-[(1-hydroxynaphthalen-2-yl) methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one

Figure 4.3(A)- Chemical structure together with IUPAC name for the modified scaffold (scaffold C).
Figure 4.3(B)- Two dimensional representation for the docking of scaffold C into MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153]. Hydrogen bonds are represented by green dashed lines.
5-[(4-hydroxynaphthalen-1-yl) methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one

Figure 4.4(A)- Chemical structure together with IUPAC name for the modified scaffold (scaffold D).
Figure 4.4(B)- Two dimensional representation for the docking of scaffold D into MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153].

Binding Energy: -8.83 kcal/mol

kI : 337.25 nM
As a final attempt to defeat the blood brain barrier and to improve the overall kinetic profile of our design, we found that it is more convenient to target the transporters system within this barrier. Several previous researches have shown that attachment of L-alanine residue to certain drugs can enhance the CNS accessibility of these drugs. The addition of such neutral amino acid residue will make the whole resultant molecule a good substrate for large neutral amino acid transporter (uptake system L). Such strategy was employed successfully for the selective delivery of several drugs into the brain and good examples in our case are L-dopa and the experimental drug M10 [107]. The attachment of L-alanine residue to scaffold A or scaffold B will make these chemicals more hydrophilic (elevates polar surface area) and this will lower their passive permeation into tissues other than the brain. Through such addition, we can enhance the selective delivery of our chemicals into the brain and minimize the unwanted side effects of our design. We expect that such modification in our design will not negatively impact MAO-B inhibition effect, iron chelation capacity or radicals quenching potential. The structural formula and docking images for the two new scaffolds (L-alanine derivatives) can be seen in figure 4.5. The application of Lipinski’s rule of five shows that these two new scaffolds (E and F) may be still able to pass gastrointestinal tract as can be seen in table 4.4.

In order to expand the scope of our possible opportunities, we are hereby presenting a new design for another possible scaffold. The new design (scaffold G) combines three essential building blocks and these are: 2-thioxo-1, 3-thiazolidin-4-one ring (for MAO-B inhibitory effect and antioxidant capacity), catechol moiety (for iron
chelation activity and radical quenching potential) and L-alanine residue (for selective delivery to the brain). We believe that the core of our research is represented in this new design (scaffold G). The docking image and virtual pharmacokinetic parameters for this scaffold can be seen in figure 4.6 and table 4.4.
2-amino-3-(8-hydroxy-7-\{[4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene]methyl\}quinolin-4-yl)propanoic acid

Figure 4.5(A)- Chemical structure together with IUPAC name for the modified scaffold (scaffold E).
Binding Energy: -8.42 kcal/mol
kI : 667.65nM

Figure 4.5(B)- Two dimensional representation for the docking of scaffold E into MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153]. Hydrogen bonds are represented by green dashed lines.
2-amino-3-(8-hydroxy-5-\{[4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene]methyl\}quinolin-2-yl)propanoic acid

Figure 4.5(C)- Chemical structure together with IUPAC name for the modified scaffold (scaffold F).
Figure 4.5(D)- Two dimensional representation for the docking of scaffold F into MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153]. Hydrogen bonds are represented by green dashed lines.
2-amino-3-(2,3-dihydroxy-4-{[4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene]methyl}phenyl)propanoic acid

Figure 4.6(A)- Chemical structure together with IUPAC name for our novel design (scaffold G).
Figure 4.6(B)- Two dimensional representation for the docking of scaffold G into MAO-B crystal, this image was generated by using LigPlot+ v.1.4.5 [153]. Hydrogen bonds are represented by green dashed lines.
Table 4.4- The application of Lipinski’s rule of five to scaffolds E, F and G. The last two conditions (number of rotatable bonds and polar surface area) were obtained from Veber’s rules for oral bioavailability of drug candidates [187].

<table>
<thead>
<tr>
<th>Physicochemical parameter</th>
<th>Standard value</th>
<th>Scaffolds E &amp; F</th>
<th>Scaffold G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>≤ 500 g/mol</td>
<td>375.42 g/mol</td>
<td>340.37 g/mol</td>
</tr>
<tr>
<td>Log P</td>
<td>≤ 5</td>
<td>1.7</td>
<td>1.24</td>
</tr>
<tr>
<td>H-bond donors (OH + NH)</td>
<td>≤ 5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>H-bond acceptors (O + N)</td>
<td>≤ 10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Number of rotatable bonds</td>
<td>≤ 10</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Polar surface area (PSA)</td>
<td>≤ 140 Å²</td>
<td>125.54 Å²</td>
<td>132.88 Å²</td>
</tr>
</tbody>
</table>
Our preliminary data indicate that compounds with a close structural formula to that of both scaffolds A and B are effective in MAO-B inhibition assay; some of these compounds were able to display an IC50 value within micro Molar range. One of these compounds, the one that closely resembles scaffold A, was superior in MAO-B inhibition to Zonisamide. It is important to mention that Zonisamide is a well-known anticonvulsant agent with antiparkinsonian effect (reversible MAO-B inhibitor); it was used as a positive control in our MAO-B inhibition assay. Based on these recent data, we are currently trying to synthesize both scaffolds A and B in our lab. Characterization of these two scaffolds and refining their structural formula appears to be a promising future plan to produce and optimize an antiparkinsonian lead compound. The newly proposed structural modification (generation of scaffolds C, D, E and F) may also expand the scope of our future plans, goals and opportunities. We believe that scaffold G is a novel design for a multifunctional antiparkinsonian agent and we hope that such design can see the light in the nearby future.
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