Monoamine oxidase inhibitory activities of heterocyclic chalcones

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ABSTRACT

TITLE

Monoamine oxidase inhibitory activities of heterocyclic chalcones.

KEYWORDS

Chalcones, monoamine oxidase inhibitors, Parkinson's disease.

BACKGROUND AND RATIONALE

Parkinson's disease is the second most common age-related neurodegenerative disease after Alzheimer's disease. The characteristic pathological feature of Parkinson's disease is the loss of neurons in the substantia nigra pars compacta (SNpc), which leads to a striatal dopamine deficiency responsible for the major symptoms of Parkinson's disease. These symptoms include tremor at rest, postural instability, bradykinesia and in the later stages of Parkinson's disease, even psychosis.

Presently, there is still no cure for Parkinson's disease and all treatments are only symptomatic. Current research is therefore directed towards the prevention of further dopaminergic neurodegeneration, while the ultimate aim is the reversal of neurodegeneration.

Monoamine oxidase (MAO) enzymes are responsible for the regulation and metabolism of monoamine neurotransmitters, such as dopamine. There are two MAO isoforms, MAO-A and MAO-B. Since MAO-B has greater activity in the basal ganglia, it is of particular importance in movement disorders, which include Parkinson's disease. The selective inhibition of MAO-B, increases dopamine available for binding, and thus reduces Parkinson's disease symptoms.

MAO inhibitors also have neuroprotective potential and thus may slow down, halt and even reverse neurodegeneration in Parkinson's disease. It is still unclear exactly how MAO inhibitors protect neurons, but one theory suggests that MAO inhibition decreases oxidative stress by reducing the formation of hydrogen peroxide, a metabolic by-product of MAO oxidation of monoamines. Normally, hydrogen peroxide is inactivated by glutathione (GSH), however, in Parkinson's disease, GSH levels are low, resulting in the accumulation of hydrogen peroxide, which then becomes available for the Fenton reaction. In the Fenton reaction, Fe^{2+} reacts with hydrogen peroxide and generates an active free radical, the hydroxyl radical. This radical depletes cellular anti-oxidants,

damage lipids, proteins and DNA. MAO inhibitors reduce the formation of hydrogen peroxide thus decreasing the formation of hydroxyl radicals and oxidative stress.

The MAO inhibitory potential of natural and synthetic chalcones have been illustrated. For example, in 1987, Tanaka and co-workers determined that natural chalcones, such as isoliquiritigenin, have MAO inhibitory activity in rat mitochondria. In 2009, Chimenti and co-workers synthesized a series of 1,3-diphenyl-2-propen-1-ones which exhibited human MAO-B (hMAO-B) selective inhibitory activity. On the other hand, Robinson and co-workers (2013), synthesized novel furanochalcones which also had hMAO-B selective inhibitory activity. A reversible, competitive mode of binding was demonstrated by these compounds. Since the effect of heterocyclic substitution, other than furan on the MAO inhibitory properties of the chalcone scaffold remains unexplored, the aim of this study was to synthesize and evaluate further heterocyclic chalcone analogues as inhibitors of hMAO.

RESULTS

Design and synthesis: Heterocyclic chalcone analogues that incorporated pyrrole, 5-methylthiophene, 5-chlorothiophene and 2-methoxypyridine substitution were synthesized using the Claisen-Schmidt condensation reaction. All compounds were characterized with ^{1}H-NMR, ^{13}C-NMR, IR, MS, and melting points were recorded. Purity was determined with HPLC analysis.

MAO inhibition studies: The 50% inhibitory concentration (IC_{50}) values and selectivity index (SI) of all compounds were determined using a fluorometric assay and kynuramine as substrate. Eight out of the ten synthesized compounds exhibited IC_{50} values < 1 µM, and can thus be considered as potent MAO-B inhibitors, while all compounds showed selectivity for the MAO-B isoform. Compound **10i** was the most potent MAO-B inhibitor with an IC_{50} value of 0.067 µM and the highest SI of 240.7. The most potent MAO-A inhibitor, compound **10f**, had an IC_{50} value of 3.805 µM. Some structure-activity relationships were derived, for example; it was concluded that heterocyclic substitution with 5-methyl-thiophene ring resulted in optimal hMAO-B inhibition, while pyrrole substitution was less favourable. Further investigation is however required as this is only a preliminary study.

Reversibility studies: To determine the reversibility of binding, the recovery of enzymatic activity after dilution of the enzyme inhibitor complexes were determined for selected compounds. Results indicated that the most potent MAO-A inhibitor, the pyrrole derivative **10f,** had a reversible mode of binding to both the hMAO-B and hMAO-A isoforms, since the enzyme activities were completely recovered by dilution of the inhibitor concentration. In contrast, enzyme activity was only partially recovered after dilution of the most potent MAO-B inhibitor **10i**, indicating that this methylthiophene derivative possibly exhibited tight binding to the hMAO-B isoform, and the inhibition caused by this compound was not readily reversed by dilution. In order to determine whether the tight binding as

exhibited by compound **10i** was due to the thiophene or phenyl moieties, reversibility of binding was also determined for the pyrrole derivative **10e**. The results showed that **10e** had a reversible mode of binding to the hMAO-B isoform, and enzyme activity was completely recovered by dilution of the inhibitor. Based on these results, it was concluded that the tight binding as exhibited by compound **10i** was due to the presence of the thiophene moiety. To confirm that compound **10i** exhibited tight, and not irreversible binding, reversibility of binding was also determined by dialysis of enzyme-inhibitor mixtures. For this purpose hMAO-B and **10i**, at a concentration of 4 × IC_{50}, were preincubated for a period of 15 min and subsequently dialyzed for 24 h. The results of this study showed that **10i** had a reversible mode of binding for MAO-B, since enzyme activity was recovered to a level of 83% after dialysis.

Mode of inhibition: To determine the mode of inhibition of compound **10f,** Lineweaver-Burk plots were constructed for the inhibition of hMAO-A and hMAO-B. The lines of the Lineweaver-Burk plots intersected at a single point at the y-axis, indicating that **10f** had a competitive mode of binding to both hMAO-B and hMAO-A isoforms.

MTT viability assay: To determine the toxicity of the chalcones for cultured cells, selected compounds were evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. The cytotoxicity of the test compounds were evaluated at concentrations of 1 and 10 µM, in HeLa cells. The results indicated that compound **10i** was non-toxic at 1 and 10 µM, with 100% and 96% cell viability remaining after 24 h exposure of the compound to the cultured cells. Compound **10f**, however, exhibited significant toxicity at 10 µM, with only 5% viable cells remaining. In contrast, compound **10e**, with the same pyrrole moiety as **10f**, was non-toxic at 1 µM and 10 µM, with 99% and 98%, cell viability remaining. It was concluded that the pyrrole moiety of **10f** was not responsible for its higher degree of cytotoxicity, which suggests that the CF_3 substituent may play a role in the higher degree of cytotoxicity observed for **10f**. Further investigation is required to determine the mode of cytotoxicity, when cultured cells are exposed to **10f**.

Docking Studies: To complete this study and rationalise the results of the MAO inhibition studies, molecular modelling was carried out and all compounds were docked into the crystal structure of hMAO-B, by using the CDOCKER module of Discovery Studio. Some insights were obtained regarding the binding of compound **10i**. This compound bound to MAO-B with the phenyl ring facing the FAD cofactor. This orientation allowed for the formation of pi-pi interaction with Tyr 398 as well as a pi-sigma interaction between the thiophene ring and Ile 199 (which is part of the gating switch of MAO-B). It is speculated that the tight binding component of hMAO-B inhibition by **10i** may, at least in part, be attributed to the interaction of this compound with the gating switch amino acid, Ile 199. The docking results also showed that most compounds interacted with Tyr 326 or Tyr 398, while interactions with Cys 172, Gln 206, Ile 199 and Tyr 435 also occurred.

In conclusion, novel heterocyclic chalcone analogues with promising MAO-B inhibitory activities were successfully synthesized and evaluated.

OPSOMMING

TITEL

Monoamienoksidase inhiberende aktiwiteite van heterosikliese chalkone.

SLEUTELWOORDE

Chalkone, monoamienoksidase-inhibeerders, Parkinson se siekte.

AGTERGROND EN RATIONAAL

Naas Alzheimer se siekte, is Parkinson se siekte die tweede mees algemene ouderdomsverwante neurodegeneratiewe siekte. Patologies word Parkinson se siekte gekenmerk deur die afsterwing van neurone in die substantia nigra pars compacta (SNpc) wat lei tot ‘n tekort aan dopamien in die striatum. Hierdie tekort is dan ook verantwoordelik vir die simptome van Parkinson se siekte. Die simptome sluit tremor tydens rus, posturale onstabiliteit, bradikinesie en selfs psigose in die latere fases van die siekte in.

Daar is tans geen kuur vir Parkinson se siekte nie en alle behandeling is slegs simptomaties van aard. Navorsing is dus tans gemik op die voorkoming van verdere degenerasie van die dopaminergiese neurone met die hoofdoel om omkering van neurodegenerasie te bewerkstellig.

Monoamienoksidase (MAO) ensieme is verantwoordelik vir die regulering en metabolisme van monoamienneuro-oordragstowwe, soos dopamien. Daar is twee MAO-isoforme, naamlik MAO-A en MAO-B. Aangesien MAO-B groter aktiwiteit in die basale ganglia het, speel dit dus ‘n belangrike rol in bewegingsteurnisse, soos Parkinson se siekte. Selektiewe inhibisie van MAO-B verhoog die dopamienkonsentrasie beskikbaar vir binding, en verlig so die simptome van die siekte.

MAO-inhibeerders bied ook die moontlikheid van neurobeskerming en mag die neurodegenerasie soos waargeneem in Parkinson se siekte vertraag, stop of moontlik selfs omkeer. Daar is onsekerheid oor presies hoe MAO-inhibeerders neurone beskerm. Een teorie is dat inhibisie van MAO oksidatiewe stres, soos veroorsaak deur waterstofperoksied, ‘n metaboliese byproduk van die oksidasie van monoamiene deur MAO, verminder. Normaalweg word waterstofperoksied deur glutatioon (GSH) geïnaktiveer, maar in Parkinson se siekte is die GSH vlakke ongewoon laag. Dit veroorsaak dat waterstofperoksied ophoop, en beskikbaar is vir die Fentonreaksie. Gedurende die Fentonreaksie reageer Fe^{2+} met waterstofperoksied en vorm ‘n aktiewe vryradikaal, die hidroksielradikaal. Hierdie radikaal put die sellulêre antioksidante uit, en beskadig lipiede, proteïene en DNA. MAO-inhibeerders verminder die vorming van waterstofperoksied, en verlaag dus ook die vorming van vry radikale en oksidatiewe stres.

Die potensiaal van natuurlike en gesintetiseerde chalkone om MAO te inhibeer is voorheen geïllustreer. In 1987, het Tanaka en medewerkers byvoorbeeld aangetoon dat natuurlike chalkone, soos isolikwiritigenien, MAO inhiberende aktiwiteit in rot mitochondria het. In 2009, het Chimenti en medewerkers ‘n reeks 1,3-difeniel-2-propen-1-one gesintetiseer, wat menslike MAO-B (mMAO-B) selektief geïnhibeer het. Aan die ander kant, het Robinson en medewerkers (2013), furaangesubstitueerde chalkoonderivate gesintetiseer wat mMAO-B ook selektief geïnhibeer het. ‘n Omkeerbare en kompeterende meganisme van binding vir die MAO-B-isoform is vir dié verbindings aangetoon. Aangesien die effek van heterosikliese substitusie, buite furaansubstitusie, op die MAO inhiberende eienskappe van chalkone nog nie ondersoek is nie, was die doel van hierdie studie om verdere heterosikliese chalkoonanaloë as inhibeerders van MAO te sintetiseer en te evalueer.

RESULTATE

Ontwerp en sintese: Heterosikliese chalkoonanaloë, gesubstitueer met pirrool, 5-metieltiofeen, 5-chlorotiofeen en 2-metoksipiridien groepe is gesintetiseer deur van ‘n Claisen-Schmidt kondensasiereaksie gebruik te maak. Karakterisering van al die verbindings is met ^{1}H-KMR, ^{13}C-KMR, IR en MS gedoen, terwyl smeltpunte ook bepaal is. ‘n HPLC-analise is uitgevoer om suiwerheid te bepaal.

MAO-inhibisiestudies: ‘n Fluorometriese toets, met kinuramien as substraat, is gebruik om die 50% inhiberende konsentrasie (IC_{50}) waardes en die selektiwiteitsindeks (SI) van al die verbindings te bepaal. Agt uit die tien gesintetiseerde verbindings het ‘n IC_{50} waarde < 1 µM getoon, en kan dus as potente MAO-B-inhibeerders geklassifiseer word. Al die verbindings was selektief vir die MAO-B-isoform. Verbinding **10i**, was die mees potente MAO-B-inhibeerder met ‘n IC_{50} waarde van 0.067 µM en het ook die hoogste SI waarde van 240.7. ‘n IC_{50} waarde van 3.805 µM is bepaal vir die mees potente MAO-A-inhibeerder, verbinding **10f**‘. ‘n Paar struktuuraktiwiteitsverwantskappe kon afgelei word, byvoorbeeld: daar is aangedui dat heterosikliese substitusie met ‘n 5-metieltiofeenrring tot optimale MAO-B inhibisie lei, terwyl pirroolsubstitusie minder gewens is. Verdere navorsing in hierdie verband word egter benodig aangesien hierdie slegs ‘n voorlopige studie is.

Omkeerbaarheidstudies: Om te bepaal of MAO-binding omkeerbaar was, is die herstel van ensiematiese aktiwiteit na verdunning van die ensiem-inhibeerder-komplekse bepaal vir geselekteerde verbindings. Die resultate het aangedui dat die mees potente MAO-A-inhibeerder, die pirroolderivaat **10f,** omkeerbaar bind aan beide die mMAO-A en mMAO-B-isoforme - die ensiemaktiwiteit het dus heeltemal herstel na verdunning van die inhibeerder konsentrasies. In teenstelling daarmee, het die ensiemaktiwiteit van die mees potente MAO-B-inhibeerder **10i**, slegs gedeeltelik herstel. Dit dui aan dat die metieltiofeenderivaat **10i,** stewig aan die mMAO-B-isoform

bind en dat die inhibisie deur die verbinding nie maklik omgekeer word wanneer dit verdun word nie. Om te bepaal of die stewige binding deur **10i**, as gevolg van die tiofeen- of fenielgroep was, is die omkeerbaarheid van 'n ander pirroolderivaat, **10e**, ook bepaal.

Die resultate van verbinding **10e** se verdunningstoets het aangedui dat die verbinding omkeerbaar aan die mMAO-B-isoform bind - die ensiemaktiwiteit is volkome herstel na verdunning van die inhibeerder. Gevolglik blyk dit dat die stewige binding soos gesien is by **10i**, toegeskryf kan word aan die teenwoordigheid van die tiofeengroep. Om te bevestig dat verbinding **10i** net stewig, en nie on-omkeerbaar bind nie, is die omkeerbaarheid van die verbinding verder bepaal deur gebruik te maak van 'n dialisetoets, waartydens die dialise van die ensiem-inhibeerdermengsel plaasvind. Die mMAO-B en **10i**, by 'n konsentrasie van 4 × IC_{50}, is gepreïnkubeer vir 15 min en daarna vir 24 h gedialiseer. Die resultate van hierdie studie het aangedui dat **10i** omkeerbaar bind aan die MAO-B-isoform, aangesien die ensiemaktiwiteit na 83% van die van die kontrole herstel het.

Meganisme van inhibisie: Om die meganisme van inhibisie van verbinding **10f** te bepaal, is Lineweaver-Burk grafieke opgestel vir die inhibisie van beide MAO-A en MAO-B. Die lyne van die Lineweaver-Burk grafieke het gekruis by 'n enkele punt op die y-as, wat aandui dat **10f** kompeterend aan beide die mMAO-B en mMAO-A-isoforme bind.

MTT- sellewensvatbaarheidstoets: Om die toksisiteit van die chalkone vir gekweekte selle te bepaal, is geselekteerde verbindings geëvalueer deur die 3-(4,5-dimetieltiasool-2-iel)-2,5-difenieltetrasolium bromied (MTT) lewensvatbaarheidstoets uit te voer. Die sitotoksisiteit van die toetsverbindings is geëvalueer by konsentrasies van 1 en 10 µM, in HeLa-selle. Die resultate het aangetoon dat verbinding **10i** nie toksies is by konsentrasies van 1 en 10 µM nie, aangesien sellewensvatbaarheid van 100% en 96%, na blootstelling van die gekweekte selle aan die verbindings vir 24 h, verkry is. Pirroolderivaat **10f**, aan die ander kant, het toksisiteit getoon by 'n konsentrasie van 10 µM, aangesien slegs 5% lewensvatbare selle na blootstelling verkry is. In teenstelling daarmee, het verbinding **10e**, wat ook gesubstitueer is met 'n pirroolgroep, geen toksisiteit getoon by 1 µM of 10 µM nie, aangesien 99% en 98%, sellewensvatbaarheid verkry is. Dit blyk dus dat dit nie die pirroolgroep is wat verantwoordelik is vir die toksisiteit van **10f** nie, maar dat die trifluorofenielgroep 'n rol speel in die waargenome sitotoksisiteit van **10f**. Verdere navorsing in hierdie verband word egter benodig.

Molekulêre modelleringstudies: Om die resultate van die MAO- inhibisiestudies te rasionaliseer en die studie af te sluit is molekulêre modellering van al die verbindings in Discovery Studio gedoen deur gebruik te maak van die CDOCKER-module. Al die verbindings is gepas in die kristalstruktuur van mMAO-B. 'n Paar insigte is verkry rakende verbinding **10i** se biologiese eienskappe. Die verbinding het aan MAO-B gebind met die fenielring aan die kant van die FAD- ko-faktor. Hierdie oriëntasie het veroorsaak dat 'n pi-pi interaksie met Tyr 398 kon vorm, asook 'n pi-sigma interaksie tussen die tiofeenring en Ile 199 (wat deel is van die hekskakelaar van MAO-B). Daar word

gespekuleer dat die stewige binding tussen **10i** en hMAO-B gedeeltelik toegeskryf kan word aan dié interaksie van hierdie verbinding met hierdie aminosuur. Die molekulêre modelleringsresultate het verder aangedui dat die meeste verbindings interaksies met Tyr 326 of Tyr 398 het, terwyl interaksies met Cys 172, Gln 206, Ile 199 en Tyr 435 ook voorgekom het.

Om saam te vat, in hierdie studie is nuwe, heterosikliese chalkoonderivate met positiewe MAO-B inhiberende aktiwiteite suksesvol gesintetiseer en geëvalueer.

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ABBREVIATIONS

3-OMD	3-O-methyldopa
5-HIAA	5-Hydroxyindole acetic acid
5-HT	Serotonin also known as (5-hydroxy-tryptamine)
6-OHDA	6-Hydroxydopamine
β-PEA	^{14}[C] β-phenylethylamine
ADP	Adenosine 5'-diphosphate
APCI	Atmospheric-pressure chemical ionization
Arg	Arginine
ATP	Adenosine 5'-triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
calcd	Calculated
CBF	Cerebrospinal fluid
$CDCl_3$	Deuterochloroform
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal fluid
Cys	Cysteine
DA	Dopamine
DDC	Dopa decarboxylase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMSO-*d6*	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPAC	Dihydroxy-phenyl acetic acid
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethyl alcohol

FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
GDNF	Glial-derived neurotrophic factor
Gln	Glutamine
GPO	Glutathione peroxidase
GSH	Glutathione
h	Hours
His	Histidine
hMAO	Human monoamine oxidase
h-MAO-A	Human monoamine oxidase type A
h-MAO-B	Human monoamine oxidase type B
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
IR	Infrared
IL	Interleukin
Ile	Isoleucine
LAT	L-amino acid transporter
L-DOPA	Levodopa also known as [(-)-3-(3,4-dihydroxyphenyl)-L-alanine]
Leu	Leucine
LRRK-2	Leucine rich repeat kinase 2
Lys	Lysine
MAO	Monoamine oxidase
MAO-A	Monoamine oxidase type A
MAO-B	Monoamine oxidase type B
min	Minutes
mp	Melting point
MPP^+	1-Methyl-4-phenylpyridinium
$MPDP^+$	1-Methyl-4-phenyl-2,3-dihydropyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Reduced nicotinamide adenine dinucleotide
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NSAIDS	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
Phe	Phenylalanine
PDB	Protein data bank
ROS	Reactive oxygen species
rt	Room temperature
SD	Standard deviation
SET	Single electron transfer
SI	Selectivity index
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-alpha gene
Trp	Tryptophan
Tyr	Tyrosine
UCHL-1	Ubiquitin C-terminal hydrolase L 1
UV	Ultraviolet
VTA	Ventral tegmental area

NMR:

δ	Dalta scale used to indicate chemical shift
br d	Broad doublet

br s	Broad singlet
br t	Broad triplet
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
J	Coupling constant in Hz
m	Multiplet
p	Pentet
ppm	Parts per million
q	Quartet
s	Singlet
t	Triplet

Biological assays:

Abs	Absorbance as read by the spectrophotometer
$Abs_{(negative\ control)}$	Absorbance of cells without treatment
$Abs_{(positive\ control)}$	Absorbance of the cells treated with 0.3% formic acid (100% cell death)
$Abs_{(sample)}$	Absorbance from spectrophotometer of the sample
[I]	Inhibitor concentration
K_i	The equilibrium constant used to indicate the reversibility of an enzyme-inhibitor complex
K_m	Michaelis-Menten constant: substrate concentration that produces half maximal velocity.
[S]	Substrate concentration
V_i	The measured initial velocity
V_{max}	Maximum velocity

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