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Neuroprotection of Amphetamine and Methylphenidate psychostimulants against dopaminergic neurotoxins in an *in vitro* Parkinson model

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. Still, it is the most common neurodegenerative movement disorder, affecting a significant percentage of the world's population. Its incidence will certainly rise with the ageing population. PD is mainly characterised by the loss of dopaminergic neurons with subsequent depigmentation of the substantia nigra pars compacta region of the brain. Overall and biochemically, it leads to a decrease in dopamine (DA) levels and is accompanied with Lewy's bodies. The pathogenesis of PD is not completely known, but it is thought that mitochondrial dysfunction, oxidative stress and neuroinflammation may be involved. In addition, there is evidence that PD has some genetic predisposition in some of its forms. PD is still a debilitating condition for which no effective and modifiable therapies have been identified to treat the disease, so research to discover new therapies is mandatory.

Psychostimulants like amphetamine (AMPH) and methylphenidate (MPH), currently used in treatment of attention deficit hyperactivity disorder and narcolepsy, are pharmacologically inhibitors of DA reuptake, and seem to interfere with the vesicular monoamine transporter 2. This dissertation aimed to evaluate their neuroprotective potential against three known dopaminergic neurotoxins, 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium ion (MPP⁺) and paraquat (PQ), which have been used as experimental toxins for inducing PD *in vitro*.

Differentiated human dopaminergic SH-SY5Y cells were the model used herein and they were exposed to therapeutic concentrations of AMPH and MPH (0.001- 10 μ M). Firstly, their cytotoxicity was assessed by neutral red (NR) incorporation and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assays after a 24 h exposure. No toxicity was found for all concentrations tested. Also, there was no mitochondrial membrane potential changes evoked by the drugs, evaluated by JC-1 probe. The 1 μ M concentration of both drugs was selected for further studies.

Regarding the toxins, 6-OHDA (31.25-500 μ M), MPP⁺ (125-1500 μ M) and PQ (100-5000 μ M) cytotoxicity was evaluated in differentiated SH-SY5Y cells by the NR uptake and MTT reduction assays, morphological analysis, and Hoechst nuclear staining. Moreover, reactive species and mitochondrial membrane potential were determined with different fluorescent probes, also at 24 h, in order to analyse their toxic potential and mechanisms behind it. 6-OHDA, MPP⁺ and PQ caused mitochondrial dysfunction and lysosome damage followed by neuronal death, after a 24 h exposure and in the case of 6-OHDA and PQ in a

concentration-dependent manner. 6-OHDA (62.5 and 125 μ M) and PQ (500 μ M) decreased mitochondrial membrane potential, and in addition 6-OHDA (62.5 and 125 μ M) promoted reactive oxygen species increase. Furthermore, the analysis by phase contrast revealed that 6-OHDA (125 μ M) and PQ (500 μ M) promoted cellular death accompanied by dendrite loss. Additionally, the analysis after Hoechst staining revealed that 6-OHDA (125 μ M), MPP⁺ (1500 μ M) and PQ (500 μ M) evoked features of apoptotic cell death.

When assessing the putative neuroprotection of AMPH and MPH towards the dopaminergic toxins, it was found that these drugs prevented PQ-induced cell death in the MTT assay, but this was not observed in the NR assay. There was no evidence that these drugs provided any protection against exposure to 6-OHDA and MPP⁺ in the MTT or NR assays. When assessing reactive species, there was no evidence that AMPH or MPH decreased the reactive species production elicited by 6-OHDA. Instead, MPH and especially AMPH, demonstrated to increase in reactive species production by themselves during the 24 h of exposure.

In conclusion, AMPH and MPH appear to protect SH-SY5Y cells from PQ-induced toxicity, but more studies need to be done to try to understand the mechanism behind this neuroprotective effect.

Keywords: amphetamine, methylphenidate, Parkinson's disease, neurotoxin dopaminergic, neuroprotection

Resumo

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum depois da doença de Alzheimer. É, no entanto, a doença neurodegenerativa do movimento mais comum, afetando uma percentagem significativa da população mundial. A sua incidência irá certamente aumentar com o envelhecimento da população. A DP caracteriza-se principalmente pela perda de neurónios dopaminérgicos com subsequente despigmentação de uma região do cérebro, a substantia nigra *pars compacta*. Na generalidade e bioquimicamente, conduz a uma diminuição dos níveis de dopamina (DA) e é acompanhada com o aparecimento de corpos de Lewi. A patogénese da DP não é completamente conhecida, mas pensa-se que a disfunção mitocondrial, o stress oxidativo e a neuro inflamação possam estar envolvidos. Além disso, há evidências que a DP tem alguma predisposição genética em algumas das suas formas. A DP é ainda uma condição debilitante para a qual não foram identificadas terapias eficazes e modificáveis para tratar a doença, pelo que a investigação para descobrir novas terapias é indispensável.

Psicostimulantes como a anfetamina (AMPH) e o metilfenidato (MPH), atualmente utilizados no tratamento do transtorno do défice de atenção e hiperatividade e narcolepsia, são farmacologicamente inibidores da recaptção de DA, e parecem interferir com o transportador vesicular da monoamina 2. Esta dissertação teve como objetivo avaliar o potencial neuroprotetor da AMPH e MPH relativamente a três neurotoxinas dopaminérgicas conhecidas, a 6-hidroxidopamina (6-OHDA), o 1-metil-4-fenilpiridínio (MPP+) e o paraquato (PQ), que têm sido utilizadas como toxinas experimentais para a indução da DP *in vitro*.

As células dopaminérgicas humanas diferenciadas SH-SY5Y foram o modelo aqui utilizado e foram expostas a concentrações terapêuticas de AMPH e MPH (0,001- 10µM). Em primeiro lugar, a sua citotoxicidade foi avaliada por ensaios de incorporação de vermelho neutro (NR) e de redução de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio (MTT) após uma exposição de 24 horas. Não foi encontrada toxicidade para todas as concentrações testadas. Além disso, não houve alterações no potencial da membrana mitocondrial, usando a sonda JC-1. Desta forma, a concentração de ambos os fármacos de 1µM foi selecionada para mais estudos.

Em relação às toxinas, a citotoxicidade da 6-OHDA (31,25-500µM), do MPP+ (125-1500µM) e do PQ (100-5000µM) foi avaliada em células SH-SY5Y diferenciadas pelos ensaios de incorporação de NR e redução MTT, pela análise morfológica, e pela coloração nuclear de Hoechst. Além disso, as espécies reativas e o potencial da membrana mitocondrial foram determinados com diferentes sondas fluorescentes, usando como

tempo de incubação também as 24 horas. Estes estudos tiveram, como objetivo, analisar o seu potencial tóxico e os mecanismos que lhe estão subjacentes. A 6-OHDA, o MPP+ e o PQ causaram disfunção mitocondrial e danos nos lisossomas seguidos de morte neuronal, após 24 horas de incubação e no caso de 6-OHDA e PQ de uma forma dependente da concentração. A 6-OHDA (62,5 e 125µM) e o PQ (500µM) diminuíram o potencial da membrana mitocondrial, e além disso a 6-OHDA (62,5 e 125µM) promoveu o aumento das espécies reativas de oxigénio. A análise morfológica feita recorrendo à microscopia de contraste de fases revelou que 6-OHDA (125µM) e PQ (500µM) promoveram a morte celular acompanhada de perda de dendrites. Além disso, a análise após a coloração Hoechst revelou que a 6-OHDA (125µM), o MPP+ (1500µM) e o PQ (500µM) evocavam características de morte celular apoptótica.

Ao avaliar a potencial neuroprotecção da AMPH e MPH contra as toxinas dopaminérgicas em estudo, verificou-se que estes fármacos apenas preveniram parcialmente a morte celular causada pelo PQ no ensaio de redução do MTT, mas isto não foi observado no ensaio de NR. Não houve qualquer demonstração que estes fármacos dessem qualquer protecção contra a 6-OHDA e o MPP+ nos ensaios de redução de MTT ou incorporação do NR. Ao avaliar as espécies reativas, não houve provas de que a AMPH ou MPH tenham diminuído a produção de espécies reativas provocada pelo 6-OHDA. Em vez disso, a MPH e especialmente a AMPH, demonstraram aumentar a produção de espécies reativas quando incubadas isoladamente durante as 24 horas de exposição.

Concluindo, a AMPH e o MPH parecem proteger parcialmente as células SH-SY5Y da toxicidade induzida por PQ, mas é necessário fazer mais estudos para tentar compreender o mecanismo por detrás deste efeito neuroprotetor.

Palavras-chave: anfetamina, metilfenidato, Doença de Parkinson, neurotoxina dopaminérgica, neuroprotecção

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List of abbreviations

$\Delta\Psi_m$ - Mitochondrial membrane potential

5-HT - 5-Hydroxytryptamine or Serotonin

6-OHDA - 6-Hydroxydopamine

ADHD - Attention-deficit hyperactivity disorder

AMPH - Amphetamine

ATP - Adenosine triphosphate

BBB - Blood-brain barrier

C_{max} - Peak plasma concentration

CNS - Central nervous system

COMT - Catechol-O-methyltransferase

DA - Dopamine

DAT - Dopamine transporter

DCFH-DA - 2',7'- Dichlorofluorescein diacetate

Fe³⁺ - Ferric iron

GSH - Glutathione

h - Hours

H₂O₂ - Hydrogen peroxide

HNE - 4-Hydroxynonenal

HO• - Hydroxyl radical

JC-1 - Tetraethylbenzimidazolylcarbocyanine iodide

LBs - Lewy bodies

LC50 - Lethal concentration 50

L-Dopa - Levodopa

MAO - Monoamine oxidase

MPH - Methylphenidate

MPP+ - 1-Methyl-4-phenyl-pyridinium ion

MPTP - 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MTT - 3-(4,5- Dimethylthiazol -2-yl)-2,5-diphenyl tetrazolium bromide

NA - Noradrenaline

NAT - Noradrenaline transporter

NMDA - N-methyl-d-aspartate

•NO - Nitric Oxide

NR - Neutral Red

PD - Parkinson's disease

PQ - Paraquat

RA - Retinoic acid

ROS - Reactive oxygen species

SNpc - Substantia nigra pars compacta

T_½ - Plasma half-time

T_{max} - Time to reach the peak plasma concentration

t-BHP- tert-Butyl hydroperoxide

TH - Tyrosine hydroxylase

TPA - 12-O-tetradecanoylphorbol-13-acetate

UPS - Ubiquitin-proteasome system

VMAT-2 - Vesicular monoamine transporter-2

1 Introduction

1.1 Parkinson's Disease

1.1.1 Epidemiology

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD) (1). It is the most common neurodegenerative movement disorder, and the number of affected patients by this disease may double by 2030 (2).

In industrialised countries, the estimated prevalence of PD is 0.3 % in the general population, 1.0% in people over the age of 60, and 3.0% in people over the age of 80. According to prospective population-based studies, the incidence of PD is estimated to be between 8 to 18 per 100,000 person-year. The most important risk factor for PD is age, with the median age of onset being 60 years (1) (late onset), but there are patients whom develop it earlier being, in those situations, young onset PD (3). In addition, male sex is considered as a significant risk factor for developing PD. Both incidence and prevalence of PD are 1.5 to 2.0 times higher in men than in women. Moreover, age at onset is 2.1 years later in women (53.4 years) than in men (51.3 years). In women, the development of symptomatic PD may be delayed by higher physiological striatal dopamine (DA) levels, possibly due to the activity of oestrogens. Additionally, women exhibit often more tremor (67%) than men (48%) which, in turn, is associated with milder motor decline and striatal degeneration (4). Furthermore, there are some non-motor symptoms more prevalent in women such as depression, anxiety and constipation, whereas men suffer more with drooling, sleepiness and sexual symptoms (5).

The mean duration of the disease after diagnosis is 15 years, although patients can live for decades with PD (1). The mortality cause is sometimes difficult to determine because the disease is not usually the main cause of death, but the symptoms of the disease sometimes lead to decreased life expectancy (6).

PD can be divided into the hereditary and sporadic forms. However, the majority of PD cases are sporadic (90%), and only about 10% of patients report a positive family history (7). In the case of sporadic PD where no well-known cause has been established, it is hypothesised that some environmental factors may be linked to the higher risk for PD, including rural living and occupational use of certain pesticides or herbicides that could elicit a three- to four-fold increase in the risk of developing PD (8, 9). Mutations in some genes, including α -synuclein (*E46K*, *A30P*, *SNCA*, and *A53T*), parkin (*PRKN*), and PD-associated protein (*DJ-1*) can lead to developing of PD at an early age (9) (age of onset <40 years) (1). Summary of the main etiological factors for PD were presented in Table 1.

Table 1. Main etiological factors for Parkinson's disease (adapted from Batías-Candia et al. 2019).

Genetic presentation of PD		Environmental presentation of PD	
Autosomal dominant	Autosomal recessive	Neurotoxins	Pesticides
<i>SNCA</i>	<i>PARK2</i>	6-OHDA	Rotenone
<i>LRRK2</i>	<i>PINK1</i>	MPTP	Paraquat
<i>VPS35</i>	<i>PARK7/DJ-1</i>	MDMA	Maneb
		Methamphetamine	

PD - Parkinson's disease; **SNCA** - α -Synuclein; **LRRK2** - Leucine-rich repeat kinase 2; **VPS35** - Vacuolar protein sorting ortholog 35; **PARK2** - Parkin RBR E3 ubiquitin protein ligase; **PINK1** - PTEN-induced kinase 1; **PARK7** - Protein deglycase DJ-1; **6-OHDA** - 6-Hydroxydopamine; **MPTP** - 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; **MDMA** - 3,4-Methylenedioxymethamphetamine

1.1.2 Clinical Symptoms

PD is characterized by a range of motor and non-motor symptoms. The cardinal motor symptoms of PD include tremor, rigidity, akinesia/hypokinesia/bradykinesia, and postural instability, being identified by the acronym TRAP. Other motor symptoms may include alteration of eye and blinking movements, diminished arm swing, gait disturbances and 'freezing', decreased voice volume (hypophonia), disturbances of speech, masked facies (hypomimia) and micrographia, amongst others (2, 8). In addition to motor dysfunction, non-motor symptoms of PD are known to have a significant impact on patients' quality of life (1), and include a great number of manifestations that are listed in Table 2.

Table 2. The non-motor symptoms of Parkinson's disease (adapted from Zesiewicz, 2019).

Neuropsychiatric non-motor symptoms	Cognitive non- motor symptoms	Dysautonomia	Sleep disorders	Sensory abnormalities
- Apathy - Anxiety - Depression - Impulse control disorders - Hallucinations - Psychosis - Mood disorders - Abulia	- Dementia - Memory loss - Executive dysfunction	- Constipation - Orthostatic hypotension - Sexual dysfunction - Urinary incontinence - Olfactory dysfunction - Altered cardiac reflexes - Sweating - Gastrointestinal dysfunction	- Insomnia - Excessive daytime sleepiness - Somnolence - Restless legs syndrome - Periodic limb movements of sleep - Sleep attacks - Rapid eye movement sleep behaviour disorder	- Pain - Fatigue - Numbness - Olfactory impairment

One of the most common non-motor symptoms is constipation with a prevalence ranging from 50% to 70%. Cardiovascular dysfunction is present in almost 60% of patients with PD. Nonetheless, one of the most feared complications of PD for patients is the development of dementia. Its prevalence is greater than 75% in individuals who survive 10 years with PD diagnosis. Furthermore, pain also occurs in PD and patients with pain are more depressed and have a lower quality of life than those without it. In fact, pain is a common condition in PD that becomes worse over time (1).

1.1.3 Pathophysiology

The pathophysiology of PD is mainly characterized by the degeneration of dopaminergic neurons with subsequent depigmentation of the substantia nigra pars compacta (SNpc) region of the midbrain. In fact, the motor symptoms become evident when 60% to 80% of dopaminergic neurons are lost (2, 3). The degeneration of neurons results in DA release decrease in the striatum, which alters the circuits by which the basal ganglia control movement (3).

Another hallmark of PD is the presence of proteinaceous aggregates in neurons known as Lewy bodies (LBs), which can alter synaptic function and cause neuronal degeneration. The abnormal deposition of intraneuronal, round, eosinophilic inclusion bodies, whose main components are α -synuclein and ubiquitin leads to formation of LBs (3, 8). α -Synuclein is the main protein marker and the biological hallmark of PD, and the toxicity of α -synuclein oligomers has been demonstrated, as they may directly bind to mitochondria membranes leading to mitochondrial fragmentation and neuronal cell death (10).

Although the pathogenesis of PD is unknown, a variety of cellular, biochemical, and molecular changes have been related to this disease (11), which will be addressed in the following subsections.

1.1.3.1 Mitochondrial dysfunction

Mitochondrial dysfunction is caused by bioenergetic problems, mutations in mitochondrial DNA, nuclear DNA gene mutations connected to mitochondria, and changes in dynamics, in size and morphology of mitochondria, impairment of transcription, and the presence of mutant proteins associated with mitochondria (12). These dysfunctions affect several cellular mechanisms that can cause cell death, including excitotoxicity induction, oxidative stress, and apoptosis (13), that will be debated next.

Mitochondria provide 90% of the adenosine triphosphate (ATP) needed for all cellular processes, including detoxifying reactive oxygen species (ROS) produced by mitochondrial respiration, controlling the cellular redox state, and regulating cytoplasmic calcium levels, where mitochondria functions as an intracellular reservoir for this ion. Because mitochondria act as both a source and a target of ROS, mitochondrial dysregulation can have a significant role in the pathogenesis of PD (14). The electron transport chain is the main source of ROS, since during reduction of oxygen to water a small percentage of radical anion superoxide is produced (15). Reduced activity of mitochondrial complex I, a major component of the electron transport chain, in the substantia nigra has been identified as a significant biochemical characteristic in the pathophysiology of PD (16, 17). Complex I has such a significant influence on oxidative phosphorylation in synaptic mitochondria that a 25% decrease in its activity had a significant impact on ATP synthesis and total energy metabolism inside the cell (18).

Several observations suggest that excitotoxicity may play a key role in PD pathology. Neuronal death as a result of excitotoxicity is due to activation of N-methyl-d-aspartate (NMDA)-type receptors by the excitatory neurotransmitter glutamate, which causes calcium

influx and activates a variety of calcium-dependent proteases (11, 19). Under normal conditions of membrane potential, calcium influx is blocked by magnesium transport, but an impairment of complex I activity in the central nervous system (CNS) leads to a loss of ATP, reversal of the sodium/potassium-ATPase and loss of resting membrane potential. This causes partial depolarization of dopaminergic cell membrane and relieves the voltage-dependent blockade of NMDA receptors by magnesium, which results in calcium influx. This increase in calcium levels results in damage to the cytoskeleton by mitochondrial dysfunction and an increase of ROS production (11, 13). Calcium is sequestered by the mitochondria, which leads to an activation of the apoptotic pathway (20), because of its intra mitochondrial increase content.

Furthermore, calcium influx activates nitric oxide ($\bullet\text{NO}$) synthase, leading to the production of $\bullet\text{NO}$, which readily reacts with superoxide radicals to produce highly reactive peroxynitrite (ONOO^-), which can decompose to the highly toxic hydroxyl radical ($\text{HO}\bullet$). $\text{HO}\bullet$ can induce DNA fragmentation, protein oxidation and lipid peroxidation. $\bullet\text{NO}$ can also inhibit mitochondrial activity and release iron, both of which can promote the production of ROS in a full cycle (11, 15).

In addition, glutamate can inhibit the uptake of cysteine which is required for the intracellular synthesis of glutathione (GSH), which reduces the antioxidant defences of cells and balances the redox equilibrium towards ROS formation (21).

1.1.3.2 Oxidative stress

Human brain cells employ 20% of the oxygen consumed by the body but constitute only 2% of the body (22). Consequently, high oxygen mitochondrial consumption, due to the great need for ATP production, can result in the increase of reactive species, including ROS (23). To counteract excessive or prolonged formation of ROS, antioxidant mechanisms are needed and a failure in these protective mechanisms can cause cell death and, in particular, neurodegeneration (23). The brain is not particularly rich in antioxidant defences when compared to other organs.

Oxidative stress seems to contribute significantly to the degeneration of dopaminergic neurons in PD. A variety of sources and processes leading to formation of ROS has been identified, including DA metabolism, iron, neuroinflammation and mitochondrial dysfunction, this last has already been mentioned in subsection 1.1.3.1. Furthermore, oxidative stress affects cellular homeostatic mechanisms, such as the ubiquitin-proteasome system (UPS) and mitophagy (15). Oxidative stress likely occurs in PD based on the following findings: reduced levels of the antioxidant GSH, increased levels

of the pro-oxidant iron, and evidence of oxidative damage to proteins, lipids, and DNA (11), which will be mentioned next as pathophysiology of PD.

GSH is a tripeptide composed of cysteine, glutamate, and glycine, with the reactive thiol group of its cysteine acting as an efficient antioxidant. GSH is synthesized in the cytoplasm but must be transported into the mitochondria where it acts also as a mitochondrial antioxidant molecule (15). The GSH system is very important for the cellular defence against ROS because (i) GSH can react directly with radicals (such as $\text{HO}^\bullet$, radical anion superoxide and $^\bullet\text{NO}$) in non-enzymatic reactions and, also (ii) can react enzymatically as an electron donor in the reduction of peroxides in GSH peroxidase reaction (reducing H_2O_2 to H_2O), with the final product of the GSH oxidation being the oxidized glutathione (GSSG). Within the cells, GSH are maintained by GSH reductase, an enzyme that reduces GSSG into GSH and thus there is a continuous action of GSH peroxidase. It should be mentioned that the GSH system is only one part of the cellular ROS defence mechanism, being that antioxidant enzymes such as superoxide dismutase and catalase are also involved (22). It has been observed that during PD, there is a decrease in GSH levels within the SNpc, being this finding the main indicator for oxidative stress during the progression of PD (24, 25). Furthermore, the elevated ratio of GSSG to GSH in PD is also consistent with the oxidative stress (26). However, under conditions of GSH depletion as in PD, as already mentioned, it may lead to oxidative stress.

Abnormally increase in total iron and ferric iron (Fe^{3+}) concentrations have been detected in patients with PD, with particularly in the substantia nigra (27-29). Indeed, one study demonstrated that iron deposition was found mainly in the SNpc in PD (29). Evidences of the role of iron in pathogenesis of the disease is its ability to produce ROS since Fe^{3+} and ferrous iron (Fe^{2+}) can react with radical anion superoxide and hydrogen peroxide (H_2O_2), respectively, in a chain reaction generating the highly reactive $\text{OH}^\bullet$, which together with DA oxidation can induce neurotoxicity (30, 31). Ferritin is the main protein responsible for storing iron in the body by keeping the iron in a non-reactive form in the cell. It is unclear whether the excess iron seen in PD brain is free or bound to ferritin (25, 32, 33). There is evidence that ferritin in PD patients is heavily loaded with iron, implying that even if ferritin levels are increased to counteract excess iron, ferritin molecules are saturated with iron. In the case of superoxide-mediated iron release from charged ferritin, there may be an increase in reactive iron available for ROS production. (34, 35). Moreover, iron promotes auto-oxidation of DA in neurons of substantia nigra, releasing H_2O_2 (36). In addition, iron catalyses the conversion of excess DA to neuromelanin, an insoluble black-brown pigment that accumulates in all dopaminergic neurons with age in humans (37). Neuromelanin also seems may play a role in PD as it is an intracellular store for iron (38). Neuromelanin can

be neuroprotective and sequester redox active ions in the cell with a high affinity for Fe^{3+} ions, but when bound to excess Fe^{3+} , it tends to become a pro oxidant and reduces Fe^{3+} to Fe^{2+} , which then gets released from neuromelanin owing to its weak affinity, which, in turn, increases the fraction of iron capable of reacting with H_2O_2 (39).

Dopaminergic neurons are particularly exposed to oxidative stress, being that certain ROS are naturally produced during the metabolism of DA, including superoxide radical anion, quinones and $\text{HO}^\bullet$. Degradation of DA can occur through auto-oxidation, in the presence of iron, or enzymatically, through the enzyme monoamine oxidase (MAO)-B, with formation of H_2O_2 , this ROS favours a depletion of the antioxidant GSH, suggesting the occurrence of oxidative stress and impairment of a critical antioxidant mechanism (40, 41). Any mechanisms that impairs the GSH oxidation-reduction cycle or that increase iron-mediated reactions in the substantia nigra would then enhance oxidative stress (41). DA quinones are a product from DA auto-oxidation, which can be oxidized to aminochrome, which is highly reactive and leads to the generation of superoxide radical and depletion of cellular nicotinamide adenine dinucleotide phosphate-oxidase (NADPH). Aminochromes can form adducts with proteins such as α -synuclein, parkin, DJ-1, and superoxide dismutase-2. Moreover, aminochromes are the precursor of neuromelanin, known to be accumulated in the SNC and that contribute to neurodegeneration by triggering neuroinflammation (15, 42) and oxidative stress as mentioned earlier on in this section.

Furthermore, quinones are chemically reactive compounds and in the presence of thiolates, form catechol thioethers as conjugation products. In biological systems, catechol thioethers are formed mainly with free cysteine, GSH, or protein-bound cysteine. Referring to the biogenic catecholamines, catecholamine most likely to enter a redox cycle like the one mentioned is DA. Catechol thioether formation appears to contribute to dopaminergic neurodegeneration through GSH depletion. Alternatively, catechol thioethers may also act directly or indirectly as endogenous neurotoxins (43, 44). Therefore, in PD the formation of catechol thioethers derived from DA and its metabolites has some importance because of the possible formation of ROS, which leads to oxidative stress, and the toxicity of the conjugates themselves.

In addition, it has been suggested that nigral DA neurons are more vulnerable to metabolic and oxidative stress because they possess particularly long and unmyelinated axons, with huge numbers of synapses which require great energy to be maintain and have elevated levels of cytosolic DA (45), that can undergo oxidation.

The brain is rich in lipids, particularly unsaturated fatty acids, which are targets for lipid peroxidation (22). Lipid damage leads to loss of integrity of the cell membrane, as well

as organelles, and increasing its permeability to ions, such as calcium, which in turn leads to excitotoxicity. Moreover, lipid peroxidation tends to produce 4-hydroxynonenal (HNE), and its levels are increased in dopaminergic cells in substantia nigra in PD. HNE is a lipophilic, α -alkenal that is highly reactive, which forms stable adducts with nucleophilic groups on proteins such as amines and thiols. HNE can activate members of the caspase family and cause DNA fragmentation, leading to apoptosis, decreased GSH levels as well inhibit complexes I and II of the mitochondrial respiratory chain, and it promotes an increase in intracellular calcium (41, 46), all mechanisms that can lead to cell death.

The main mechanism whereby the cells degrade and eliminate damaged and undesirable proteins is the UPS (47). The efficient elimination of these undesirable materials by the proteasome, during oxidative stress is viewed as a defensive mechanism, as degradation reduces the risk of oxidised proteins forming toxic aggregates (15). Mutations in the genes for ubiquitin carboxy-terminal hydrolase L1 and parkin, that are associated to PD and are components of the UPS, can indicate a role for UPS in pathogenesis of PD (47). Furthermore, UPS is involved in the degradation of damaged mitochondria, reducing the formation of ROS. In addition, α -synuclein, the main component of LBs, is a substrate of the UPS, when oxidatively damaged and aggregated impairs proteasomal function. Thus, evidence for impaired UPS with structural proteasome changes in the SNC of PD patients has been reported (15).

1.1.3.3 Neuroinflammation

There is evidence that neuroinflammation plays a role in the pathophysiology of PD, mediated mainly by activated microglia (48, 49). Glial cells do not generate electrical impulses and they were considered as supporting cells for neurons. This group includes oligodendrocytes, astrocytes, and microglia and they regulate neuronal activity (50). Microglia are phagocytic cells, components of the innate immune system of the CNS, that normally have a resting phenotype but get activated in response to brain injury or immunological challenge (48). Furthermore, the activated microglia that surround dopaminergic neurons are generally pro-inflammatory (51). Dopaminergic neuronal death releases oxidized DNA, lipids and proteins such as aggregated α -synuclein, which activates microglia into the pro-inflammatory phenotype. This, activation results in increased cytokine formation, increased production of reactive oxygen and nitrogen species, and decreased secretion of trophic factors responsible for the normal maintenance of neuronal viability, such as tumour necrosis factor- α , $\bullet$ NO, and interleukin 1 β derived, which can modulate the neuroinflammatory process in PD (50, 52).

Thus, neuronal death exacerbated by microglia leads to increased activation of microglial cells, resulting in a neurotoxic vicious cycle (48). Because the midbrain includes more microglial cells than other brain areas, these cells would be more harmful to dopaminergic neurons (53).

1.1.3.4 Genetic factors

The finding of genes associated to familial forms of PD, such as *SNCA*, *LRRK2*, *PARK7*, *PARKIN* and *PINK1* among others can provide a useful insight into the disease's mechanism by identifying certain molecular pathways and their consequences at the cellular level (3, 15). Therefore, characteristics of these genes will be discussed next.

SNCA, the gene encoding for α -synuclein, was the first gene linked to autosomal dominant PD, and some pathogenic *SNCA* mutations have been identified: A53T, E46K, and G51D (3, 8). The A53T and A30P mutations promote the formation of protofibrils *in vitro* (54), and the E46K mutation accelerates the formation of fibrils, which are observed as LBs, compared to the A53T and A30P mutations (55). In general, people with *SNCA* mutations have early onset of disease, with most cases developing symptoms when patients are 40–60 years old and presenting the classical PD motor symptoms. However, patients with A53T and E46K mutations also exhibit early cognitive impairment and pathologically show degeneration of the SNC and extensive accumulation of LBs (3).

LRRK2 codes for leucine-rich repeat kinase 2, a protein that is expressed in the brain, lungs, kidneys, and the immune system. Mutation in *LRRK2* also causes autosomal dominant PD, being those gene mutations the second most common genetic risk factor for sporadic PD, being associated with 1% of sporadic PD cases and 4% of hereditary PD (1, 3). It has been suggested that this mutation leads to increased kinase activity (56). The neuropathology of *LRRK2* patients is heterogeneous, some of them do not have LBs and have instead tau inclusions (3).

PARK7 codes for DJ-1, a protein that acts as a ROS sensor/scavenger, protease, transcriptional regulator, and RNA binding protein in the mitochondria (57). Mutations in the *DJ-1* gene are linked to autosomal recessive, early-onset PD. According to some studies, knocking down DJ-1 promotes cell death caused by oxidative stress as well as other insults such as proteasome inhibition, endoplasmic reticulum stress, and promotes calcium entry (15).

PARKIN codes for E3 ubiquitin-protein ligase parkin (Parkin), and *PINK1* codes for a kinase of the same name. Mutations in the Parkin and PINK1 genes are associated to

early-onset autosomal recessive PD. Parkin and PINK1 genes have been related to a cellular pathway that involves the preferential degradation in lysosomes of dysfunctional mitochondria via macro-autophagy, a process termed mitophagy. Loss of function of these genes leads to impairs mitophagy, resulting in an accumulation of dysfunctional mitochondria (3, 58).

These are just a few examples of genetic factors, and there are many others (Table 1). The most common cause of familial PD is mutations in the parkin gene, and an increasing number of studies show that stress factors related with sporadic PD promote parkin accumulation in the insoluble fraction. The accumulation of α -synuclein could contribute to the PD pathogenesis by promoting alterations in parkin and tubulin solubility, which may compromise neural function by damaging the neuronal cytoskeleton. These findings provide new insight into the potential nature of pathogenic α -synuclein and parkin interactions in PD (59), but other genes may not be overlooked.

1.1.4 Symptomatic treatments

The treatment of PD is symptomatic and is predominantly focused on the dopaminergic pathway while there are no disease-modifying treatments for PD (8). In fact, there are several treatments available for PD symptoms, including surgical procedures, occupational therapy, physiotherapy, and other support services (60), but none is able to restore the full function of the patient.

1.1.4.1 Levodopa

Levodopa (L-dopa), the metabolic precursor of DA (61), has proved to be a successful therapy being considered the gold standard for the treatment of PD and the most effective drug to treat motor symptoms (8, 60). However, most non-motor symptoms usually do not improve greatly after L-dopa (60). L-dopa can cross the blood-brain barrier (BBB) and be converted to DA by decarboxylation primarily within the presynaptic terminals of dopaminergic neurons in the striatum (8). Its' entry in the brain via the BBB is mediated by a membrane transporter for aromatic amino acids (61). The DA produced is responsible for the drug's therapeutic efficacy in PD. After release, DA is either transported back into dopaminergic terminals via the presynaptic uptake mechanism or metabolised via the actions of MAO and catechol-O-methyltransferase (COMT) (61).

When taken orally, L-dopa is absorbed rapidly from the small bowel by the transport system for aromatic amino acids. Drug concentrations in the plasma usually peak between

0.5 and 2 hours (h) and the half-life in plasma is short (1-3 h) (61). Furthermore, over 99% of the L-dopa is catabolized peripherally and never reaches the brain. The one that reaches the brain, is mostly converted by aromatic L-amino acid decarboxylase to DA inside the dopaminergic neurons (61). To improve the pharmacokinetic profile of L-dopa and brains' availability, a combination with a decarboxylase inhibitor (benserazide or carbidopa) is used, which also avoids other side effects such as confusion, sleepiness, hallucinations, and impulse control disorders (8).

Finally, the L-dopa most significant limitation relates to the development of motor complications such as dyskinesia, fluctuations, dystonia and wearing-off (8). L-dopa long-term use is related with the development of debilitating motor complications in about 30% of PD patients after 2 to 3 years of use, and in more than 50% of PD patients after five years (45).

1.1.4.2 Monoamine oxidase B inhibitors

The two isoforms of MAO (MAO-A and MAO-B), which oxidize monoamines, are both present in the periphery and CNS, and metabolize monoamines of intestinal origin and of the CNS. MAO-B is the predominant type in the striatum and is responsible for the majority of the oxidative metabolism of DA in the brain (61). MAO-B inhibitors' therapeutic potential is based on their ability to reduce DA metabolism and thus prolong and enhance dopaminergic stimulation (by increasing synaptic DA concentrations) (8, 45). Irreversible MAO-B inhibitors, such as selegiline and rasagiline prevent L-dopa degradation in the brain and are also recommended in combination with L-dopa (8). Safinamide is a new alternative for treating motor fluctuations in mid- and late-stage of PD being a reversible MAO-B inhibitor. It has additional features such as voltage-dependent sodium channel blockage, calcium channel modulation, and glutamate release inhibition. It has been proven to improve symptom management of motor function in advanced PD (8, 45).

1.1.4.3 Catechol-O-methyl transferase inhibitors

COMT is responsible for the catabolism of L-dopa as well as of DA. COMT transfers a methyl group from the donor S-adenosyl-L-methionine, producing the pharmacologically inactive compounds 3-O-methyldopa (from L-dopa) and 3-O-methoxytyramine (from DA) (61). Thus, its inhibition may be useful in PD. The principal therapeutic action of the COMT-inhibitors is to prevent the peripheral conversion of L-dopa to 3-O-methyl-dopa, especially

in the liver. That inhibition increases both the plasma half-life of L-dopa and the percentage of each dosage that reaches the CNS (8, 61). Therefore, the inhibition of these enzymes is used as an adjuvant therapy in combination to L-dopa to mitigate motor fluctuations (8). Available COMT inhibitors include tolcapone (which has been linked to severe liver failure and should be used with caution), entacapone, and opicapone (which has recently been approved in Europe) (8).

1.1.4.4 Dopamine agonists

DA-receptor agonists, unlike L-dopa, do not require enzymatic conversion to an active metabolite, do not produce potentially toxic metabolites, do not compete with other substances for active transport to across the BBB, and do not rely on the functional capacities of the nigrostriatal neurons (61). DA agonists directly stimulate the postsynaptic D₁, D₂ and D₃ receptors in the striatum without the requirement of further metabolism within the dopaminergic neurons (8). However, DA agonists are not as effective as L-dopa in reversing motor symptoms. On the other hand, DA agonists are linked with a decreased risk of dyskinesia. They may be administered as monotherapy or in combination with L-dopa in the early stages of the disease (8), but their efficacy decreases when the number of neurons also decrease. DA agonists include ropinirole and pramipexole which are administered orally. Rotigotine is given as a transdermal patch and apomorphine, which has a short duration of activity, can be administered subcutaneously (8, 61).

1.1.4.5 Other compounds

Since PD is also seen as a disequilibrium between the dopaminergic system and the cholinergic system, antimuscarinic drugs (e.g., benztropine, trihexyphenidyl, biperiden, orphenadrine) are given and show to cause improvements in some motor symptoms such as tremor but they induce deficits in cognitive function. Amantadine is an NMDA glutamate receptor antagonist with anticholinergic activity, being used to reduce motor symptoms and complications of PD (8).

At present, several drugs are being investigated to discover potential new treatments, and more efficacious therapies are needed urgently.

1.2 Toxin-Induced models for the study of PD

Neurotoxins remain the most common tools to induce selective neuronal death in both *in vitro* and *in vivo* systems. Among the various known experimental models of PD (62), *in vitro* models have been successfully used to study putative neuroprotective agents (63). The pharmacologically induced models mostly used to study PD in the laboratory experimental paradigms will be presented next, where some of their characteristics will be described.

1.2.1 6-Hydroxydopamine

The neurotoxin 6-hydroxydopamine (6-OHDA) has some structural similarities with DA and noradrenaline (NA) and leads to apoptosis of catecholaminergic cells. 6-OHDA can enter both dopaminergic and noradrenergic neurons and cause damage to the catecholaminergic pathways in the CNS and peripheral nervous system through its high affinity for catecholaminergic membrane transporters, the DA-transporter (DAT) and NA-transporter (NAT) (62, 64). The chemical structure of 6-OHDA is shown in Figure 1.

The main mechanism by which 6-OHDA induces neuronal damage is mainly due to its ability to cause severe oxidative stress. As already mentioned, 6-OHDA is similar to DA and shows great affinity for DAT. Once inside neurons, 6-OHDA accumulates in the cytosol of mitochondria and is rapidly oxidized to H_2O_2 and paraquinone, species that are highly toxic to mitochondria and inhibit electron transport chain activity by blocking complex I, resulting in increased ROS generation. 6-OHDA auto-oxidation pathway is shown in Figure 1. 6-OHDA can destroy catecholaminergic structures by a combined effect of ROS and quinones (65-67). There is also evidence that the availability of reduced GSH is decreased following 6-OHDA exposure (68).

6-OHDA *in vivo* models reproduce the major cellular processes implicated in PD, including oxidative stress, neurodegeneration, neuroinflammation, and neuronal death by apoptosis (69). However, 6-OHDA has some disadvantages like the fact that it induces an acute effect, which is quite different from the slow and progressive nature of human PD, and it is not able to induce LBs formation (9). Furthermore, 6-OHDA is not able to cross the BBB, hence failing to accumulate within the brain parenchyma to meaningful neurotoxic concentrations, so it must be applied directly into the brain (e.g. striatum) to induce damage

(62). Nonetheless, 6-OHDA remains the more developed and well-studied model of PD (9) *in vitro*.

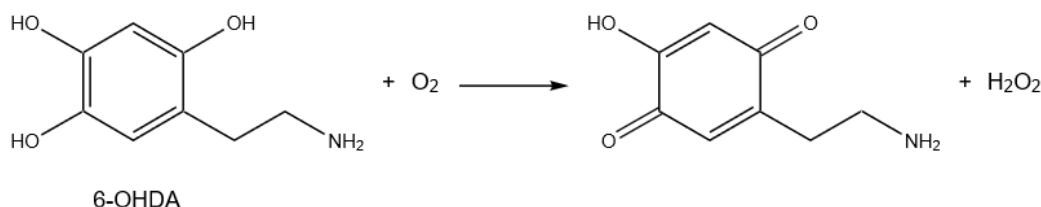


Figure 1. The chemical structure of 6-hydroxydopamine (6-OHDA) and its auto-oxidation into para-quinone and hydrogen peroxide.

1.2.2 MPTP

Unlike 6-OHDA, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is highly lipophilic and crosses the BBB. It can be administered systemically or directly into the CNS (9). Once inside of CNS, in glial cells MPTP is metabolized by MAO to 1-methyl-4-phenyl-2,3-dihydropyridium (MPDP⁺), which is then oxidized to 1-methyl-4-phenyl-pyridinium ion (MPP⁺) (Figure 2). MPP⁺ has a high affinity for DAT, allowing it to enter dopaminergic neurons easily and selectively. It then accumulates within the mitochondria, interfering with complex I of the mitochondrial transport chain, thereby inhibiting its activity. This reduces ATP cellular stores, promoting reactive ROS formation which induces apoptotic death (9, 70).

The MPTP-based model appears to be able to reproduce some of the features associated with PD, in humans and monkeys such as tremor, rigidity, freezing and slowness of movement, depending on the administration regime. Chronic exposure to MPTP promotes apoptosis of dopaminergic neurons (71). Rodents, such as mice and rats, are less vulnerable MPTP neurotoxicity than primates: MPTP-induced DA depletion requires a higher dose and the permanent behavioural symptoms of parkinsonism rarely appear. The emergence of parkinsonian behaviour in rats needs a total blocking of the DA response by dopamine D₂ antagonists, and relative DA depletion is insufficient (72).

Neurotoxins such as 6-OHDA and MPP⁺ are inappropriate for systemic administration because they do not cross the BBB. However, their entrance into brain synaptosomes is identical in primates and rats, making them beneficial in *in vitro* neuronal cultures. Cultures of dissociated foetal mesencephalic neurons (primary cultures of dopaminergic neurons),

catecholaminergic cell line (e.g., rat pheochromocytoma PC12) and DA-neuron-derived cell lines (e.g., human SH-SY5Y) are useful for researching precise mechanisms of dopaminergic neuronal degeneration and screening new pharmacological compounds using these neurotoxins (73, 74).



Figure 2. The chemical structures of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenyl-pyridinium (MPP+) ion.

1.2.3 Rotenone

The organic pesticide rotenone is commonly used as an insecticide or to kill or sample fish populations in lakes and reservoirs (75). Its chemical structure is shown in the Figure 3. Rotenone contains a lipid component, so it crosses biological membranes easily, not requiring DAT. Moreover, rotenone is not sequestered into synaptic vesicles (76). Rotenone appears to accumulate in some neuronal organelles, such as mitochondria where it impairs oxidative phosphorylation through inhibition of complex I, like MPP+ and 6-OHDA (9), which increases the rate of mitochondrial ROS release, leading to cell apoptosis (70). When administered systemically, rotenone, unlike 6-OHDA and MPP+, causes selective damage in the striatum but not in the substantia nigra (77). Furthermore, in rotenone -based models, increased iron deposits and formation of LBS with ubiquitin in nigral cells has been observed (78). Moreover, the rotenone model presents some limitations such as high related death rate and low reproducibility (79, 80).

Although rotenone, MPP+ and 6-OHDA are being currently used to study PD using *in vivo* and *in vitro* approaches, neither of these substances fully reproduce all the clinical features observed in PD. The lack of an adequate cellular and/or animal model in the laboratory to fully study PD has led to the search for other neurotoxic compounds (70).

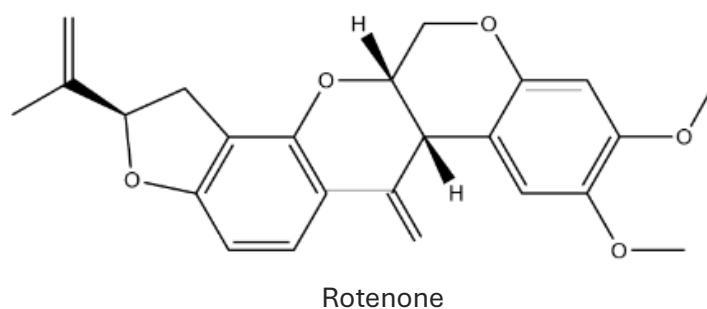


Figure 3. The chemical structure of rotenone.

1.2.4 Paraquat

The potent herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride, PQ) (Figure 4) has recently received great attention as a possible inducer of PD, as its' occupational exposure, especially in agriculture, has been associated with Parkinsonism (81). Furthermore, a large majority of experimental studies with PQ focus on its effects on the liver, lungs, and kidney, probably because the toxicity induced by this herbicide in these organs is responsible for death following acute exposure. However, it has also been shown that patients who died of PQ intoxication have severe brain damage (82, 83).

PQ can cross the BBB and affect the dopaminergic system, although in a slow and limited way (84). It appears that PQ is transported into dopaminergic neurons via DAT due to the structural similarity between PQ and MPP⁺, which also reaches striatal cells (85, 86). Several mechanisms have been postulated for PQ-induced neurotoxicity such as excitotoxicity, increase of ROS production and inhibition of mitochondrial complex I. However, the mechanisms by which PQ promotes neurotoxicity remain to be fully elucidated (86-88).

Several mechanisms have been postulated for PQ-induced neurotoxicity, such as excitotoxicity, increased ROS production and inhibition of mitochondrial complex I. However, the mechanisms by which PQ promotes neurotoxicity are yet to be fully elucidated.

In summary, in neurons PQ triggers an oxidative stress-related vicious cycle, resulting in mitochondrial dysfunction and protein aggregation, with consequent formation of LBs. The production of pro-oxidant mediators (e.g. Jun N-terminal kinase activation) also occurs (9). However, the mechanisms by which PQ promotes neurotoxicity are not yet fully elucidated (70).

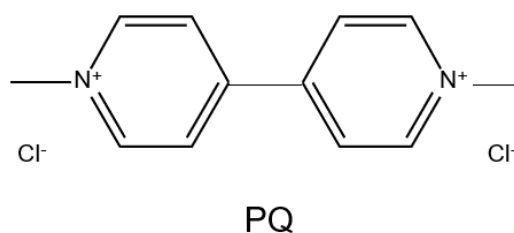


Figure 4. The chemical structure of paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride, PQ).

More research is needed to achieve an ideal laboratory model of PD, which should demonstrate easily detectable PD-associated motor deficits, gradual and selective loss of DA neurons with the progression of ageing, and LB-type inclusions. This will definitely contribute to a better understanding of the mechanisms of dopaminergic neuronal degeneration in PD and also to the development of new therapies to improve symptom management and stop disease progression. (72). Still, the advantages and limitations of PD inducing toxins are summarized in Table 3.

Table 3. Advantages and limitations of neurotoxic chemicals used in in vitro and in vivo for Parkinson's disease

Advantages		Limitations
6-OHDA	- Reproduces a specific loss of substantia nigra. - Oxidative stress is mediated by hydrogen peroxide, quinones and paraquinones, among others. - Inhibits ETC complex I.	- Not able of producing LBs.
MPTP	- Systemic administration is possible. - Induces a specific loss of substantia nigra. - Inhibits ETC complex I.	- Neurological and clinical features depend on the administration regime (acute or chronic). - Not able of producing LBs.
ROT	- Systemic administration is possible. - Reproduces loss of striatum. - Inhibits ETC complex I. - Induces LBs formation.	- Low reproducibility. - High mortality.
PQ	- Systemic administration is possible. - Reproduces a specific loss of substantia nigra - Oxidative stress is mediated by ROS production. - Induces LBs formation.	- The specificity of dopaminergic neuronal damage has caused some controversies.

6-OHDA - 6-hydroxydopamine; **MPTP** - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; **ROT** - rotenone; **PQ** - paraquat; **ETC** - electron transport chain; **LBs** - Lewy bodies

1.3 Amphetamine and Methylphenidate as antiparkinsonian drug candidates

Amphetamine (AMPH) and methylphenidate (MPH) have been studied regarding several CNS diseases. Treatments with AMPH, a CNS stimulant have been shown to increase neuronal plasticity, enhance neuronal growth, decreased oxidative stress, and improvement of motor function in animals with CNS injury (89-92). In addition, one study in rats showed an increase of both GAP-43 (a growth-associated protein) and synaptophysin proteins (important proteins in identification of axonal sprouting and synaptogenesis) when d-AMPH was administered (93). On the other hand, several clinical trials have been shown that in stroke, traumatic brain injury and in some symptoms of PD, AMPH and MPH can be valuable treatment options. Improvement in activities of daily living, that can mean improvement in the patient's quality of life, in motor and cognitive functions were the main results found after AMPH and MPH on patients after stroke (94-97). Patients following a traumatic brain injury were treated with MPH and as main results there were improvements in memory and cognitive functions (98-101). MPH treatment, in patients with PD showed some improvements in motor symptoms (102-106).

This dissertation used these two drugs in protocols *in vitro* as potential neuroprotective agents against dopaminergic toxins, so they need to be reviewed regarding their pharmacodynamic and pharmacokinetic characteristics.

1.4 Amphetamine

AMPH was discovered in 1910 as racemic α -methylphenethylamine, but it was first synthesized only in 1927. AMPH belongs to the family of drugs called the ' β -phenylethylamines' and one of the reasons for developing racemic AMPH was its structural similarities to the biologically active molecule, ephedrine (107). AMPH-like compounds are derived from the β -phenylethylamine core structure and are characterised dynamically and kinetically by rapidly crossing the BBB, resisting brain biotransformation, and releasing monoamine neurotransmitters from nerve terminals. The presence of the α -methyl group on the AMPH molecule protects the amine group from being oxidized by MAO enzymes and increases the capacity of the AMPH molecule to cross membranes, because of its amphipathic nature (108). AMPH is a potent psychostimulant that can increase alertness, wakefulness, sleeplessness, vitality, and self-confidence while decreasing fatigue and appetite (60). Its peripheral effects include an increase in blood pressure and gastrointestinal hypermotility (109). AMPH is used in the clinical practice as CNS stimulant

in children and adults with attention deficits and hyperactivity disorder (ADHD) and in narcolepsy (110).

The amphetaminic structure of AMPH permits the substitutions at the aromatic ring and at the α and β carbons of the aliphatic chain and at the amine terminal, to originate a diverse range of AMPH isomers (60). AMPH is a molecule with a single chiral centre, occurs in two optically active forms, namely the dextro- (or d-) and levo- (or l-) isomers or enantiomers (107). Figure 5 shows the similarity among AMPH and monoamine neurotransmitters. The enantiomers show different pharmacological action and body distribution. For example, the (S)-(+)-AMPH, appears to have stronger pharmacological action as a stimulant and hyperthermic agent than the (R)-(-)-enantiomer (111). Studies in rats and humans reveal that d-AMPH is more potent than l-AMPH (107). Nonetheless, most available AMPH formulations contain both isomers (110).

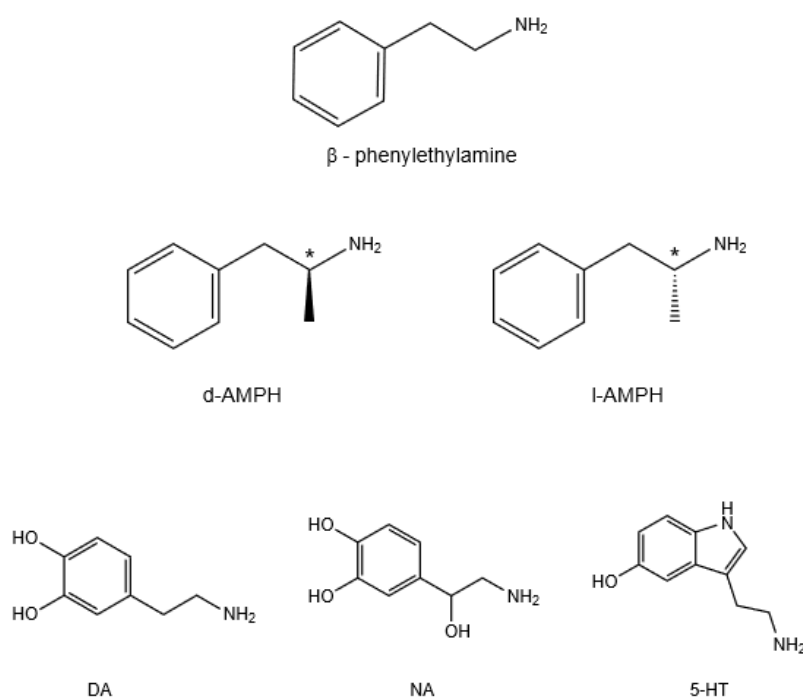


Figure 5. The chemical structure of β -phenylethylamine, amphetamine (AMPH) isomers (* represent the chiral carbon) and monoamines: dopamine (DA), noradrenaline (NA) and serotonin (5-HT).

1.4.1 Pharmacodynamics

AMPH acts as a substrate for the membrane transporters of monoamines, due to the structural similarity with monoamine neurotransmitters: NA, DA, and serotonin (also known as 5-hydroxytryptamine, 5-HT) (107), as shown in figure 5. Moreover, AMPH is an indirect monoamine agonist that induces the release of NA, DA, and 5-HT from presynaptic terminals in the CNS and in the periphery. AMPH can enter in the nerve terminals by passive entry or via a reuptake transporter, acting as inhibitor of monoamine reuptake. Once within the neuron, AMPH reverses the direction of the membrane transporter, promoting the efflux of NA, DA, and 5-HT to the synaptic cleft (60).

There are four mechanisms by which AMPH enhances monoamine availability in the synaptic cleft: (i) competition with endogenous monoamines for transport to pre-synaptic terminals via DAT, NAT and 5-HT-transporter, resulting in a inhibition of monoamine reuptake, that (ii) causes a displacement of monoamines from the cytosolic pool to the presynaptic terminal, by inhibiting the vesicular monoamine transporter-2 (VMAT-2), which causes an inhibition of vesicular uptake, (iii) this decrease in vesicular monoamines content results in increased cytoplasmic monoamines concentrations, leads to reverse transport action in the reuptake transporters, therefore engaging an efflux of monoamines from the presynaptic terminal to the synaptic cleft and (iv) the inhibition of MAO, which results in increased amount of monoamines that are available for retro-transport into the synaptic cleft (Figure 6), reinforcing the initial steps of transport (107, 112).

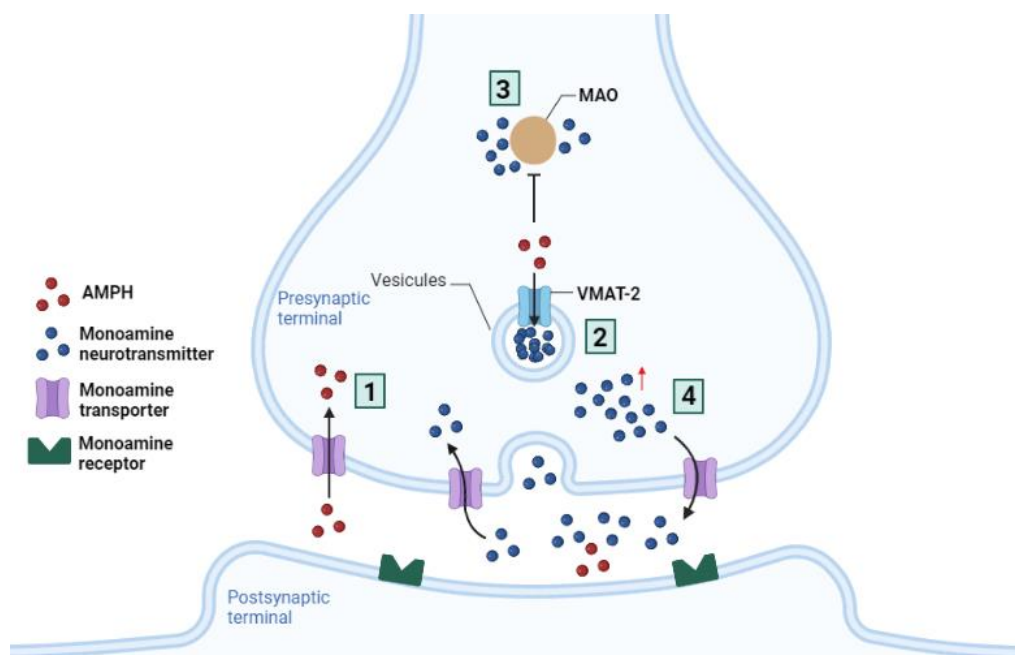


Figure 6. Mechanism of action of amphetamine (AMPH). AMPH inhibits: (1) monoamine transporters, transmembrane proteins that are responsible for the reuptake of dopamine, noradrenaline and serotonin from the synaptic cleft to the presynaptic terminal and uses it to enter the neuron; (2) Vesicular monoamine transporter-2 (VMAT-2), leading to increased monoamines release within synaptic vesicles increasing the cytoplasmic pool of neurotransmitters; (3) Monoamine oxidase (MAO), causing an increase in presynaptic levels of monoamines. (4) Furthermore, its' action leads to monoamine transporters transport reversal of monoamines to the synaptic cleft. The significant increase in neurotransmitters in the synaptic cleft improves monoaminergic transmission.

1.4.2 Pharmacokinetics

AMPH has high oral bioavailability, high volume of distribution (around 4 L/kg), and low plasma protein binding (less than 20%). Its plasma half-time ($T_{1/2}$) ranges between 6 and 12 h, and both hepatic and renal elimination occur. Hepatic metabolism is extensive, although a significant percentage of the drug remains unchanged at elimination (113). AMPH is a chemically weak basic drug, with pKa values around 9.9 and relatively low molecular weight. AMPH-type stimulants easily diffuse across cell membranes and lipid layers, as well as to tissues or biological substrates with a lower pH than blood (60).

1.4.2.1 Absorption

AMPH is used by different routes, usually orally either as the S-(+)-enantiomer (d-AMPH) or as the racemic mixture (i.e. AMPH sulphate) (60). Most of the oral dose (>90%) is absorbed (110). AMPH when administered orally, it is rapidly absorbed with the peak plasma concentrations (C_{max}) generally attained within 4 h after ingestion (T_{max}), and both area under the plasma concentration-time curve in the 24 h following administration and C_{max} are proportional to the dose administered, and no significant differences between the two enantiomers occurs (60). AMPH does not seem to be a substrate or inhibitor of the enteric efflux P-glycoprotein transporter (110).

1.4.2.2 Distribution

In general, AMPH has low protein binding (often less than 20%) and high volume of distribution, which favours its easy diffusion across lipid layers, cell membranes and to tissues with more acidic pH than blood (60), as stated. AMPH isomers are estimated to be approximately 16% to 20% bound to plasma proteins (110). AMPH-drug abusers appear to have greater volume of distribution comparing with drug-free individuals (6 vs 4 L/kg), which is probably due to tissue sequestration. This sequestration leads to the development of pharmacokinetic tolerance towards the drug (60). S-(+)- and R-(-)-AMPH enantiomers have identical protein binding and distribution volumes, but the $T_{1/2}$ of (S)-(+)-AMPH is shorter than that of (R)-(-)-isomer due to the rapid metabolism of the more pharmacologically active enantiomer. In humans, AMPH appears to follow one-compartment pharmacokinetics (60).

1.4.2.3 Metabolism

AMPH is metabolized mainly via (i) N-dealkylation, oxidation and deamination into the corresponding benzoic acid derivatives, which are then conjugated with glycine and excreted as the corresponding hippuric acids and (ii) hydroxylation in position 4 of the aromatic ring, forming 4-hydroxyamphetamine, followed by phenol group conjugation with sulphate or glucuronic acid (113). AMPH can produce a reactive intermediate during the hydroxylation step, which then interacts with GSH to form the (GSH-S-yl)-p-hydroxy-AMPH adduct (114). The minor metabolic pathway involves the oxidation at the β -carbon of the side chain, which results in the formation of norephedrine, which is then oxidized in the aromatic ring to 4-hydroxynorephedrine (113). The metabolism of AMPH is presented in Figure 7.

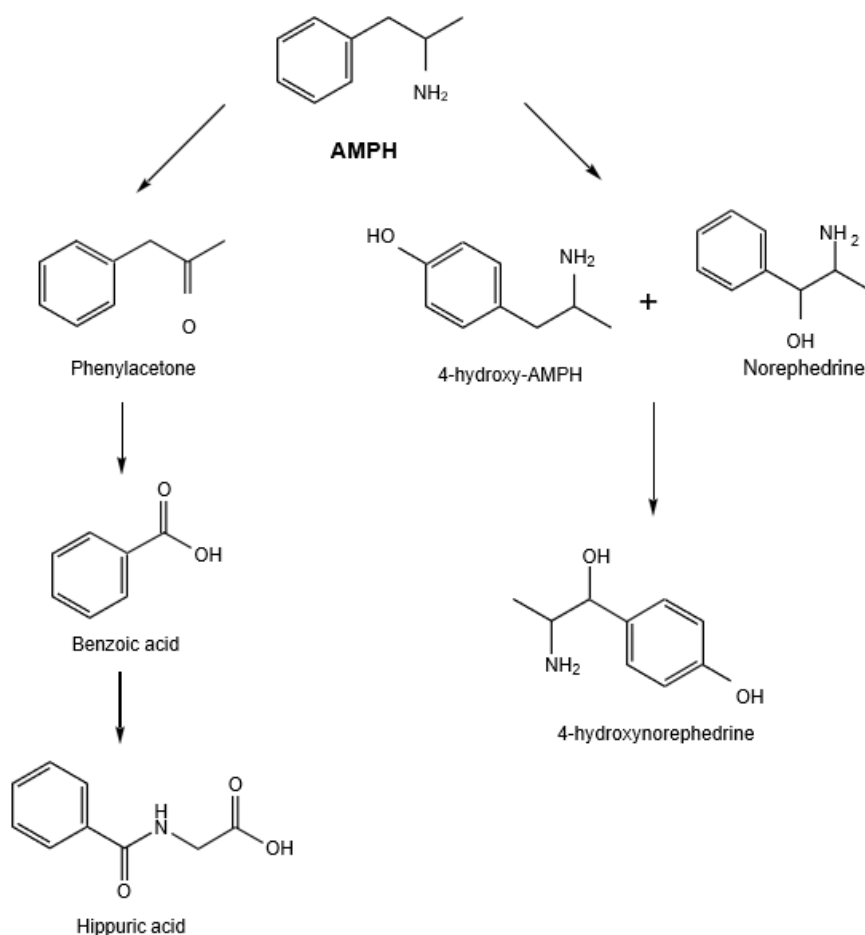


Figure 7. Metabolism of amphetamine (AMPH) and its principal metabolites.

AMPH deamination seems to be catalysed by CYP450 isoenzymes belonging to the CYP2C subfamily (115). The CYP2D6 is one of several enzymes involved in the biotransformation of AMPH, namely it catalyses the hydroxylation of the benzene ring (60, 110). This isoenzyme is expressed polymorphically in humans, and subjects classified as poor metabolisers are more susceptible to the acute toxic effects of AMPH derivatives or to abuse liability (60), because its concentrations remain higher.

It appears that d-AMPH is metabolized faster than l-AMPH, resulting in different exposure to each isomer when administered as the racemate mixture. The significant difference in the disposition of the AMPH enantiomers is evident in the $T_{1/2}$, with d-AMPH having a $T_{1/2}$ 1-2 h shorter than l-AMPH (110).

1.4.2.4 Excretion

AMPH is eliminated by the kidneys in a pH-dependent way, being the $T_{1/2}$ of AMPH and related substances highly dependent on the acidity of the urine. At normal urine pH, 30%-40% of an administered dosage of AMPH is eliminated as unaltered parent compound, with hippuric acid being the second most abundant urinary metabolite (110). As AMPH-derived compounds are weak basic substances, renal excretion is increased by urinary acidification and decreased by urinary alkalinisation. Therefore, some AMPH consumers consume large quantities of bicarbonate to prolong the drug's effects and decrease elimination (60).

1.5 Methylphenidate

MPH is a ritalinic acid psychostimulant, first synthesised in 1944. It is clinically effective in treating both adult and childhood ADHD as well as narcolepsy (116).

MPH is a piperidine-derived molecule with two chiral centres, which give rise to four possible isomers: d-threo (R,R), l-threo (S,S), d-erythro (R,S) and l-erythro (S,R)-MPH (117) (Figure 8). The first marketed preparation was a mixture of all four isomers (80 : 20) racemic erythro- and threo-MPH (117). However, the d/l-erythro MPH isomers increased cardiovascular toxicity while having minimal influence on the targeted CNS stimulant effects. Thus, they were removed from the formulation to improve the safety for MPH formulations (118). As a result, currently the drug is marketed as a racemic mixture of l-threo MPH and d-threo MPH (50 : 50 mixture) (117, 119), being the d-threo-MPH isomer more potent than the l-enantiomer (117).

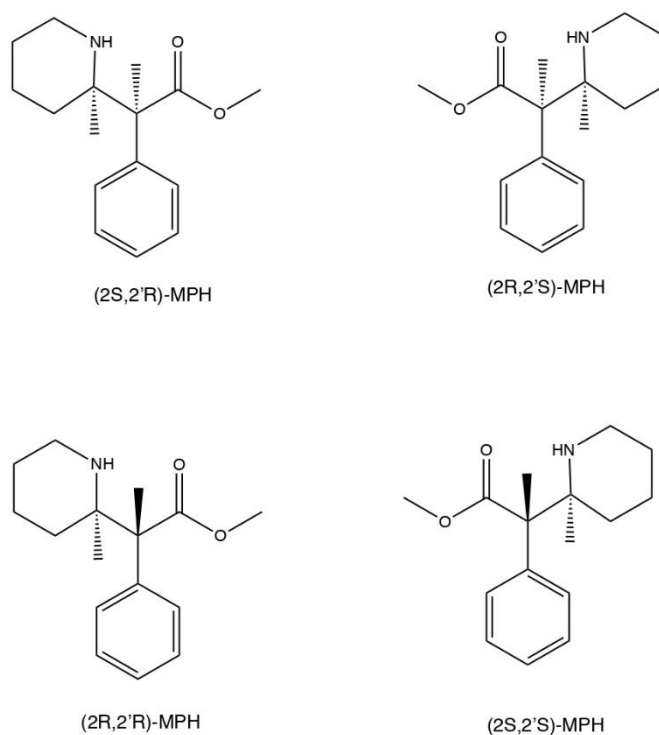


Figure 8. The chemical structures of methylphenidate (MPH) isomers.

1.5.1 Pharmacodynamics

MPH seems to accumulate in the brain mostly through passive diffusion (120). In the presynaptic cell membrane, MPH inhibiting the DAT, resulting in a blocking reuptake of DA that will cause an increase in extracellular DA levels. This increase leads to a prolonged and/or intensified DA postsynaptic signal. In addition, MPH also binds and inhibits the NA transporter, and shows low affinity for the 5-HT transporter (116). Orally administered, MPH may bind to and occupy more than 50% of DAT in the human brain at therapeutic doses of 0.3-0.6 mg/kg (121).

In the CNS, VMAT-2 is a transporter that moves cytoplasmic DA into synaptic vesicles for storage and eventual exocytotic release, avoiding oxidative deamination of the monoamine in the cytoplasm or its non-vesicular release to the cleft. A single administration of MPH redistributes VMAT-2 inside nerve terminals from the synaptosomal membranes to the cytoplasm, promoting sequestration of DA into the vesicles. This alteration increases the DA content available for vesicular release (122, 123).

The therapeutic effects of MPH are postulated to be mediated by DA and NA efflux increases from neurons and DA and NA reuptake inhibition from the synaptic cleft (116, 117).

1.5.2 Pharmacokinetics

MPH is extensively absorbed, but has a low oral bioavailability (107). The low protein binding, high lipid solubility, and high volume of distribution result in a rapid uptake into the CNS (124, 125). The stereoselective profile, administration route, and drug formulation all have an impact on MPH's pharmacokinetic properties (125), that will be discussed next.

1.5.2.1 Absorption

MPH can be administered by several routes: intraperitoneal, intravenous, or oral. In the case of oral administration, the absorption of MPH is essentially complete and rapid (121). Despite extensive absorption, the oral bioavailability of MPH is low, because of an extensive and stereospecific 'first-pass' hepatic metabolism. This first passage effect through the liver leads predominantly to the hydrolysis product ritalinic acid (119), 'destroying' roughly 80% of the MPH dose (120). $C_{\max}$ occurs 1 to 3 h ($T_{\max}$) after oral administration, being significantly variable between individuals (126), with a $T_{1/2}$ of approximately 2.6 to 3 h (120).

Results from intravenous and oral administration showed that absolute bioavailability of d-threo-MPH and l-threo-MPH was found to be 23% and 5%, respectively, demonstrating differences in presystemic metabolism between the two enantiomers (127). In children, the bioavailability of MPH ranges from 10 to 50 % (128).

Furthermore, MPH should be taken 30 to 45 minutes before meals because the presence of food may interfere with the drug's absorption, reducing its bioavailability even further (126).

1.5.2.2 Distribution

MPH has a low degree of protein binding and a high lipid solubility. It is rapidly distributed and relatively large amounts may pass the BBB (129). Positron Emission tomographic studies with marked [11C] d, l-threo-MPH were carried out to understand the distribution of MPH in the brain. One study in rats revealed that a higher uptake of d-threo was observed in the striatum, with the C_{max} at 5 to 15 minutes, but not in the cerebellum after intravenous administration of d-isomer when compared with l-isomer (129). In humans, another study reveals that 8% of intravenously administered dose (0.5 mg/kg) enters the brain, with the striatum having the greatest concentrations of d-isomer and the cortex and cerebellum having relatively low concentrations (124). MPH has fast peak brain uptake (T_{max} <10 minutes), but the clearance relatively slow (elimination $T_{1/2}$ = 90 minutes) (124, 130). d-threo MPH appears to accumulate substantially more in the striatum than in any other brain area. In another hand, l-threo MPH demonstrated a homogenous distribution in brain (130).

1.5.2.3 Metabolism

In humans, the main metabolic pathway of MPH is de-esterification reaction, to form the corresponding carboxylic acid metabolite, known as ritalinic acid, which is pharmacologically inactive (125, 127). Representing minor pathways (less than 2 %) the aromatic hydroxylation, microsomal (cytochrome-P450) oxidation and conjugation with glucuronic acid have been reported to form the p-hydroxy-MPH, oxo-MPH and conjugated metabolites, p-hydroxy-ritalinic acid and oxo-ritalinic acid, respectively. There is no indication that any of these MPH metabolites has a significant role in its pharmacological activity (126, 127). The main metabolic routes for MPH are represented in Figure 9.

In rats, aromatic hydroxylation appears to be the main metabolic route, since p-hydroxy-MPH, p-hydroxy-ritalinic acid, and a glucuronide conjugate of p-hydroxy-ritalinic acid were identified in about half of the urine metabolites. Products of de-esterification and microsomal oxidation were also identified (125).

The metabolism of MPH is stereoselective (117), as it occurs in other pharmacokinetic phases. The endoplasmic reticulum human carboxylesterase 1 (CES1A1) has catalytic efficiency to MPH enantiomers but with different affinities for each. CES1A1 has higher catalytic affinity for l-threo-MPH than for d-threo-MPH (131). The threo enantiomers are mainly hydrolysed in the ester group by the CES1A1 to d- or l-threo- ritalinic acid, which has a half-life of 3 to 4 h (132). CES1A1 appears to be the major responsible

for 'first-pass' stereoselective metabolism of MPH (131). Polymorphisms in the human *CES1* gene, encoding for a significantly less active enzyme (p.Gly143Glu and p.Asp260fs variants) have previously been reported. This can provide significant changes in pharmacokinetics by decreasing MPH metabolism. However, the frequency of these variants is low (133).

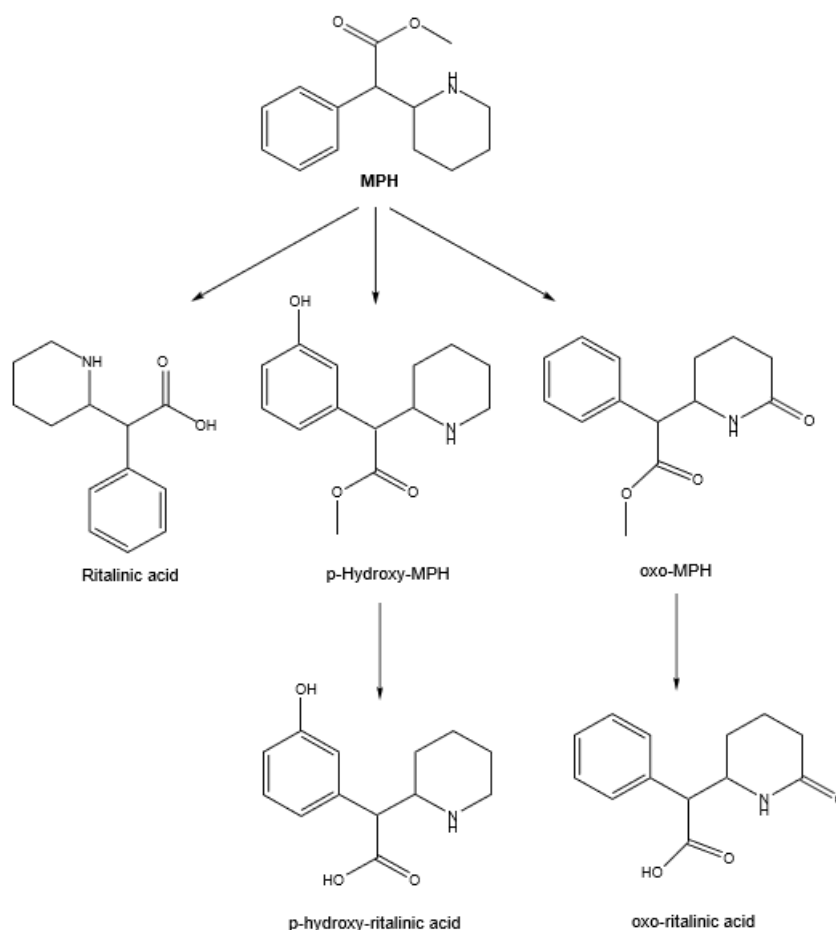


Figure 9. The main metabolic pathways methylphenidate (MPH) and its metabolites.

1.5.2.4 Excretion

In humans, when administered orally and based on radioactive marked MPH, 50% and 90% of a dose is excreted in the urine until 8 to 48 h, respectively. In relation to faecal elimination, this up to 24 and 48 h contributed for 1.5 and 3.3% of MPH radioactive marked, respectively (125). MPH is mainly metabolised to RA, as state and 70–75% of which is excreted in the urine (134). Urine contains 2- to 3-fold more l-threo-ritalinic acid than d-threo-ritalinic acid. The enantioselective 'first-pass' metabolism, rather than excretion, has been linked to these observations (135). The renal clearance of d- and l-threo-MPH following oral or intravenous administration did not reveal significant differences (127).

2 Aims

PD is considered the second most common neurodegenerative disorder worldwide. Moreover, current treatments do not cure or stop the progression of disease. Therefore, new pharmacological therapies with neuroprotective potential are needed.

The main aim of this dissertation was to evaluate whether AMPH and MPH can promote neuroprotection against dopaminergic neurotoxins *in vitro* and to study the putative mechanisms involved. For this purpose, an experimental work was done using SH-SY5Y differentiated cells, dopaminergic cells derived from human neuroblastoma with the specific objectives of:

- Obtain concentration-toxicity curves for the modular dopaminergic neurotoxins **MPP+**, **6-OHDA** and **PQ**.
- Evaluate the **neurotoxic potential of therapeutically relevant AMPH and MPH concentrations**.
- Evaluate the **neuroprotective potential of non-toxic AMPH and MPH concentrations** against dopaminergic neurotoxins MPP+, 6-OHDA and PQ.
- Evaluate the involvement of **oxidative stress** in the mechanism of toxicity/neuroprotection.
- Evaluation of **mitochondrial membrane potential** in the presence of drugs and toxins under study.

3 Materials and methods

In this section, all materials, and reagents as well as all laboratory procedures were performed to develop laboratory work are presented.

3.1 Materials and reagents

Materials used for cell culture came from several suppliers: sterile pipettes were obtained from Nerbe plus (Winsen, Germany), 48-well plates from TPP (Trasadingen, Switzerland) and 25 cm² flasks from Corning (New York, USA).

Regarding reagents, antibiotic (10.000 U/ml Penicillin and 10 mg/ml Streptomycin), heat inactivated foetal bovine serum (FBS) and Hanks' balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺ were obtained from Pan biotech (Aidenbach, Germany). Phosphate-buffered saline (PBS) was obtained from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium (DMEM) high glucose, neutral red (NR) solution (0.33%), trypan blue solution (0.4%), trypsin-EDTA solution, sodium bicarbonate, dimethyl sulfoxide (DMSO), PQ, 6-OHDA hydrochloride, MPP⁺ iodide, tert-butyl hydroperoxide (t-BHP) solution, retinoic acid (RA), 12-O-tetradecanoylphorbol-13-acetate (TPA), bisBenzimide trihydrochloride (Hoescht dye) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were obtained from Merck (St. Louis, USA).

Acetic acid glacial (97%) was obtained from Fisher Chemical (UK) and alcohol (99.9%) was obtained from Fábrica do álcool (Torres Novas, Portugal). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (98%) was obtained from Alfa Aesar (Kandel, Germany). Threo-methyl- α -phenyl-2-piperidineacetate hydrochloride (MPH) and AMPH were obtained from Tocris (Bristol, United Kingdom). Tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Abcam (UK). Neurite Outgrowth Staining Kit was obtained from Thermo Fisher Scientific (Massachusetts, USA).

3.2 SH-SY5Y cell line

SH-SY5Y cell line is thrice cloned subline of the SK-N-SH cell line, originally established from a bone marrow biopsy of a metastatic neuroblastoma in the 1970s (136, 137). The SK-N-SH cell line contains cells with three different phenotypes: intermediary (I type), Schwannian (S type) and neuronal (N type). The SH-SY5Y cell line contains only cells with type N, i.e. neuronal (137). The human neuroblastoma SH-SY5Y cell line is an *in vitro* model commonly used in studies related to neurotoxicity, oxidative stress, and neurodegenerative diseases. Moreover, this cell line is widely used in the study of PD (138). An important characteristic of SH-SY5Y cells is that it can proliferate in culture for long periods (137). The main characteristics of the SH-SY5Y cell line are presented in Table 4.

The SH-SY5Y cell line has several characteristics that make it valuable as a model of dopaminergic neurons (137), such as, (i) the ability to synthesize DA and NA, given that the cells express tyrosine hydroxylase (TH), enzyme responsible for the transformation of tyrosine to L-dopa, the precursor of DA, which is then converted to NA by DA- β -hydroxylase (136, 139), (ii) they express DAT, a membrane uptake transporter expressed in dopaminergic neurons within the CNS (140), (iii) despite their low levels of DA receptors, they respond to DA agonists (137).

Because the SH-SY5Y cell line has the ability to synthesize both DA and NA as stated, they may have a catecholaminergic phenotype. The use of an oncogenically altered cell line with catecholaminergic rather than exclusively dopaminergic properties remains a controversial issue in the PD area (136). According to some studies, undifferentiated SH-SY5Y express only immature neuronal markers and lack mature neuronal markers (141, 142), and thus may not represent an appropriate model for the study of PD, which mainly affect differentiated dopaminergic neurons (143). The use of undifferentiated SH-SY5Y cells as a model for dopaminergic neurons also has shown some problems such as low expression of DA synthesizing enzymes (144), and a continuous division state, which can be a bias to distinguish whether there is neuroprotection or if neurotoxic agents effect the rate of cell proliferation or induce death (144, 145).

Differentiation of SH-SY5Y with certain differentiating agents results in a more dopaminergic neuronal phenotype (137). Furthermore, the treatment with differentiation-inducing agents enables SH-SY5Y cells to become morphologically similar to mature primary neurons (146) and the different treatment agents (e.g. RA, phorbol esters, dibutyl cyclic adenosine monophosphate) result in a variety of neuronal phenotypes (e.g. cholinergic, dopaminergic, noradrenergic) (147). After differentiation, cells stop proliferating,

become a stable population, and exhibit extensive neurite outgrowth with morphological similarity to living neurons in the human brain (148). Differentiated SH-SY5Y cells also express a variety of neuronal specific markers, including growth-associated protein (GAP43), receptors for neurotrophic factors, neuropeptides, neurosecretory granula neuron-specific enolase (NSE), neuronal nuclei (NeuN), and also contain vesicle proteins such as synaptophysin, and neuronal-specific cytoskeletal proteins, including microtubule associated protein (MAP), Tau and neurofilament proteins (149).

Some studies reported that SH-SY5Y with RA differentiation followed by treatment with TPA significantly elevates production of DA synthetic enzymes (150), the expression of D2 receptors (151), and its G-proteins (152). Another study reported that the differentiation with RA followed of treatment with TPA significantly increases the content of TH and DAT, when compared with undifferentiated or just differentiated cells with RA. On the other hand, combined treatment with RA and TPA reduces VMAT levels (144). Our group has published results revealing that SH-SY5Y cells that underwent RA/TPA treatment for 6 days presented, both morphological (fusiform shape and extended processes) and biochemical (significant increase in DAT and TH expression analysed by Western blot) changes indicative of a well-accomplished differentiation into a dopaminergic phenotype (153). Also, RA/TPA treatment increased the ability to uptake and release DA in SH-SY5Y cells (154).

Table 4. Characteristics of SH-SY5Y cell line.

Advantages	Disadvantages
Expresses DAT.	Limited synthesis of DA and NA because of the deficiency in dihydroxyphenylalanine decarboxylase.
Expresses DA- β -hydroxylase and tyrosine hydroxylase.	Proliferation rate of cells in an undifferentiated state.
Exhibits specific uptake of NA.	Time and cost required to achieve neuronal differentiation.
Expresses neurofilaments proteins.	
Expresses muscarinic, opioid, and nerve growth factor receptors.	
Treatment with several agents may cause differentiation.	
After differentiation, presents a functionally mature neuronal nature.	

DAT- Dopamine transporter; **DA** - Dopamine; **NA** - Noradrenaline

3.2.1 SH-SY5Y cell culture and differentiation

SH-SY5Y cells (European Collection of Authenticated Cell Cultures (ECACC)) were obtained from Sigma-Aldrich (St. Louis, USA), and were cultured in 25 cm² flasks. Cells were grown to confluence in DMEM medium supplemented with 10% FBS plus 1% of antibiotic (10.000 U/ml Penicillin and 10 mg/ml Streptomycin). Cells were incubated in a humidified atmosphere of 5% CO₂ – 95% air at 37°C. The medium was replaced once a week. The cells were used between passages 18 and 27 for the experiments.

After confluence, the cells were dissociated with trypsin - EDTA solution and subcultured in 48 well-plates. In all experiments, cells were seeded at a density of 25.000 cells/cm² (250 µL of 100 cells/µL, i.e., 25.000 cells/well). To ascertain the cell density of the cell suspension before seeding, cells were counted using a Neubauer chamber after the use of trypan blue solution in cellular suspension. To obtain a dopaminergic phenotype, and induce neuronal differentiation, the cells were differentiated with RA at a final concentration of 10µM for 3 days soon after seeding. At the end of the third day, another differentiating factor was added, and complete medium contained 80nM TPA for another 3 days. After 6 days of differentiation, the medium was replaced with new warm DMEM medium, and the cells were exposed to the drugs and/or toxins of interest for 24 h. The protocol for SH-SY5Y cells trypsinization, differentiation and seeding is shown in Figure 10.

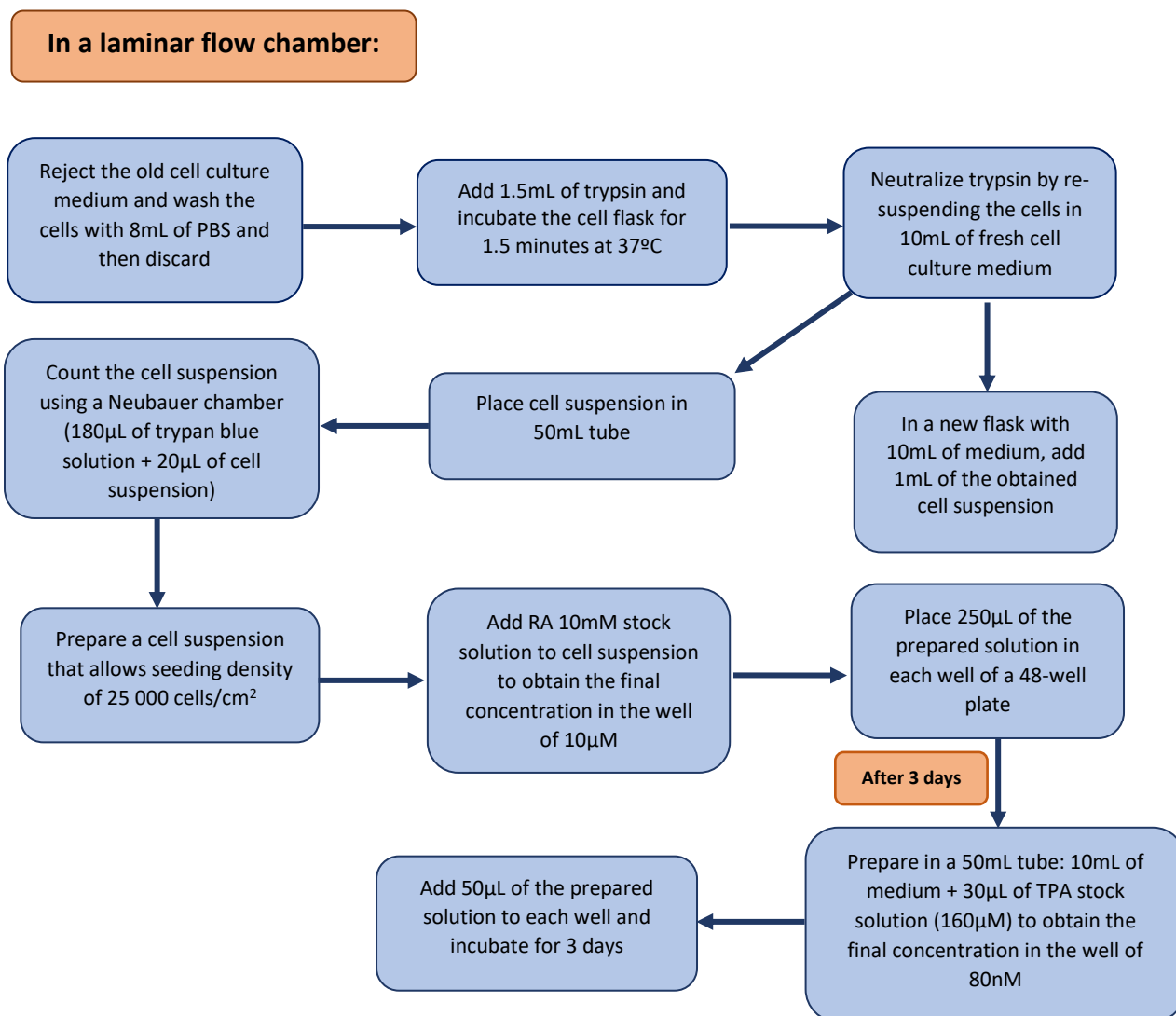


Figure 10. The experimental protocol for SH-SY5Y cells trypsinization, seeding and differentiation.

3.3 Cytotoxicity evaluation

Following the differentiation protocol, the medium was removed and replaced with new warm medium, as stated. Cells were then exposed to AMPH or MPH (concentration ranges 0.001 - 10.0µM), PQ (concentration ranges 100-5000µM), 6-OHDA (concentration ranges 31.25 – 500µM) or MPP+ (concentration ranges 125-1500µM) for 24 h. The drugs were solubilized in distilled sterilized water and stored at -20°C. However, in the case of 6-OHDA and MPP+, the solutions were prepared freshly immediately before use, because of their chemical instability. To avoid any effect of the solvent, the control wells were exposed

with the same volume of sterile water as that used for the drug/toxin treatment wells. After the exposure time-period, the NR uptake and the MTT reduction assays were conducted.

3.3.1 MTT reduction assay

MTT assay is a colorimetric assay that reflects mostly the mitochondrial activity of the cells (155). MTT is a water-soluble yellow dye that is quickly absorbed by viable cells and degraded mostly by mitochondrial dehydrogenases by cleaving the tetrazolium ring resulting in a water-insoluble blue formazan crystals (156), which can be solubilized in an organic solvent or detergent (157). Thus, when measuring the formazan concentration, any variation in the number of viable cells can be detected. Most viable cells mitochondrial activity is constant, thus an increase or decrease in the number of viable cells is linearly related to mitochondrial activity (157), although changes in their activity can be found without viability compromise.

3.3.1.1 Experimental protocol

After a 24 h of exposure to compounds in 48-well culture plates, without removing the cell culture medium 25µL of MTT 5mg/ml solution was added to each well (dilution of 1:10). Cells were incubated at 37°C, 5% CO₂ for 1.5 h. After this incubation period, the cell culture medium was removed, and the formed formazan crystals were dissolved in 200µL of DMSO. The plates were placed in a microplate shaker, protected from light for 15 minutes in order to obtain a homogeneous solution. The absorbance of formazan was measured at 570 nm, whose value was subtracted to the values obtained in the reference wavelength of 690 nm in the microplate reader (Biotech Synergy HT, Vermont, USA).

The results were expressed in percentage of control, which was set to 100%. The results were done in quadruplicate *per* independent experiment.

3.3.2 Lysosomal neutral red uptake assay

The NR assay is one of the most used in cytotoxicity tests with many environmental and biomedical applications (158), and is based on the incorporation of the NR dye into lysosomes of viable cells (158, 159). NR is a weakly cationic dye that penetrates in cell membranes by non-ionic passive diffusion and concentrates in the lysosomes, where it

binds by electrostatic hydrophobic bonds to anionic and/ or phosphate groups of the lysosomal matrix (158).

The uptake of NR depends on the cell's ability to maintain pH gradients through ATP production. The dye has a net charge close to zero, allowing it to penetrate the cell membranes at physiological pH. Inside the lysosomes, a pH lower than that of the cytoplasm is maintained through a proton gradient and in these the dye becomes charged and is retained within the lysosomes. When the pH gradient is reduced or if the cell dies, the dye is not retained. The amount of dye retained is proportional to the number of viable cells. Thus, lysosomal integrity, with concomitant binding of NR dye, is a highly sensitive indicator of cellular viability (158). NR is extracted from the viable cells using an acidified ethanol solution, and the absorbance of the solubilized dye is quantified (158).

3.3.2.1 Experimental protocol

After a 24 h of exposure to compounds in 48-well culture plates, the medium was discarded and 250µL culture medium with 33µg/mL of NR was added to each well. Cells were incubated at 37 °C, 5 % CO₂ for 1.5 h. Then, the medium was discarded and 250µL of warmed HBSS (+/+) was added to each well. HBSS (+/+) was then removed and 200µL of solubilization solution (1 % acetic acid in 50 % ethanol) was added to each well to extract NR dye inside the cells. The content of each well was carefully mixed in a microplate shaker, protected from light, until obtaining a homogeneous solution. The absorbance was measured at 540nm and 690nm (reference wavelength) in a microplate reader (Biotech Synergy HT, Vermont, USA).

The results were expressed in percentage of control, which was set to 100%. The results were done in quadruplicate *per* independent experiment.

3.4 Neuroprotection evaluation

For neuroprotection experiments, cells were incubated with AMPH (1µM) and MPH (1µM) for 30 minutes before exposure to the toxins, at 37°C, 5 % CO₂. After this incubation period toxins were added at concentrations that elicited about 25% or 50% of cell death, in the MTT assay. In detail, PQ (300 and 500µM), 6-OHDA (62.5 and 125µM) were used to evaluate the potential of the neuroprotectors under study and after a 24 h exposure. In the case of MPP⁺ a higher and a lower concentration were selected (250 and 1500µM) since

no dose-dependent response was seen. The assays carried away were the NR uptake and the MTT reduction assays as described above.

3.5 Reactive oxygen species levels (DCFH-DA assay)

ROS are involved in pathologies such as inflammatory, cardiovascular, and neurodegenerative diseases, cancer, and aging (160). The main ROS formed in biological systems are H_2O_2 , superoxide radical anion, $\text{HO}^\bullet$ and peroxy radicals ($\text{ROO}^\bullet$) (161). ROS can damage and react with many cellular components such as proteins, lipids and DNA (160), as mentioned in the introduction section. There is usually a balance between ROS generation and its elimination by the antioxidant systems, in a healthy normal cell. Oxidative stress occurs when there is an unbalance between the oxidant and antioxidant systems (161), as mentioned in the introduction section. Therefore, the detection of ROS plays a key role in scientific research. Because of easy data processing, relative high resolution and sensitivity, methods based on fluorescent probes, such as DCFH-DA, have been widely used in intracellular ROS detection (162). DCFH-DA does not have fluorescence and can freely penetrate cell membrane, but inside the cell, DCFH-DA is hydrolysed by intracellular esterase to produce dichlorodihydrofluorescein (DCFH). DCFH can be oxidized by ROS, such as H_2O_2 , $\text{HO}^\bullet$ and $\text{ROO}^\bullet$, and also by reactive nitrogen species (RNS) like $\bullet\text{NO}$ and ONOO^- , to produce fluorescent 2',7'-dichlorofluorescein (DCF). The fluorescence intensity of DCF is used to quantify the ROS/RNS levels (162, 163).

3.5.1 Experimental protocol

The DCFH-DA probe was used to evaluate intracellular ROS/RNS production, and SH-SY5Y cells were incubated with DCFH-DA 200 μM (15 μL of stock solution: 20mM in DMSO) at 37°C, 5 % CO_2 for 30 minutes and protected from light. After this pre-incubation period, the medium was removed and 250 μL of new warm cell culture medium were added. Cells were then treated with AMPH (1 μM) and MPH (1 μM) for 30 minutes, at 37°C. After this period, the toxins were added PQ (300 μM), 6-OHDA (62.5 and 125 μM) and MPP+ (250 and 1500 μM). Cells were exposed to tert-butyl hydroperoxide 200 μM as a positive control for ROS production.

The fluorescence was immediately measured in a microplate reader (Biotech Synergy HT, Vermont, USA) with the excitation and emission wavelengths of 485nm and 530nm, respectively, and thereafter at 1, 2, 3, 4, 5, 6, and 24 h. The results are expressed

as DCFH-DA oxidation regarding the fluorescence signal at the lowest time-point measured. All conditions were performed in triplicate *per* experiment.

3.6 Mitochondrial Membrane Potential Assay

Mitochondria are key organelles for the generation of ATP through oxidative phosphorylation, during which the electrons are transferred from donors to acceptors. This electron transport chain creates an electrochemical gradient through a series of redox reactions between the two mitochondrial membranes. This electrochemical gradient promotes ATP production and creates the mitochondrial membrane potential ($\Delta\Psi_m$), which is a key parameter for evaluating mitochondrial function (164). Mitochondria are involved in the apoptotic process of cells, so $\Delta\Psi_m$ is an important parameter that may be used as used as an indicator of cell health. $\Delta\Psi_m$ is oriented to induce inward transport of cations and outward transport of anions, promoting cation accumulation between the mitochondrial membranes. $\Delta\Psi_m$ decreases during apoptosis is related with the opening of mitochondrial permeability pores and the loss of the electrochemical gradient (165), which unleashes the release of apoptotic markers.

The cyanine dye JC-1 has been used to detect $\Delta\Psi_m$ in healthy and apoptotic cells across multiple cell types, including myocytes, neurons and endothelial cells (166). JC-1 is a lipophilic, cationic dye which naturally exhibits green fluorescence and can enter mitochondria, where it accumulates (166). JC-1 monomers emit green fluorescence with a maximum at 530 nm and J-aggregates emit orange-red fluorescence with a maximum at 595 nm (167). Thus, the JC-1 dye enters and accumulates in the mitochondria of healthy cells with normal $\Delta\Psi_m$, and forms red fluorescent J-aggregates. By contrast, in apoptotic or unhealthy cells the JC-1 dye reaches the mitochondria but to a lesser degree since the interior of the mitochondria is less negative due of increased membrane permeability and associated loss of electrochemical potential. Under these condition, JC-1 does not reach a concentration high enough to cause the formation of J aggregates, retaining its original green fluorescence (166).

3.6.1 Experimental protocol

Differentiated cells seeded in a 48 well plate were incubated with AMPH (1 μ M), MPH (1 μ M), 6-OHDA (62.5 or 125 μ M) or MPP+ (250 or 1500 μ M), at 37°C, 5 % CO₂. For the evaluation of $\Delta\Psi_m$, following 24 h of exposure, JC-1 was added (final concentration of 20 μ M) to each well and mixed gently. Then cells were incubated for 15 minutes at 37°C, protected from light. Following the incubation period, the medium was removed and 250 μ L of warm HBSS (+/+) were added to each well. To continue the washing step, the plate was centrifuged for five minutes at 400g. The supernatant was again removed and 250 μ L of warm HBSS (+/+) were added. Then, fluorescence was measured at two fluorescence readings: excitation and emission at 535nm and 595nm, respectively, and excitation and emission at 485nm and 535nm, respectively in a microplate reader (Biotech Synergy HT, Vermont, USA). The results are determined as the ration between aggregate form ($\lambda_{excitation}=535$ and $\lambda_{emission} = 595$ nm) and monomer conformation ($\lambda_{excitation}=485$ and $\lambda_{emission} = 535$ nm) and expressed as percentage of the average of control values.

3.8 Microscopic evaluation of the cells

3.8.1 Phase contrast microscopy

Morphological changes in SH-SY5Y differentiated cells after a 24-h exposure period to 6-OHDA (125 μ M), MPP+ (1500 μ M) and PQ (500 μ M) were assessed by capturing phase-contrast microphotographs in a Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera (Japan).

3.8.2 Hoechst nuclear staining

To assess alterations in cellular nuclear morphology, after a 24-h exposure period to 6-OHDA (125 μ M), MPP+ (1500 μ M) and PQ (500 μ M) cells were stained with the nuclear dye Hoechst 33342 (final concentration 10 μ M) for 10 min at 37°C (protected from light). Cells were then observed, and microphotographs were taken with a microscope Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera (Japan) using a fluorescent filter ($\lambda_{excitation\ maximum} = 346$ nm and $\lambda_{emission\ maximum} = 460$ nm).

3.9 Statistical analysis

The data obtained were presented as mean $\pm$ standard error (SD) of different independent experiments, being the concentration-toxicity curves data the only exception, which were presented as mean and 95 % confidence interval (CI 95 %). To evaluate the normality of the data distribution four tests were performed: Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test for data with parametric distribution in at least one test. For data with non-parametric distribution, one-way ANOVA on ranks (Kruskal–Wallis test) was performed, followed by Dunn's comparisons test. In the case of data concerning ROS production, two-way ANOVA repeated measurements was performed, followed by Tukey's multiple comparisons test. For 6-OHDA and PQ, the lethal concentration 50 (LC50) values were obtained by analysis of the MTT and NR concentration-toxicity curves. Curves were fitted using least squares as the fitting method. More details of the statistical analysis are provided in the legend of the figures and table. Statistical significance was considered with a *p* value < 0.05. All statistical analysis were performed using the GraphPad Prism 8.3.0 software program (San Diego, CA, USA).

4 Results

4.1 Assessment of AMPH and MPH therapeutic concentrations toxicity

4.1.1 AMPH and MPH did not reveal cytotoxicity in differentiated SH-SY5Y cells

AMPH and MPH cytotoxicity of therapeutic relevant concentrations (0,001 - 10 μ M) was evaluated by the NR uptake and MTT reduction assays, 24 h after exposure, to select a non-cytotoxic working concentration to be used in the subsequent studies. In the NR uptake and MTT reduction assay, (Figure 11 and 12) no significant cytotoxicity was detected for any of the tested concentrations of AMPH or MPH.

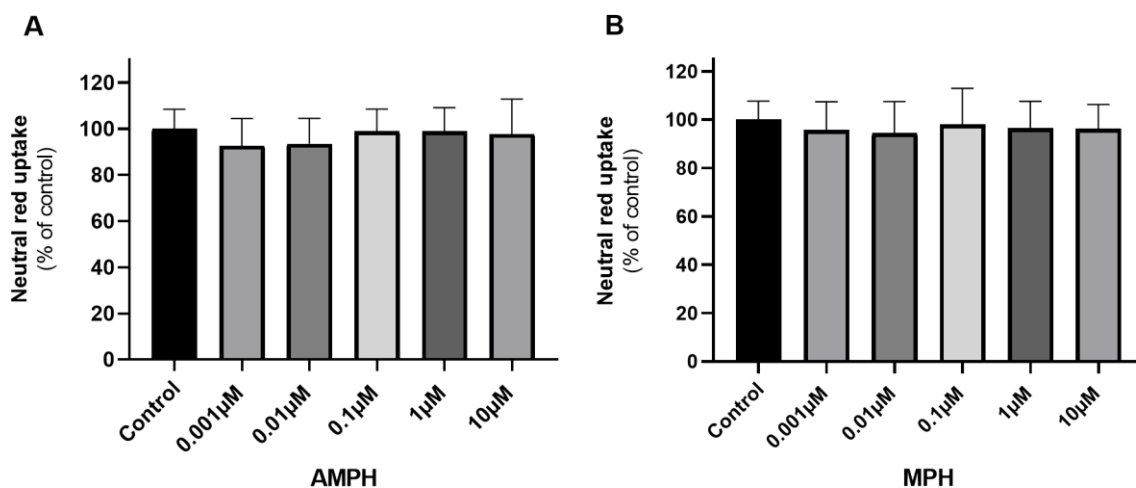


Figure 11. Amphetamine (AMPH) (0.001 - 10 μ M) (A) and Methylphenidate (MPH) (0.001 - 10 μ M) (B) cytotoxicity in differentiated SH-SY5Y evaluated by the neutral red (NR) uptake assay, 24 h after exposure. Results are presented as mean $\pm$ SD from 7 independent experiments (N=26 to 27). Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test.

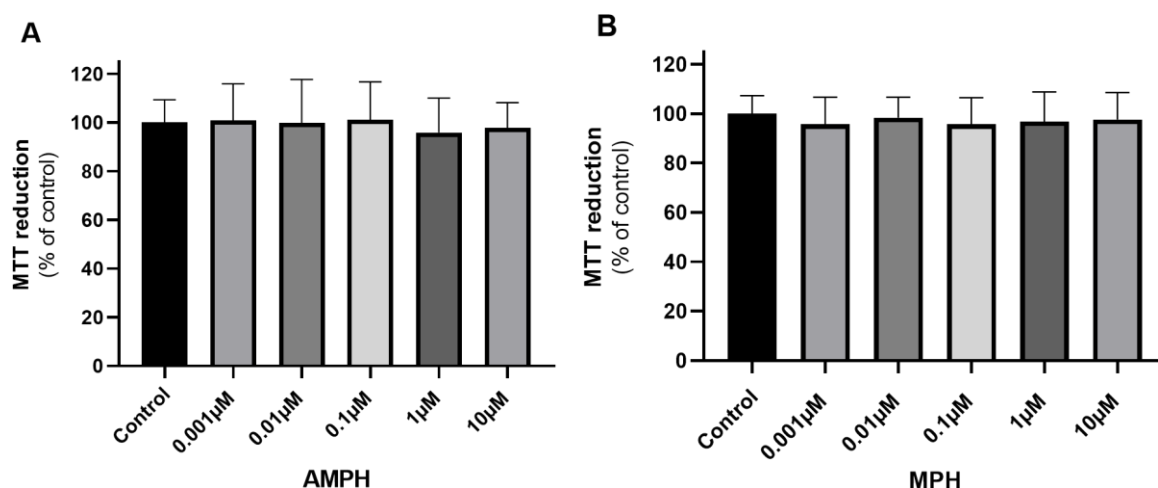


Figure 12. Amphetamine (AMPH) (0.001 - 10 μ M) (A) and Methylphenidate (MPH) (0.001 - 10 μ M) (B) cytotoxicity in differentiated SH-SY5Y evaluated by the MTT reduction assay, 24 h after exposure. Results are presented as mean $\pm$ SD from 7 independent experiments (N=24 to 28). Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test.

4.1.2 AMPH and MPH did not induce changes in mitochondrial membrane potential in differentiated SH-SY5Y cells

Evaluation of the $\Delta\Psi_m$, with the JC-1 probe, on differentiated SH-SY5Y cells following exposure to AMPH 1 μ M or MPH 1 μ M for 24 h, showed that both drugs do not induce significant changes in $\Delta\Psi_m$ when compared to control cells, as observed in Figure 13.

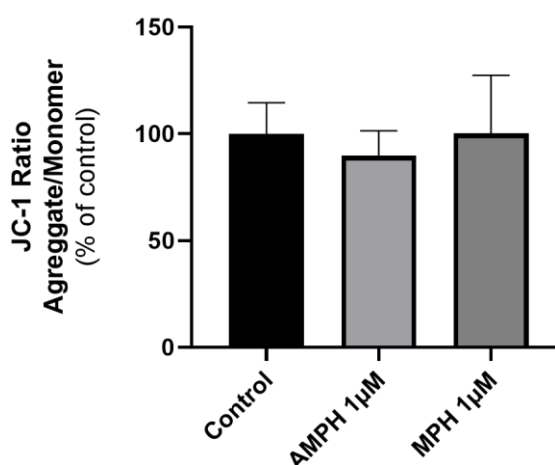


Figure 13. Mitochondrial membrane potential evaluated by JC-1 probe in differentiated SH-SY5Y cells incubated with 1 μ M of AMPH or MPH after a 24-h exposure. Results are presented as mean $\pm$ SD of 7 independent experiments (N=21). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test.

Taking into account these results, and the affinity of the drug to monoamine transporters, the working concentrations of 1μM for both AMPH and MPH was selected, for the subsequent neuroprotection experiments.

4.2 Toxicological evaluation of neurotoxins

4.2.1 6-OHDA induced a concentration-dependent neurotoxicity in differentiated SH-SY5Y cells

In the NR uptake assay (Figure 14), 6-OHDA induced significant cytotoxicity: it caused significant decrease in NR uptake from the 62.5μM concentration onwards, when compared to control cells (deionized sterile water incubated, $100 \pm 11.84\%$). 6-OHDA evoked toxicity was concentration-dependent in the NR viability assay.

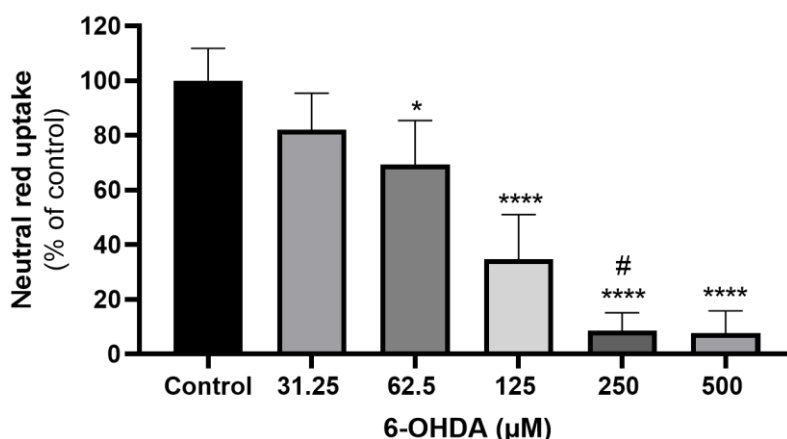


Figure 14. 6-Hydroxydopamine (6-OHDA) (31.25 - 500μM) cytotoxicity in differentiated SH-SY5Y evaluated by the neutral red (NR) uptake assay, 24 h after exposure. Results are represented as mean $\pm$ SD from 7 independent experiments (N=26 to 27). Statistical analysis was performed using one-way ANOVA on ranks (Kruskal-Wallis test) followed by Dunn's multiple comparisons test. (* $p < 0.05$, **** $p < 0.0001$ vs. Control; # $p < 0.05$ vs. previous lower concentration tested).

In the MTT reduction assay (Figure 15), 6-OHDA also induced significant cytotoxicity and caused a significant decrease in MTT reduction from the 62.5μM concentration onwards, when compared to control cells (deionized sterile water incubated, $100 \pm 5.33\%$). As in NR uptake assay, no significant cytotoxicity was detected for 6-OHDA 31.25μM. 6-OHDA evoked toxicity was concentration-dependent in the MTT viability assay.

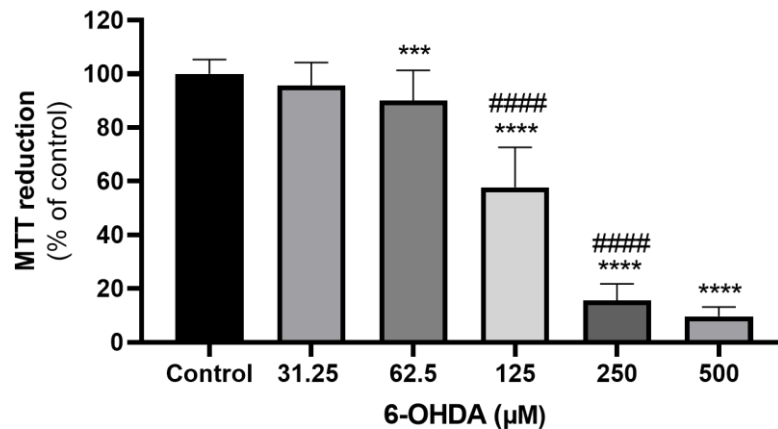


Figure 15. 6- Hydroxydopamine (6-OHDA) (31.25 - 500μM) cytotoxicity in differentiated SH-SY5Y cells evaluated by the MTT reduction assay, 24 h after exposure. Results are presented as mean $\pm$ SD of 7 independent experiments (N=27 to 28). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (**p < 0.001, ****p < 0.0001 vs. Control; ####p < 0.0001 vs. previous lower concentration tested).

4.2.2 6-OHDA decreased mitochondrial membrane potential in differentiated SH-SY5Y cells

Evaluation of the $\Delta\Psi_m$, with the JC-1 probe, in differentiated SH-SY5Y cells following 24-h exposure to 6-OHDA (62.5 and 125μM), showed that both concentrations induce a steep decrease in $\Delta\Psi_m$ to 22.93 ± 10.95 % and 16.65 ± 9.31 %, respectively when compared to control cells (100 ± 9.21 %), as observed in the figure 16. This $\Delta\Psi_m$ decrease was concentration-independent.

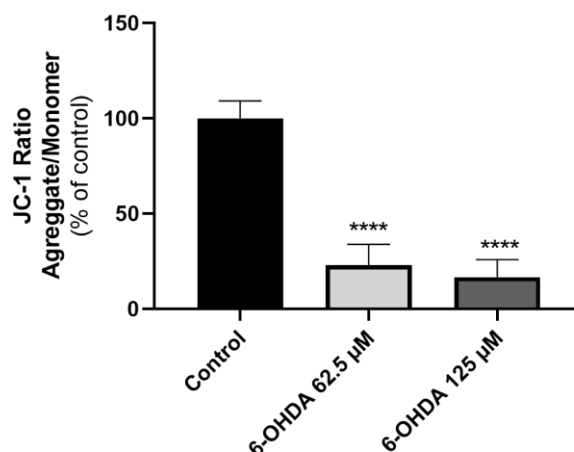


Figure 16. Mitochondrial membrane potential evaluated by JC-1 probe in differentiated SH-SY5Y cells incubated with 62.5 or 125μM of 6-OHDA after a 24-h exposure. Results are presented as mean $\pm$ SD of 2 independent experiments (N=6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (****p < 0.0001 vs. Control).

4.2.3 MPP⁺ induced neurotoxicity in differentiated SH-SY5Y cells

Cytotoxic effects induced by MPP⁺ (125 - 1500 μ M) in differentiated SH-SY5Y cells were evaluated by NR uptake and MTT reduction assays, after a 24-h exposure.

In the NR uptake assay (Figure 17), all tested concentration of MPP⁺ induced significant cytotoxicity and caused a significant decrease in NR uptake. The highest concentration tested evoked a decrease of about 9% in the cell viability, when compared to control cells (deionized sterile water incubated, 100 $\pm$ 3.23%). This cytotoxicity was concentration-independent.

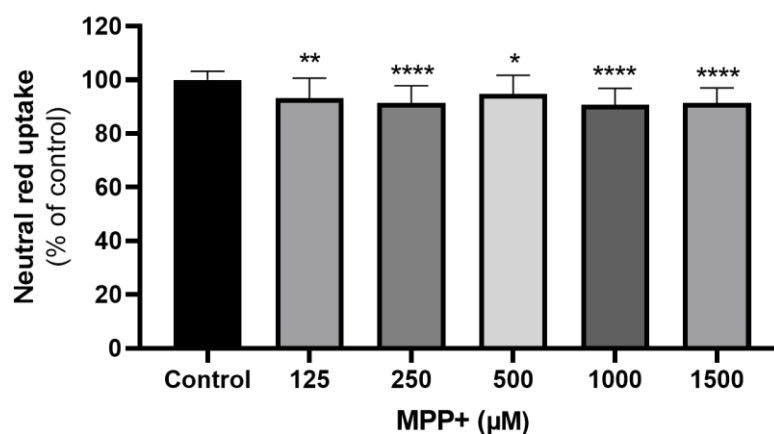


Figure 17. 1-Methyl-4-phenyl-pyridium ion (MPP⁺) (125 - 1500 μ M) cytotoxicity in differentiated SH-SY5Y cells evaluated by the neutral red (NR) uptake assay, 24 h after exposure. Results are represented as mean $\pm$ SD from 6 independent experiments (N=24). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (* p < 0.05; **** p < 0.0001 vs. Control).

In the MTT reduction assay (Figure 18), MPP⁺ also induced significant cytotoxicity and caused a significant decrease in MTT reduction. The highest concentration tested evoked a decrease of about 16% in the cell viability, when compared to control cells (deionized sterile water incubated, 100 $\pm$ 3.67%). As in the NR assay, this cytotoxicity was concentration-independent.

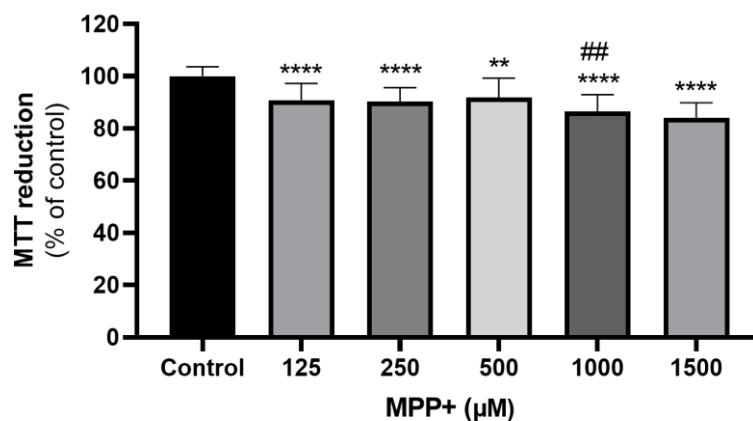


Figure 18. 1-Methyl-4-phenyl-pyridium ion (MPP+) (125 - 1500μM) cytotoxicity in differentiated SH-SY5Y cells evaluated by the MTT reduction assay, 24 h after exposure. Results are presented as mean $\pm$ SD of 5 independent experiments (N=19 to 20). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (** $p < 0.01$; **** $p < 0.0001$ vs. Control; #### $p < 0.01$ vs previous lower concentration tested).

4.2.4 MPP+ increased mitochondrial membrane potential in differentiated SH-SY5Y cells

Evaluation of the $\Delta\Psi_m$, with the JC-1 probe, in differentiated SH-SY5Y cells after a 24-h exposure of MPP+ (250 or 1500μM), showed that MPP+ 1500μM induce a statistically significant increase in $\Delta\Psi_m$ to 135.8 ± 23.82 % when compared to control cells ($100 \pm 18.28\%$), as observed in the figure 19.

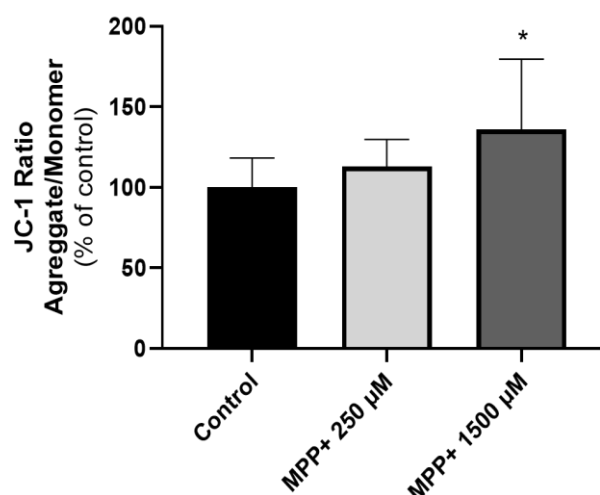


Figure 19. Mitochondrial membrane potential evaluated by JC-1 probe in differentiated SH-SY5Y cells incubated with 250 or 1500μM of MPP+ after a 24 h exposure. Results are presented as mean $\pm$ SD of 3 independent experiments (N=9). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$ vs. Control).

4.2.5 PQ induced a concentration-dependent neurotoxicity in differentiated SH-SY5Y cells

Cytotoxic effects induced by PQ (100 - 5000 μ M) in differentiated SH-SY5Y cells were evaluated by the MTT reduction and NR uptake assays, after 24-h exposure.

In the NR uptake assay (Figure 20), PQ induced significant cytotoxicity: it caused significant decrease in NR uptake from 200 μ M concentration onwards, when compared to control cells (deionized sterile water incubated, 100 $\pm$ 10.60%).

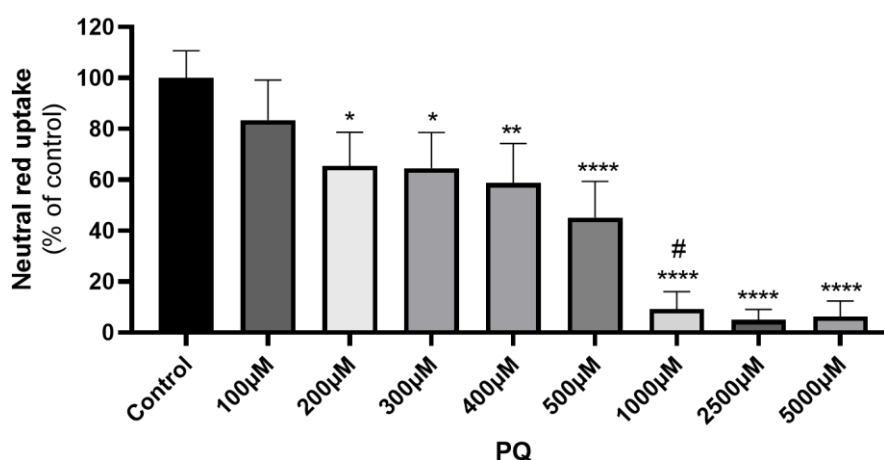


Figure 20. Paraquat (PQ) (100 - 5000 μ M) cytotoxicity in differentiated SH-SY5Y evaluated by the neutral red (NR) uptake assay, 24 h after exposure. Results are represented as mean $\pm$ SD from 4 to 7 independent experiments (N=16 to 43). Statistical analysis was performed using one-way ANOVA on ranks (Kruskal-Wallis test) followed by Dunn's multiple comparisons test. (* p < 0.05, ** p < 0.01, **** p < 0.0001 vs. Control; # p < 0.05 vs previous lower concentration tested).

In the MTT reduction assay (Figure 21) PQ also induced significant cytotoxicity and caused a significant concentration-dependent decrease in MTT reduction, when compared to control cells (deionized sterile water incubated, 100 $\pm$ 7.69%).

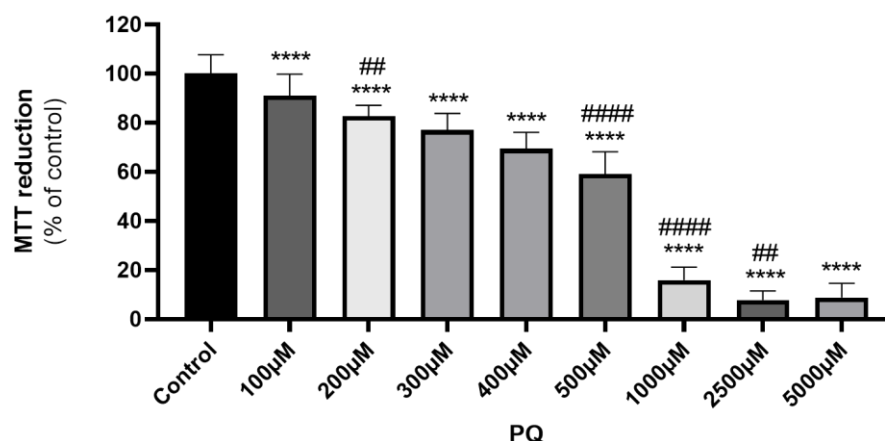


Figure 21. Paraquat (PQ) (100 - 5000µM) cytotoxicity in differentiated SH-SY5Y cells evaluated by the MTT reduction assay, 24 h after exposure. Results are presented as mean $\pm$ SD of 4 to 6 independent experiments (N=16 to 39). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (**** p < 0.0001 vs. Control; ## p < 0.01, #### p < 0.0001 vs previous lower concentration tested).

4.2.6 Determination of the LC 50 for PQ and 6-OHDA

As it was observed that PQ and 6-OHDA toxicity occurred in a concentration-dependent manner in differentiated SH-SY5Y cells in MTT reduction and NR uptake assays, so the LC50 values for both cytotoxicity assays were calculated through analysis of the fitted concentration-toxicity curves, at 24 h exposure. LC50 value represents the concentration at which each compound induces 50 % of lethality. Calculated values are presented in Table 5.

The concentration-toxicity curves for PQ and 6-OHDA in both MTT reduction and NR uptake assay for the determination of LC50 are shown in Figure 22 and 23, respectively.

For the MTT reduction assay, the calculated LC50 values for PQ and 6-OHDA were 585.8 and 133.6µM, respectively. Regarding data from the NR uptake assay the calculated LC50 values for PQ and 6-OHDA were 526.7 and 133.6µM, respectively.

Due to these obtained LC50 results, the concentration of PQ chosen for the neuroprotection assays were 500 and 300µM and for 6-OHDA was 125µM.

In the case of MPP+ it was not possible to draw a concentration-toxicity curve with the concentrations tested. Therefore, in this case the concentrations used in the neuroprotection assays were 1500 and 250µM, a higher concentration and an intermediate concentration, which cause similar cytotoxicity.

Table 5. LC₅₀ after a 24-h exposure obtained by analysis of the MTT and NR concentration-toxicity curves. Curves were fitted using least squares as the fitting method. The data, as LC₅₀ and confidence interval 95 %, are presented in μM .

	MTT reduction assay		NR uptake assay	
	6-OHDA	PQ	6-OHDA	PQ
LC ₅₀ [Mean (95 % CI) μM]	133.6 (126.3 - 141.9)	585.8 (562.3 - 611.6)	105.7 (93.69 - 117.5)	526.7 (485.5 - 569.8)

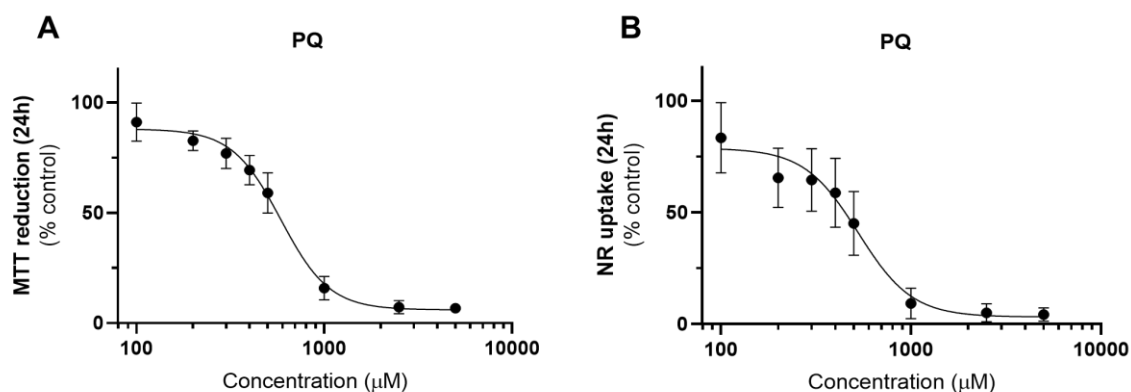


Figure 22. The concentration-response curves for PQ. Differentiated SH-SY5Y cells were exposed to 100, 200, 300, 400, 500, 1000, 2500 and 5000 μM of PQ for 24 h and the MTT reduction (A) and NR uptake (B) assays were performed.

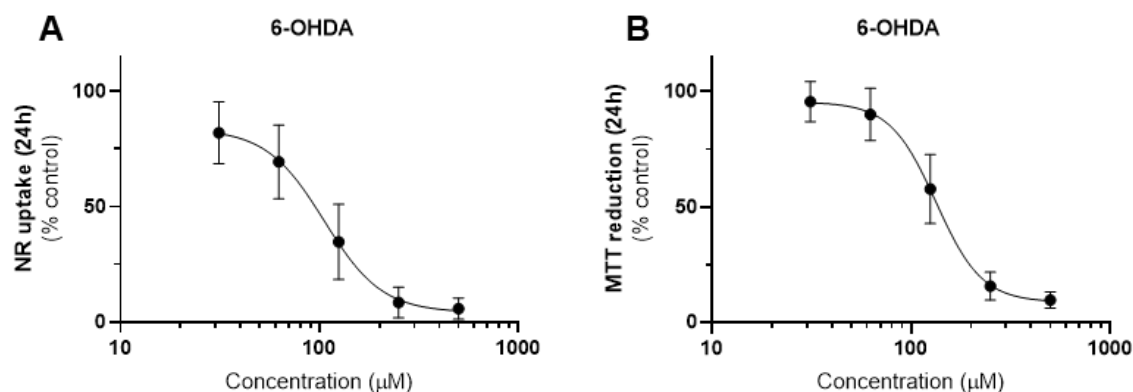


Figure 23. The concentration-response curves for 6-OHDA. Differentiated SH-SY5Y cells were exposed to 31.25, 62.5, 125, 250 and 500 μM of 6-OHDA for 24 h and the MTT reduction (A) and NR uptake (B) assays were performed.

4.2.6 PQ decreased mitochondrial membrane potential in differentiated SH-SY5Y cells

Evaluation of the $\Delta\Psi_m$, with the JC-1 probe, of differentiated SH-SY5Y cells after a 24-h exposure of PQ (300 or 500 μ M) for 24 h, showed that PQ 500 μ M induced a decrease in $\Delta\Psi_m$ to 50.08 ± 12.17 % when compared to control cells (100 ± 15.05 %), as observed in the figure 24.

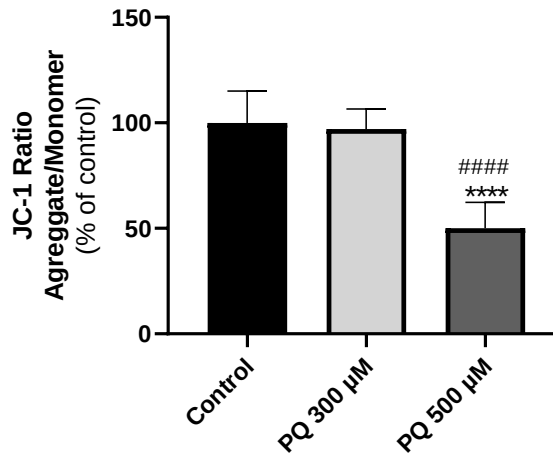


Figure 24. Mitochondrial membrane potential evaluated by JC-1 probe in differentiated SH-SY5Y cells incubated with 62.5 or 125 μ M of 6-OHDA after a 24-h exposure. Results are presented as mean $\pm$ SD of 2 independent experiments (N=6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (**** $p < 0.0001$ vs. Control; ##### $p < 0.0001$ vs. PQ 300).

4.2.8 Morphological cellular analysis of differentiated SH-SY5Y cells exposed to 6-OHDA, MPP+ and PQ

Cells were morphologically assessed after 24-h exposure to 6-OHDA 125 μ M, MPP+ 1500 μ M and PQ 500 μ M by phase-contrast microscopy and after Hoechst staining to ascertain the microscopic features associated with neurotoxins-induced cytotoxicity (Figure 25). In particular, both 6-OHDA and PQ promote cellular death accompanied by dendrite loss, in comparison to control cells. Meanwhile, by phase-contrast evaluation the MPP+ caused no morphological differences in comparison to controls.

Representative micrographs of SH-SY5Y cells exposed to 6-OHDA, MPP+ and PQ stained with Hoechst 33342 are presented in Fig. 25 D, F and H, respectively, depicting chromatin condensation (green arrows) and the presence of pyknotic nuclei (red arrows), that were almost absent in control cells. Of note, that all toxins evoke nuclear features that are usually present in apoptotic cell death, but these are mostly present in cells exposed to 6-OHDA and PQ. Despite MPP+ not evoking changes in phase contrast pictures, changes and features of nuclear changes were seen by Hoechst staining.

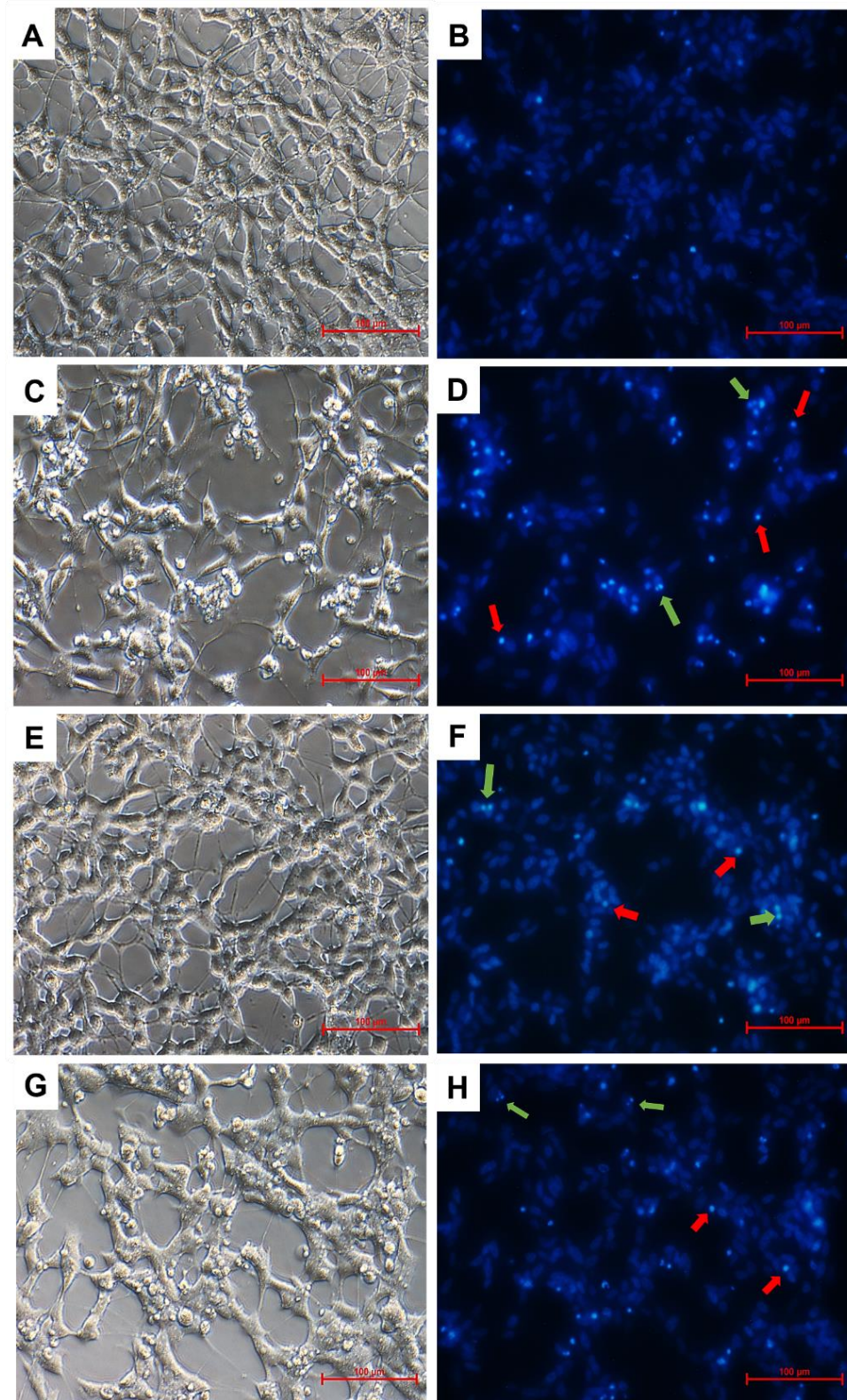


Figure 25. Morphological analysis by phase contrast microscopy (left side) of differentiated SH-SY5Y cells exposed for 24 h to 6-OHDA 125μM (C), MPP+ 1500μM (E), PQ 500μM (G) and the control group (A). Microphotographs from Hoechst nuclear staining (right side) were taken after a 24-h exposure period to 6-OHDA 125μM (D), MPP+ 1500μM (F), PQ 500μM (H) and control group (B). Inserted red arrows point the pyknotic nuclei, whilst green arrows indicate chromatin condensation. Representative microphotographs were taken of randomly chosen fields in 48-well plates (scale bar 100μm).

4.3 Neuroprotection evaluation

4.3.1 AMPH and MPH do not prevent 6-OHDA toxicity in SH-SY5Y cells

In the NR uptake assay, cells exposed to 6-OHDA 125 μ M revealed significant cytotoxicity, caused a decrease in NR uptake, when compared to control ($100 \pm 6.74\%$), which was not avoided by the pre-incubation with AMPH 1 μ M or MPH 1 μ M (Figure 26).

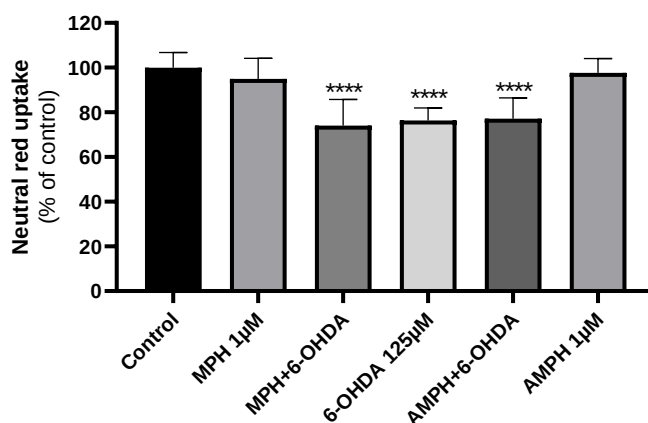


Figure 26. Cellular cytotoxicity evaluated by the neutral red (NR) uptake assay in differentiated SH-SY5Y cells pre-incubated with 1 μ M of AMPH or MPH and then incubated with 125 μ M of 6-OHDA for 24 h. Results are presented as mean $\pm$ SD of 4 independent experiments (N=15 to 16). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (**** $p < 0.0001$ vs. Control).

In the MTT reduction assay, cells exposed to 6-OHDA 125 μ M induced significant cytotoxicity, when compared to control ($100 \pm 3.15\%$), which was not prevented by the pre-incubation with AMPH 1 μ M or MPH 1 μ M (Figure 27).

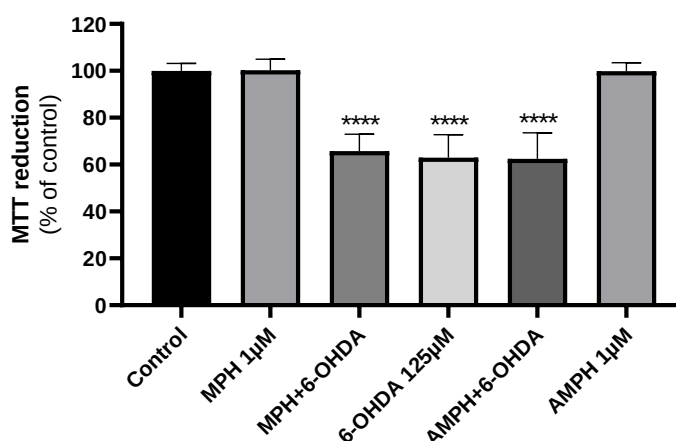


Figure 27. Cellular cytotoxicity evaluated by the MTT reduction assay in differentiated SH-SY5Y cells pre-incubated with 1μM of AMPH or MPH and then incubated with 125μM of 6-OHDA for 24 h. Results are presented as mean $\pm$ SD of 4 independent experiments (N=13 to 16). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (**** $p < 0.0001$ vs. Control).

4.3.1.1 Evaluation of ROS production

The production of ROS was measured with the fluorescent probe DCFH-DA after cells exposure to 6-OHDA (62.6 and 125μM), AMPH 1μM, MPH 1μM and 6-OHDA 62.5 or 125μM pre-incubated with AMPH 1μM or MPH 1μM for a 24-h exposure. The results are presented in Figures 28 and 29. As a positive control t-BHP 200μM was used.

As shown in the Figure 28, cells treated with AMPH 1μM evoked a significant production of ROS when compared to control levels, after 1, 2, 3, 4, 5 and 6 h exposure, but this effect was no longer observed at 24 h. On other hand, after 24 h, cells exposed to 6-OHDA 62.5μM and 6-OHDA 62.5μM pre-incubated with AMPH 1μM or with MPH 1μM, induced a significant production of ROS when compared to control cells.

As shown in the Figure 29, cells treated with MPH 1μM induced a significant production of ROS when compared to control cells at 0, 1, 2, 4, 6 and 24 h after exposure. Meanwhile, cells treated with AMPH 1μM induced a significant production of ROS at all time-points until 24-h exposure. Indeed at 24-h, cells exposed to 6-OHDA 125μM, 6-OHDA 125μM pre-incubated with AMPH 1μM or MPH 1μM, showed a significant production of ROS when compared to control cells.

The results suggest that 6-OHDA (62.5 and 125μM) promote an increase in ROS levels, but on the other hand AMPH and MPH alone can also induce its formation.

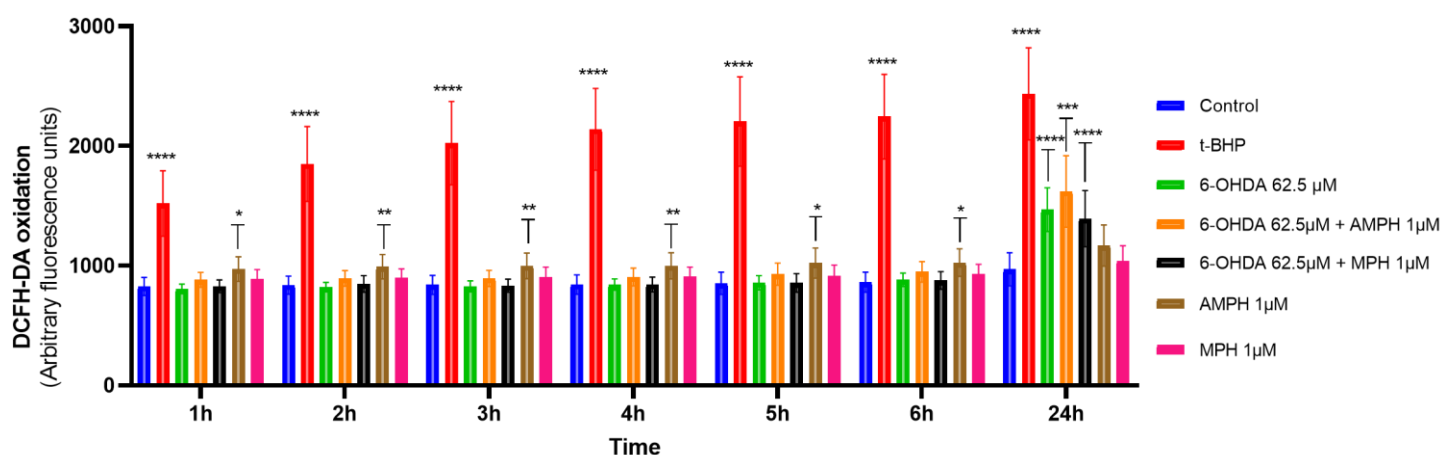


Figure 28. Assessment of reactive oxygen species (ROS) production, using the 2',7'-dichlorodihydrofluorescein diacetate probe, on differentiated SH-SY5Y cells exposed to 6-OHDA 62.5 μ M, AMPH 1 μ M, MPH 1 μ M and 6-OHDA 62.5 μ M pre-incubated with AMPH 1 μ M or MPH 1 μ M until 24-h exposure. Results are presented as mean $\pm$ SD of 4 independent experiments (N=12). Statistical analysis was performed using two-way ANOVA repeated measurements followed by Tukey's multiple comparisons test. (* p < 0.05, ** p < 0.01, *** p < 0.001 **** p < 0.0001 vs. Control).

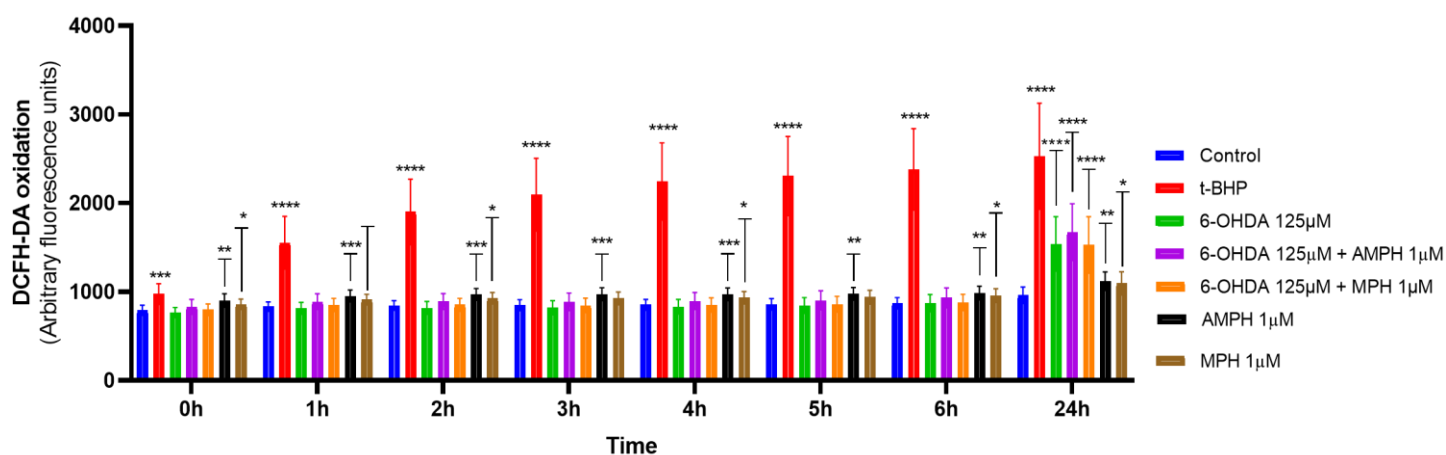


Figure 29. Assessment of reactive oxygen species (ROS) production, using the 2',7'-dichlorodihydrofluorescein diacetate probe, on differentiated SH-SY5Y cells exposed to 6-OHDA 125 μ M, AMPH 1 μ M, MPH 1 μ M and 6-OHDA 125 μ M pre-incubated with AMPH 1 μ M or MPH 1 μ M until 24-h exposure. Results are presented as mean $\pm$ SD of 5 independent experiments (N=15). Statistical analysis was performed using two-way ANOVA repeated measurements followed by Tukey's multiple comparisons test. (* p < 0.05, ** p < 0.01, *** p < 0.001 **** p < 0.0001 vs. Control).

4.3.2 AMPH and MPH do not prevent MPP+ toxicity in SH-SY5Y cells

In the NR uptake assay, MPP+ 250 μ M induced a significant cytotoxicity, when compared to control ($100 \pm 3.24\%$), which was not avoided by the pre-incubation with AMPH 1 μ M or MPH 1 μ M (Figure 30 A). Likewise, cells exposed to MPP+ 1500 μ M also induced a significant cytotoxicity, when compared to control ($100 \pm 3.24\%$), which was not avoided by the pre-incubation with AMPH 1 μ M or MPH 1 μ M. In fact, cells exposed to MPP+ 1500 μ M and pre-incubated with AMPH 1 μ M caused higher cytotoxicity when assessed by the NR uptake assay when compared to MPP+ 1500 μ M alone. It should be noted that in these experiments cells exposed to AMPH 1 μ M induced a small but significant cytotoxicity, when compared with control ($100 \pm 3.24\%$) (Figure 30 B).

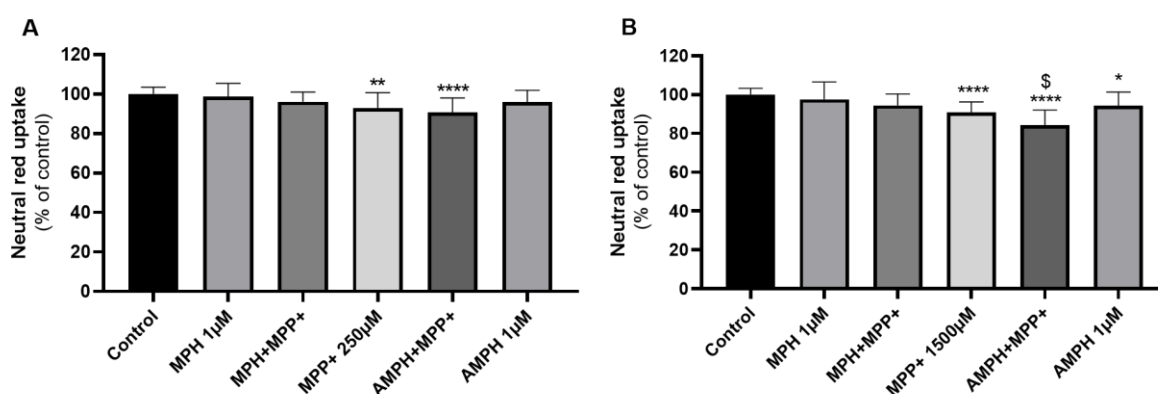


Figure 30. Cellular cytotoxicity evaluated by the NR uptake assay in differentiated SH-SY5Y cells pre-incubated with 1 μ M of AMPH and MPH and then incubated with 250 μ M (A) or 1500 μ M (B) of MPP+ for 24 h. Results are presented as mean $\pm$ SD of 5 (A) to 6 (B) independent experiments ($N=20$ to 24). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs. Control; \$ $p < 0.05$ vs. MPP+ 1500 μ M).

In the MTT reduction, MPP+ 250 μ M induced a significant cytotoxicity, when compared to control ($100 \pm 5.65\%$), which was not avoided by the pre-incubation with AMPH 1 μ M or MPH 1 μ M (Figure 31 A). Likewise, cells exposed to MPP+ 1500 μ M also induced a significant cytotoxicity, when compared to control ($100 \pm 4.72\%$), which was not avoided by the pre-incubation neither with AMPH 1 μ M or MPH 1 μ M (Figure 31 B).

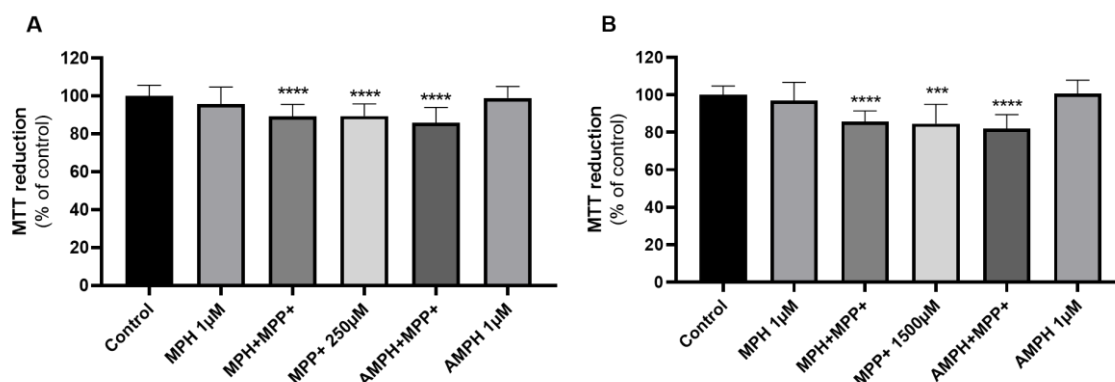


Figure 31. Cellular cytotoxicity evaluated by the MTT reduction assay in differentiated SH-SY5Y cells pre-incubated with 1µM of AMPH and MPH and then incubated with 250µM (A) or 1500µM (B) of MPP+ for 24 h. Results are presented as mean ± SD of 6 independent experiments (N=20 to 24). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (A) and one-way ANOVA on ranks (Kruskal-Wallis test) followed by Dunn's multiple comparisons test (B). (**p < 0.01, ***p < 0.001, ****p < 0.0001 vs. Control).

4.3.2.1 MPP+ did not evoke an increase in ROS levels

The production of ROS was measured with the fluorescent probe DCFH-DA after cells exposure to MPP+ (250 or 1500µM), AMPH 1µM, MPH 1µM and MPP+ 250 or 1500µM pre-incubated with AMPH 1µM or MPH 1µM. The results are presented in Figures 32 and 33. As a positive control t-BHP 200µM was used.

The results clearly suggest that MPP+ (at 250 and 1500µM) do not promote increased ROS levels at all time-points evaluated. As shown in the Figure 32, cells treated with MPH 1µM induced a significant production of ROS when compared to control cells, after 4, 5 and 24-h exposure, and AMPH 1µM induced a significant production of ROS at all time-points until 24-h exposure. Likewise, in graph of Figure 33, cells treated with MPH 1µM induced a significant production of ROS after a 1 and 24-h after exposure, and AMPH 1µM at all time-points until 24-h exposure, when compared to control cells.

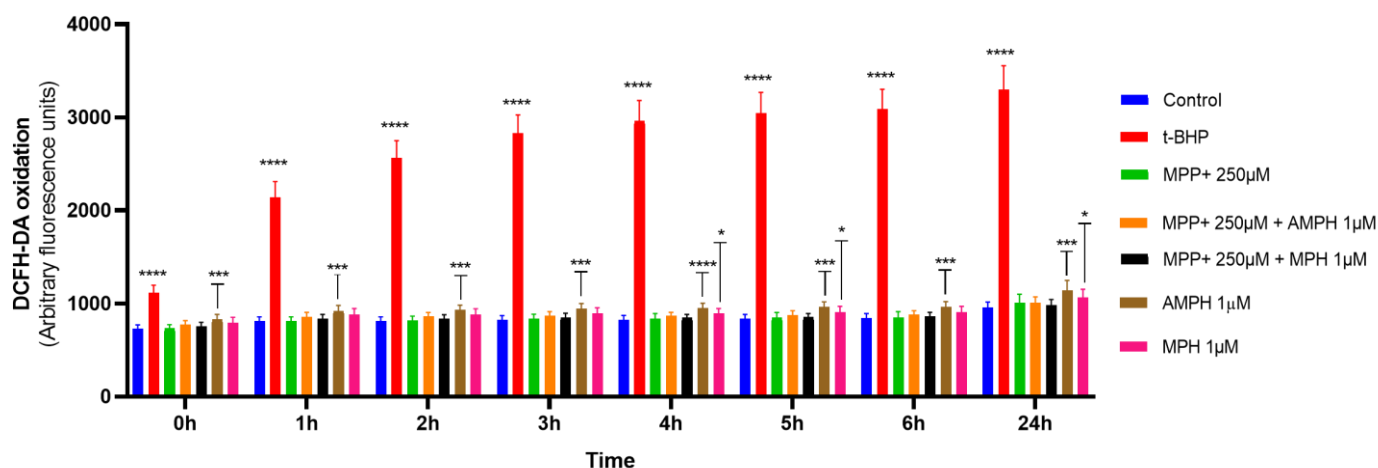


Figure 32. Assessment of reactive oxygen species (ROS) production, using the 2',7'-dichlorodihydrofluorescein diacetate probe, on differentiated SH-SY5Y cells exposed to MPP+ 250µM, AMPH 1µM, MPH 1µM and MPP+ 250µM pre-incubated with AMPH 1µM or MPH 1µM for a 24-h exposure. Results are presented as mean $\pm$ SD of 4 independent experiments (N=12). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. (* $p < 0.05$, *** $p < 0.001$ **** $p < 0.0001$ vs. Control).

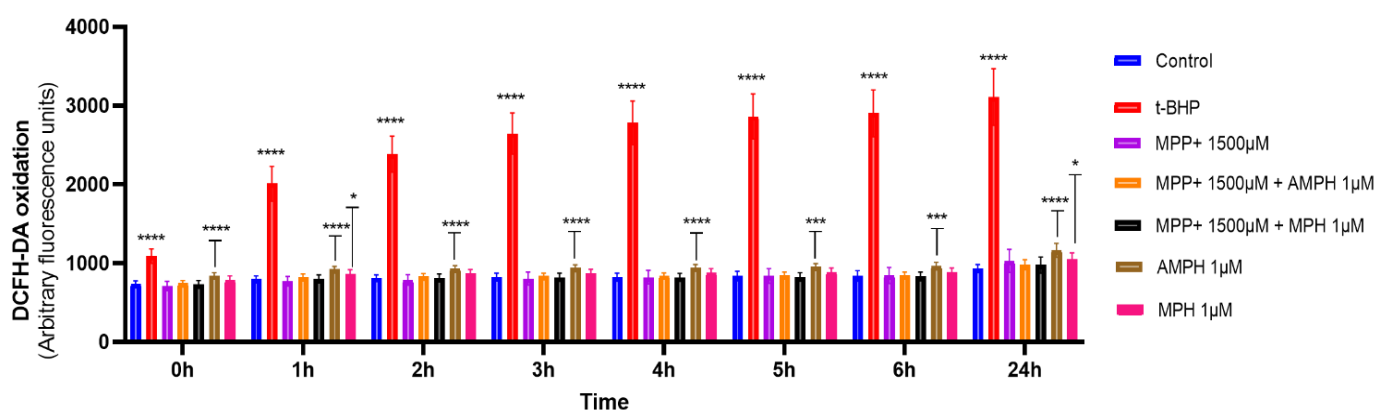


Figure 33. Assessment of reactive oxygen species (ROS) production, using the 2',7'-dichlorodihydrofluorescein diacetate probe, on differentiated SH-SY5Y cells exposed to MPP+ 1500µM, AMPH 1µM, MPH 1µM and MPP+ 1500µM pre-incubated with AMPH 1µM or MPH 1µM for a 24-h exposure. Results are presented as mean $\pm$ SD of 4 independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. (* $p < 0.05$, *** $p < 0.001$ **** $p < 0.0001$ vs. Control).

4.3.3 AMPH and MPH partially protect SH-SY5Y cells against PQ-induced neurotoxicity

In NR uptake assay, PQ 300 μ M induced significant cytotoxicity, when compared to control ($100 \pm 10.53\%$), which was not avoided by the pre-incubation with AMPH 1 μ M or MPH 1 μ M (Figure 34 A). Cells exposed to PQ 500 μ M induced significant cytotoxicity, when compared to control ($100 \pm 6.76\%$), which was not avoided by the pre-incubation with AMPH 1 μ M or MPH 1 μ M (Figure 34 A).

It should be noted that, unexpectedly, AMPH 1 μ M also caused a small, but significant, decrease in NR uptake when compared with control cells (Figure 34 A-B).

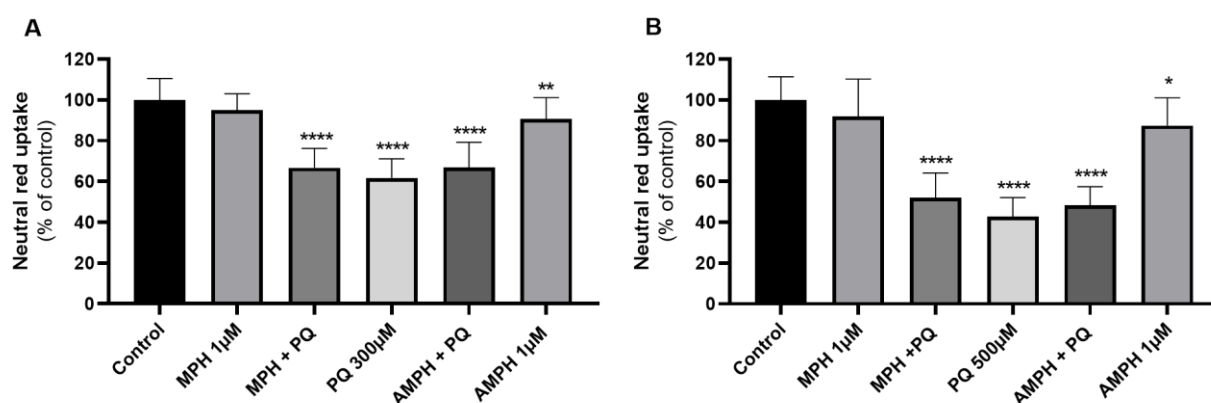


Figure 34. Cellular cytotoxicity evaluated by the neutral red (NR) uptake assay in differentiated SH-SY5Y cells pre-incubated with 1 μ M of AMPH or MPH and then incubated with 300 μ M (A) or 500 μ M (B) of PQ for 24 h. Results are presented as mean $\pm$ SD of 6 independent experiments (N=24 to 32). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs. Control).

In the MTT reduction assay, PQ 300 μ M induced significant cytotoxicity, causing a decrease in MTT reduction when compared to control ($100 \pm 6.59\%$), which was partially avoided by the pre-incubation with either AMPH 1 μ M or MPH 1 μ M (Figure 35 A). Cells exposed to PQ 500 μ M showed signs of significant cytotoxicity, when compared to control ($100 \pm 6.70\%$), which was partially avoided by the pre-incubation with MPH 1 μ M, but not by the pre-incubation with AMPH 1 μ M (Figure 35 B).

Therefore, in the MTT reduction assay, AMPH and MPH partially protected the cells from cytotoxicity induced by PQ 300 μ M, while MPH was responsible for the partial protection of cells treated with PQ 500 μ M.

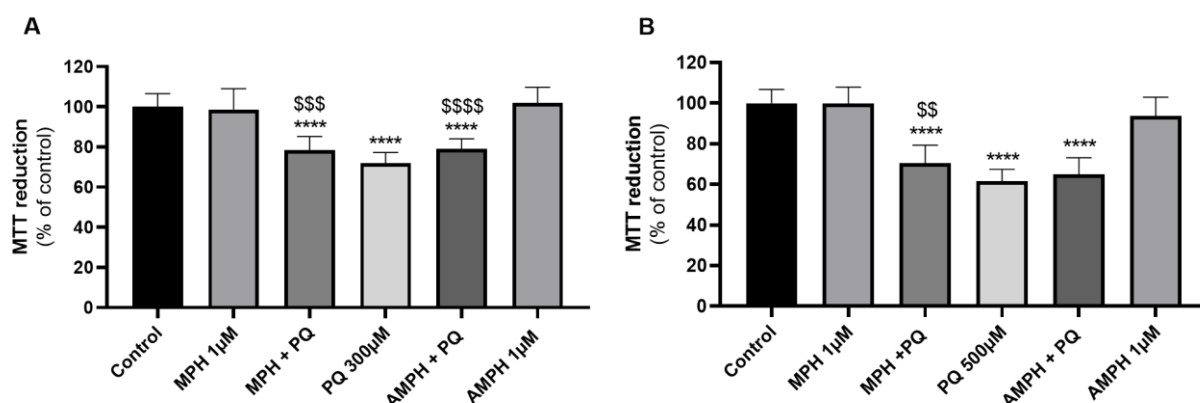


Figure 35. Cellular cytotoxicity evaluated by the MTT reduction assay in differentiated SH-SY5Y cells pre-incubated with 1µM of AMPH and MPH and then incubated with 300µM (A) and 500µM (B) of PQ for 24 h. Results are presented as mean $\pm$ SD of 6 (A) to 7 (B) independent experiments (N=24 to 32). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. (**** $p < 0.0001$ vs. Control; ** $p < 0.01$ vs PQ 500 µM; *** $p < 0.001$, **** $p < 0.0001$ vs PQ 300 µM).

4.3.3.1 PQ did not evoke an increase in ROS levels

The production of ROS was measured with the fluorescent probe DCFH-DA after cells exposure to PQ (300 and 500µM), AMPH 1µM (C), MPH 1µM and PQ 300µM pre-incubated with AMPH 1µM or MPH 1µM for a 24-h exposure. The results are presented in Figure 36. As a positive control was used t-BHP 200 µM,

PQ alone did not induce a significant increase in ROS levels at any of the time-points tested. The results suggest that PQ alone (300 and 500µM) does not promote production of ROS.

Cells treated with AMPH 1µM induced significant production of ROS when compared to control cells, after 1 to 6h exposure, but this effect was no longer observed at 24 h. Also, it should be mentioned that MPH 1µM induced significant production of ROS 1 h after exposure. In the case of cells exposed to PQ 300µM pre-incubated with AMPH 1µM or MPH 1µM, there was a significant increase in ROS levels, after 2 to 6 h exposure and 1 to 4 h exposure, respectively.

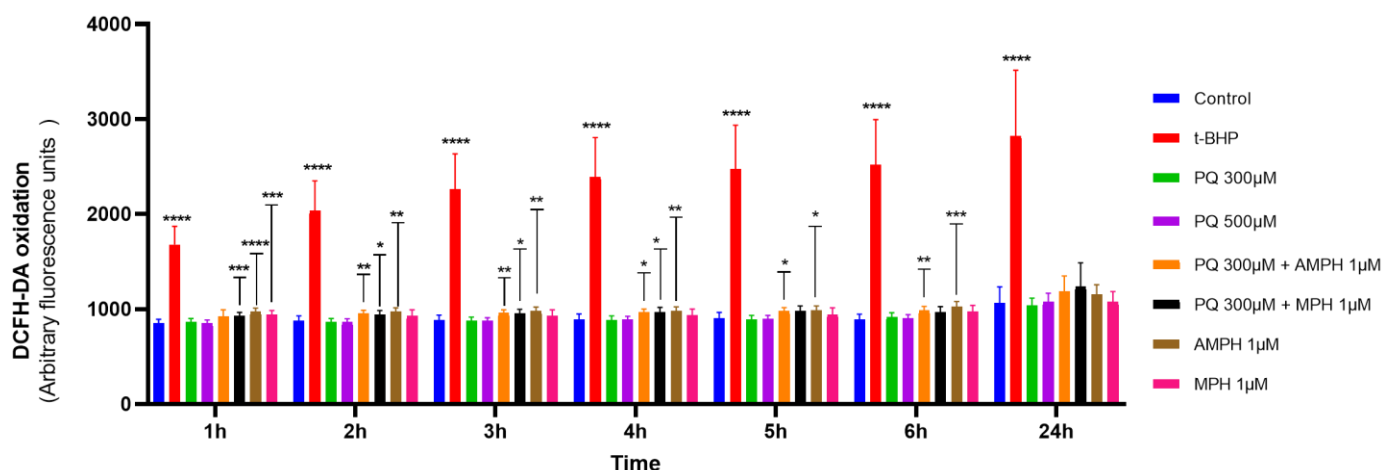


Figure 36. Assessment of reactive oxygen species (ROS) production, using the 2',7'-dichlorodihydrofluorescein diacetate probe, on differentiated SH-SY5Y cells exposed to PQ (300 and 500µM), AMPH 1µM, MPH 1µM and PQ 300µM pre-incubated with AMPH 1µM or MPH for a 24-h exposure. Results are presented as mean $\pm$ SD of 4 independent experiments (N=12). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. (* p < 0.05, ** p < 0.01, *** p < 0.001 **** p < 0.0001 vs. Control).

4.3.2.2 Microscopic analysis of SH-SY5Y cells exposed to PQ and pre-incubated with AMPH or MPH

Since MPH and AMPH afforded neuroprotection against PQ toxicity in differentiated SH-SY5Y cells, microscopic analysis of morphological cellular features were evaluated with the neurite outgrowth staining kit. For this, cells were exposed to PQ 300µM, AMPH 1µM, MPH 1µM, and PQ 300µM pre-incubated with AMPH 1µM or MPH 1µM, for 24 h, after which cells were stained. Microphotographs of the red fluorescent cells membranes stain, which stained cell bodies and membranes of neurite outgrowth are presented in Figure 37. It was possible to see that PQ toxicity was accompanied by cellular and neurite loss, in comparison to control cells Figure 35B. Meanwhile in cells pre-incubated with AMPH 1µM or MPH 1µM there was a partial protection against the neurite loss evoked by exposure to PQ 300µM Figure 37D e 37F.

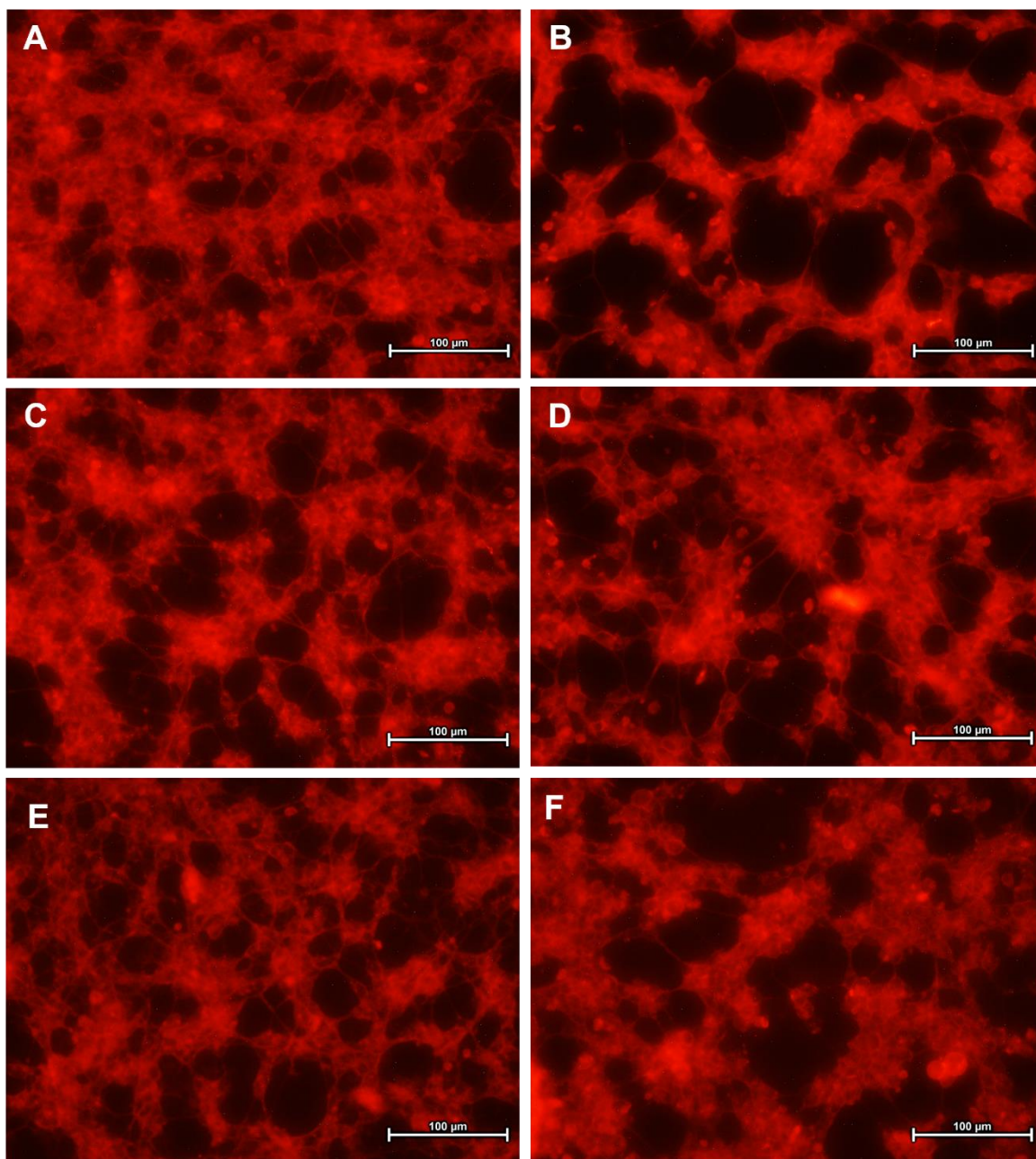


Figure 37. Microphotographs of differentiated SH-SY5Y cells stained with the neurite outgrowth staining kit after a 24 h exposure period to PQ 300 μ M (B), AMPH 1 μ M (C), MPH 1 μ M (E), PQ 300 μ M pre-incubated with AMPH 1 μ M (D) or MPH 1 μ M (F), and the control group (A). Representative microphotographs were taken of randomly chosen fields in 48-well plates (scale bar 100 μ m).

5 Discussion

This study used human neuroblastoma SH-SY5Y differentiated cells. In order to obtain a dopaminergic neuronal phenotype, cells were differentiated using RA and TPA, as previously described by our group (154). The use of RA and TPA for the differentiation of SH-SY5Y cells has been shown to promote a reduced mitotic rate and enhancement of the dopaminergic phenotype (144). In accordance, Ferreira *et al.* showed that differentiated SH-SY5Y cells had an enhanced ability to uptake and retain DA, which indicates that cells express higher levels of dopaminergic transporters (154). Moreover, Barbosa *et al.* showed higher TH levels (153). Therefore, this *in vitro* model represents a valuable tool for studying potential drugs for PD treatment.

As AMPH and MPH psychostimulants target the dopaminergic transporter and, therefore, the dopaminergic neurons, differentiated SH-SY5Y cells represent a valuable model to study their effects. Moreover, as this dissertation aims to focus on PD, which is mainly a dopaminergic affecting pathology, the dopaminergic phenotype of the cells is the best suited for *in vitro* studies.

5.1 Assessment of AMPH and MPH therapeutic concentrations neurotoxic potential

Clinical use of drugs in humans typically involves oral administration of relatively low doses. To mimic the low concentrations attained in human plasma and brain, the following range of concentrations 0.001, 0.01, 0.1, 1 and 10 μ M of both AMPH and MPH were tested in differentiated SH-SY5Y cells in this dissertation. The selection of these concentrations in the case of MPH was based on the evidence that concentrations of 1 to 100nM correlate well with patients' serum values upon 1mg/kg MPH oral intake (168), while 10nM - 1 μ M concentrations correlate well with a mixed AMPH salts extended-release formulation of 30mg (169). Both these doses are clinically relevant. These tested concentrations are well below the AMPH 1mM concentration which evoked cellular viability loss in differentiated SH-SY5Y cells (170). Moreover, these concentrations showed in a previous study that MPH evokes neurite outgrowth in differentiated SH-SY5Y cells (168). Despite this previous knowledge, it was necessary to determine the cellular viability and $\Delta\Psi_m$ of the cells incubated to both AMPH and MPH at these concentration ranges and assess if they are potentially toxic to differentiated SH-SY5Y cells. Cytotoxicity assays are widely used as *in vitro*. The NR uptake impairment, which evaluates the lysosomal function and MTT

reduction assay, which evaluates mitochondrial function, and the lactate dehydrogenase (LDH) leakage assay are the most common assays used for the detection of cytotoxicity (171). It should be noted that the NR assay and the MTT seem to be the most sensitive in detecting cytotoxic events in comparison to LDH leakage (171), which reports the rupture of cytoplasmic membranes.

In this work the NR and MTT assays were performed (**Figure 11** and **12**), and as expected no significant differences in cell viability were observed for any of the concentrations tested of either AMPH or MPH following a 24 h-exposure. In fact, all concentrations showed no cytotoxicity when compared to control cells, with consistent results in all experiments. Thus, the drugs at the concentrations tested do not appear to cause mitochondrial dysfunction or damage in lysosomes. One could even speculate even that with longer exposure periods they would lead to toxicity, but that was not performed.

Evaluation of the effects of the drugs on $\Delta\Psi_m$, with the JC-1 probe revealed that following a 24-h incubation period neither AMPH nor MPH caused a significant effect on $\Delta\Psi_m$ (**Figure 13**). A normal $\Delta\Psi_m$ is important for maintaining the function of the respiratory chain, thus with a full potential in generating ATP and is normally a sign that the cell is healthy (165) Thus, these clinically relevant concentrations of both AMPH and MPH are deemed non-toxic in several assays and can be tested *in vitro* as potential neuroprotectors.

5.2 Modular dopaminergic toxins neurotoxicity assessment

In this study, the neurotoxic potential of three toxins the 6-OHDA, MPP⁺ and PQ was assessed by the NR uptake and the MTT reduction assays and the possible interference with $\Delta\Psi_m$, with the purpose of subsequently using these toxins as an *in vitro* model of PD. Based on the previous knowledge, these neurotoxins are viewed as experimental toxicants to study the mechanisms of PD *in vitro* (62), and some of their characteristics were described in subsection 1.2.

The cytotoxic profile of 6-OHDA was analysed through the NR uptake and MTT reduction assays (**Figure 14** and **15**), after a 24-h exposure. The results demonstrated that the neurotoxicity induced by 6-OHDA had a concentration-dependent pattern, with significative toxicity from the 62.5 μ M concentration onwards. Importantly, it was observed that the NR uptake assay was overall more sensitive than the MTT reduction assay towards the toxicity of 6-OHDA. Correlating these results to the obtained LC₅₀ values for both cytotoxicity assays, the LC₅₀ in the MTT reduction assay was about 1.26 times higher, respectively, than the LC₅₀ in the NR uptake assay (**Table 5**). Other studies have shown

similar results to those herein reported. A study performed by Jordán *et al.* reported that 6-OHDA induced a marked cytotoxicity, assessed by the MTT reduction assay, in undifferentiated SH-SY5Y cells, following an exposure in the range of 25 - 1000 μM for 24 h. In addition, treatment of undifferentiated SH-SY5Y cells with 6-OHDA 100 μM resulted in morphological changes typical of apoptosis such as cell shrinkage, rounding and detachment from the plate (observed under phase contrast microscopy), and the staining of nuclei with Hoechst 33342 dye showed chromatin condensation and fragmentation, when compared with control (172). Another study in undifferentiated SH-SY5Y cells exposed to different concentrations of 6-OHDA (10 - 1000 μM) for 24 h demonstrated that the toxin induced a concentration-dependent cytotoxicity when determined by the MTT reduction assay, and LC50 was estimated to be 116.7 μM (173). In concordance, in this dissertation, it was observed under phase contrast microscopy that 6-OHDA 125 μM promoted cellular death accompanied by dendrite loss. Moreover, when cells were stained with Hoechst 33342 dye, chromatin condensation and the presence of pyknotic nuclei were observed, both being signs of apoptosis (**Figure 25**). Morphologically, apoptotic cell death is a process of condensation and fragmentation of the nucleus (including pyknosis) with blebbing of the plasma membrane to form membrane-bound apoptotic bodies that contain nuclear fragments, organelles, and condensed cytosol (174). From the microscopic observation it was possible to observe signs that suggest an important role for apoptosis in the cytotoxicity elicited by 6-OHDA.

Mitochondrial alterations, including variations in $\Delta\Psi\text{m}$, are main events that induce apoptosis. In apoptotic cells, there is an increase in membrane permeability due to opening of the mitochondrial permeability transition pores and there is a decrease in the negative charge inside the mitochondria due to the loss of electrochemical potential (165). These changes may be important and result in the generation of ROS. In this dissertation, assessment of $\Delta\Psi\text{m}$ was performed in cells previously exposed to 6-OHDA 62.5 μM and 125 μM , for 24 h (**Figure 16**). Both concentrations depolarised the mitochondria. Silva *et al.* observed in SH-SY5Y cells that 6-OHDA (100 μM , after a 3-h exposure) promoted a depolarization of the mitochondria (173). The collapse of the $\Delta\Psi\text{m}$ herein can lead to trigger events such as apoptosis, which in this case is consistent with the changes observed in cells stained by Hoechst 33342 (**Figure 25**). It should be noted that a significant loss of $\Delta\Psi\text{m}$ causes a depletion of energy with possible subsequent death of cell. These events are probably explained by the fact that 6-OHDA promotes a direct inhibition of mitochondrial complexes I, which can lead to ROS formation. Additionally, 6-OHDA can accumulate in cells and undergo a non-enzymatic auto-oxidation, promoting the ROS formation such as, radical anion superoxide and H_2O_2 , *per se*. This work revealed that 6-OHDA (62.5 and

125 μ M) produces a significant increase in ROS levels after a 24-h exposure in the DCFH-DA assay (**Figure 28 and 29**). The loss of $\Delta\Psi_m$ and ROS accumulation are probably linked and can explain the apoptotic fate of cells exposed to 6-OHDA.

The cytotoxic profile of MPP⁺ was also analysed through the NR uptake and MTT reduction assays, at 24h (**Figure 17 and 18**). The results obtained from NR uptake and MTT reduction assays demonstrated that MPP⁺ reduced cell viability significantly when compared to the control, from the concentration 125 μ M onward. The results demonstrated that the neurotoxicity induced by MPP⁺ does not occur in a concentration-dependent manner. In fact, for the concentration range tested (125 - 1500 μ M), in both assays, cytotoxicity was only about 10%. The study by Martins *et al.* showed that after exposure of differentiated SH-SY5Y cells to MPP⁺ (100, 500, and 1000 μ M), there was no increase in cellular damage at any time-point or concentration tested, assessed by LDH release assay. On the other hand, that work showed that MPP⁺ caused a significant mitochondrial dysfunction after 24 h of exposure to 500 and 1000 μ M concentrations (around 10%). However, it was only after a 72-h exposure that MTT metabolism decreased significantly for all concentrations tested, especially for 1000 μ M (around 30%) (70). Therefore, the possible reason for the low toxicity induced by MPP⁺ in this dissertation, might have been the short time of exposure (24 h). Thus, longer periods of exposure should be needed to observe higher levels of toxicity for this toxin, such as 48h or 72-h of exposure, but that was not the aim of the present work. Still, since it has been shown that differentiation of SH-SY5Y cells with RA and TPA gives them higher ability to accumulate DA and higher resistance towards DA neurotoxicity (154), that should have happened here. Indeed, Cheung *et al.* demonstrated that differentiation of SH-SY5Y cells with RA conferred greater resistance to MPP⁺ toxicity (149).

Reports justify MPP⁺ toxicity from the fact that MPP⁺ accumulates within the mitochondria, and interferes with complex I of mitochondrial transport chain, inhibiting its activity (175). This can result in a decrease in ATP stores and promote the formation of ROS, leading to possible cell death. Morphological evaluation of cells exposed to MPP⁺ (1500 μ M) for 24 h by phase-contrast revealed no significant changes compared to controls. However, when stained with dye Hoechst 33342, chromatin condensation and the presence of pyknotic nuclei were observed, which are signs of apoptosis (**Figure 25**). Following, the assessment of $\Delta\Psi_m$, in cells previously exposed to MPP⁺ (250 and 1500 μ M) for 24 h, showed a significant increase of $\Delta\Psi_m$ for both concentrations when compared with the control cells (**Figure 19**). Whether that increase of $\Delta\Psi_m$ is transient or long lasting is unknown. In fact, a long-lasting rise of $\Delta\Psi_m$ may evoke loss of cell viability, and therefore might be the trigger for apoptosis and toxicity (165). Meanwhile, these changes in

mitochondria were not accompanied by ROS production, since the DCFH-DA assay showed that MPP⁺ (250 and 1500 μ M) did not promote a significant production of ROS after a 24-h exposure (**Figure 32 and 33**). Due to the previous knowledge of the toxicity mechanisms of MPP⁺, these were unexpected results. As analysed above, MPP⁺ causes mitochondrial dysfunction and damage to lysosomes, however to a reduced extent, revealing that at the concentrations tested MPP⁺ do not promote high toxicity, at least in differentiated SH-SY5Y cells at the 24 h time-point. So, it can be hypothesised that this evidence may have conditioned the observed results obtained in the DCFH-DA assay and $\Delta\Psi_m$.

The cytotoxic profile of PQ was analysed through the MTT reduction and NR uptake assays, both performed at 24h (**Figure 20 and 21**). The results demonstrated that neurotoxicity induced by PQ occurs in a concentration-dependent manner, with cytotoxicity starting at 100 μ M in the MTT reduction assay, and in the NR uptake assay from the 200 μ M concentration onwards. Martins *et al.*, which used the same cells, did not observe differences, after a 24-h incubation with PQ, on the LDH leakage assay, however in the MTT reduction assay, mitochondrial dysfunction was observed after 24 h of exposure for the 500 μ M and 1000 μ M concentrations, and after 48 h for the 100 μ M concentration. These results led the authors to hypothesize that PQ toxicity was due to the disruption of the mitochondrial electron transport chain (70). A study conducted by Yang *et al.* in differentiated SH-SY5Y revealed that 0.5 and 1mM PQ significantly decreased cell viability to 56.2% and 42.8% of the control, assessed by trypan blue exclusion after a 48-h exposure (176). Fujimori *et al.* reported that differentiated SH-SY5Y cells exposed to PQ 500 μ M for 24 h had their cell viability decreased around 50% cell, when that was measured using cell counting reagent SF (177). These reports are in fact consistent with the results reported in this thesis (LC₅₀ = 585 μ M and 526 μ M in MTT and NR assay, respectively) (**Table 5**). It should be noted different cell lines seem to present different susceptibility towards PQ-induced cytotoxicity. Zerlin *et al.* reported that A549 cells (a human alveolar basal epithelial cells) exposed to 0.3mM PQ for 24 h had their cell viability decreased significantly, and that after the same incubation period with 0.7mM had a cell viability decrease of around 80% (178). Silva *et al.* evaluated PQ cytotoxicity in Caco-2 cells (a human colorectal adenocarcinoma cell line) by the MTT reduction assay and it was observed that after a 24 and 48-h exposure the LC₅₀ was approximately 1000 and 500 μ M, respectively (179). In a study with RBE4 cells (immortalized rat brain microvessel endothelial cells), the cell viability was not affected by PQ 1mM after 24 and 48-h exposure. In fact, cell viability was significantly reduced only at PQ concentrations higher than 2.5mM (180). It seems therefore that neurons present a higher susceptibility towards PQ neurotoxicity.

Importantly, was observed that the NR uptake assay was overall more sensitive than the MTT reduction assay to reveal the toxicity of PQ. Correlating these results to the obtained LC50 values for both cytotoxicity assays, the LC50 in the MTT reduction assay was about 1.11 times higher than the LC50 in the NR uptake assay. However, it should be noticed that in the MTT reduction assay the lowest concentration that caused significant differences in cell viability was 100 μ M, but in the NR significant toxicity was only observed for the 200 μ M (**Table 5**).

Assessment of $\Delta\Psi_m$ performed in cells previously exposed to PQ 300 and 500 μ M, for 24 h, demonstrated that depolarization of the mitochondria occurs for PQ 500 μ M, not for the 300 μ M concentration. This provides evidence that depolarization of the $\Delta\Psi_m$ occurred in a concentration-dependent manner. This collapse of the $\Delta\Psi_m$ can lead to events such as apoptosis, which in this case is consistent with the changes observed in the stained cells by Hoechst 33342 dye, such as, chromatin condensation and the presence of pyknotic nuclei (**Figure 25**). One study reported that differentiated SH-SY5Y cells treated with PQ 0.5mM significantly reduced $\Delta\Psi_m$ to 69.8% and 54.3% of control, after a 24 and 48-h exposure, respectively (176). This evidence supports the idea that PQ promotes an inhibition of mitochondrial complex I, which may lead to increased ROS production. One of the suggested mechanisms for PQ-induced neurotoxicity is oxidative stress, leading to the production of ROS such as radical anion superoxide, $\bullet$ OH and H₂O₂, which increase lipid peroxidation, increase oxidation of proteins and disrupts iron availability (176). However, herein using DCFH-DA assay, PQ (300 and 500 μ M) did not promote an increase of ROS production after 24-h exposure (**Figure 36**). This was an unexpected result, perhaps a longer exposure time or higher concentrations would be necessary to observe ROS production, or even look for ROS using another fluorescent probe. However, a study using the DCFH-DA probe in differentiated SH-SY5Y cells showed at 24 and 48-h exposure that PQ 0.5mM significantly increased ROS levels to 129.8% and 151.1%, respectively, when compared with control (100%) (176). In addition, Xiuli *et al.* reported that PQ exposure caused a dose-dependent increase in ROS production, assessed by the DCFH-DA assay, and significant increases were observed for PQ 10 and 100 μ M, in immortalized human embryonic neural progenitor cells (hNPCs) (181).

In conclusion, PQ, MPP⁺ and 6-OHDA toxicity involve mitochondrial dysfunction and damage to lysosomes. PQ and 6-OHDA promoted loss of the $\Delta\Psi_m$, which means that in the presence of these toxins there is a loss of electrochemical potential, the negative charge inside the mitochondria decreases, which causes an increase in membrane permeability. Furthermore, 6-OHDA leads to a significant increase in ROS levels. It should be noted that PQ is more stable in solution than MPP⁺ and 6-OHDA, which are photosensitive and may

lose activity when in solution with longer exposure periods for this, working solutions should be freshly prepared (information provided by the manufacturer). Therefore, PQ may represent several advantages over MPP⁺ and 6-OHDA to study PD mechanisms *in vitro*.

5.3 Neuroprotection assays analyses

This dissertation study tried to understand whether AMPH and MPH could partially prevent the toxicity induced by the previously mentioned toxins. The reduction of MTT and NR uptake by the cells were evaluated and then the results obtained from the toxin alone and pre-incubated with AMPH or MPH were compared. In addition, the production of ROS was analysed to understand if the data obtained can be complementary to the results of the cellular viability assays.

The presynaptic activity of DAT seems to play an important role in function and maintenance of the dopaminergic system. Neurotoxins such as 6-OHDA and MPP⁺ enter in the cell via DAT, and it also seems that PQ uses this transporter, leading to selective degeneration of dopaminergic neurons (182). As an example, mice without DAT are resistant to MPTP toxicity (183) and mice with increased DAT show increased toxicity to MPTP (184). Therefore, a blockade or interference of DAT should prevent toxins such as those mentioned, from entering in cells and reduce the death of dopaminergic neurons, and since AMPH and MPH interfere with that transporter, it was then used. AMPH and MPH could only partially prevent PQ-induced neurotoxicity. In the MTT reduction assay, both AMPH and MPH conferred partial protection against PQ 300µM toxicity, whereas only MPH conferred partial protection against PQ 500µM toxicity (**Figure 35**). This evidence was not demonstrated in the NR uptake assay, which might be due to differences in sensibility between NR and MTT assays, or different cellular targets. In this case, it appears that AMPH and MPH significantly protected against the mitochondrial dysfunction caused by PQ, but not from lysosomal integrity disruption evoked by PQ.

As stated, AMPH enters neurons via DAT competing with monoamines for transport into the cell and MPH is a DAT inhibitor. DAT inhibition by the drugs may decrease the entry of PQ into the cell, partially preventing/blocking its toxic effects. This is supported by the evidence that the blockage of DAT by MPH prevents the neurotoxic effect of known dopaminergic toxicants, like MPP⁺ (185), which however was not seen in this dissertation. Since PQ is structurally very similar to MPP⁺, it is expected that PQ entrance into the dopaminergic neuron might occur through the DAT (176). In accordance, Martins *et al.* showed that a specific DAT inhibitor, GBR 12909, conferred partial protection against PQ-

induced neurotoxicity in differentiated SH-SY5Y cells (70). In addition, another study using GBR12909 reported that it completely protected differentiated SH-SY5Y cells against the cytotoxicity of PQ (0.05 - 0.5mM) and partially attenuated the toxicity of 1mM concentration (176). It is important to mention that the question of PQ entrance by DAT is still controversial, as there are conflicting opinions such as those of Richardson and co-workers (186), who state that PQ-induced toxicity is independent of DAT expression or those of Ramachandiran and co-authors (187), who state that PQ does not require a functional DAT for dopaminergic toxicity. Moreover, if this partial protection would be caused by interference in the entrance, possibly both assays would be changed and not only MTT reduction assay, thus other mechanisms should be in play.

As mentioned earlier, PQ did not seem to promote ROS generation after 24-h exposure, which was probably due to lower time, concentration, or model cellular used. Also, controversially, in some time-points there was a significant production of ROS for PQ (300µM) pre-incubated with AMPH (1µM) or MPH (1µM) (**Figure 36**). This might have been due to the fact that there was a tendency for AMPH alone to elicit ROS production, and in some cases MPH as well. Perhaps an analysis of these results over longer exposure period would provide more information.

The cytotoxicity results were similar between cells exposed to 6-OHDA 125µM alone or pre-incubated with AMPH or MPH in both NR uptake and MTT reduction assays (**Figure 26 and 27**). AMPH and MPH did not provide any protection against 6-OHDA-induced neurotoxicity. This can be explained by the fact, that both 6-OHDA and AMPH or MPH alone promoted significant ROS generation after 24 h exposure. Consequently, cells exposed to 6-OHDA pre-incubated with AMPH, or MPH also showed a significant increase in ROS levels. It is hypothesised that AMPH and MPH could have aggravated ROS production in the presence of 6-OHDA 125µM (**Figure 29**). For the lower concentration of 6-OHDA 62.5µM, there was also a significant increase in ROS production, for both the 6-OHDA (62.5µM) alone and when pre-incubated with AMPH or MPH (**Figure 28**). In addition, since ROS production elicited by 6-OHDA can occur outside cells, the blocking of DAT by these protectors, might not avoid oxidative stress events evoked by this toxin. An *in vivo* by Fleming *et al.* demonstrated a neuroprotective effect of MPH against 6-OHDA. Rats receiving daily intrastriatal infusions of 6-OHDA developed deficits in the forelimb use and revealed a partial loss of dopaminergic cells in the substantia nigra. Co-administration of MPH led to a complete rescue from the behavioural and neurochemical effects of 6-OHDA, such as sparing of DA neurons (188), but that protective aspects were not found herein.

Regarding MPP⁺, the cytotoxicity results were similar between cells exposed to MPP⁺ (250 and 1500 μ M) alone or pre-incubated with AMPH or MPH in both the NR uptake and MTT reduction assays (**Figure 30** and **31**). Thus, AMPH and MPH did not provide protection against MPP⁺-induced neurotoxicity. As previously demonstrated, MPP⁺ does not appear to induce severe mitochondrial dysfunction nor extensive damage to lysosomes, which may have conditioned the neuroprotection evaluation conferred by the drugs in test. Furthermore, as previously mentioned, MPP⁺ did not promote significant ROS generation after a 24-h exposure, therefore it is not surprisingly that when incubated with AMPH or MPH there was also no significant change in ROS levels compared to control (**Figure 32** and **33**).

In the literature there is a study reporting MPH neuroprotective effects against MPP⁺ neurotoxicity. Ludolph *et al.* reported that treatment with MPH (1 μ M to 1mM) had no toxic effects in human embryonic kidney cells (HEK-293) stably expressing the human DAT (HEK-hDAT cells) or in dopaminergic neurons of rat embryonic (E14.5) mesencephalic primary cultures, after a 72-h treatment and when it was evaluated by the MTT assay. MPP⁺ treatment induced a toxic effect to HEK-hDAT cells and dopaminergic primary neurons. To show the functionality of the DAT in HEK-hDAT cells, they were exposed to the neurotoxin MPP⁺, which decreased dramatically the viability below 25%, in the concentrations of 0.1 and 10 μ M, after 72 h. Co-administration of MPH with 3.3 μ M MPP⁺ prevented the cytotoxicity in a dose-dependent manner from MPH (1 to 1000 μ M), at 72-h. In mesencephalic cultures, the treatment with 3.3 μ M MPP⁺ for 72 h resulted in shortened neurites; but when those cultures were treated with 3.3mM MPP⁺ together with 10 μ M MPH for 72 h, this neurotoxic effect was abolished. The neurotoxic effects of MPP⁺ could be significantly reduced by co-administration of MPH, an effect that is most likely explained by MPH blocking effect on DAT (185).

Overall, there was a tendency for AMPH to produce ROS. Perhaps this can be explained by the fact that AMPH within cells promotes VMAT-2 inhibition, which leads to inhibition of vesicular DA uptake and MAO inhibition, and this leads to an increase in cytoplasmic DA. It is known that auto-oxidation of DA can lead to ROS formation, so this increase in cytoplasmic DA may be harmful for the cell (108). In the case of MPH, ROS production was not so marked and, in some time-points did not even occur. As MPH is a DAT inhibitor, there is an inhibition of DA reuptake leading to a decrease in DA levels within the cell (121), the drug appears to affect VMAT-2, which may promote DA sequestration within synaptic vesicles (122). In other words, MPH, by decreasing cytoplasmic DA levels, decreases the amount of free DA avoiding in this way processes, such as its auto-oxidation or degradation by MAO that generate ROS.

6 Conclusions

In order to study PD *in vitro*, differentiated dopaminergic SH-SY5Y cells were exposed to dopaminergic neurotoxins 6-OHDA, MPP⁺ and PQ to assess their toxicological profile. Moreover, it was aimed to avoid their toxicity with the putative neuroprotectors AMPH or MPH in a short-term paradigm. First, the mechanisms of the toxins are different, while PQ and 6-OHDA have a concentration dependent toxicity, MPP⁺ although used in higher concentrations, did not show that pattern. Nevertheless, all three toxins exhibited some features of cell death by apoptosis. Overall, the three toxins showed neurotoxicity to differentiated SH-SY5Y cells after a 24 h of exposure. Moreover, although oxidative stress is a hallmark of PD, only 6-OHDA promoted it in these experimental conditions. However, there is no indication that both PQ or MPP⁺ promoted oxidative stress, but perhaps higher concentrations or longer exposure times would be required for this to be observed. Regarding $\Delta\Psi_m$, the neurotoxins were dissimilar in the effect provoked. Cells exposed to 6-OHDA and PQ revealed a decrease in $\Delta\Psi_m$ while cells exposed to MPP⁺ revealed an increase, but all seem to affect the $\Delta\Psi_m$. These toxins showed significant toxicity effects on cells, so they were used in subsequent studies.

AMPH and MPH at therapeutic concentrations did not show significant cell cytotoxicity, also the selected 1 μ M for neuroprotection studies also did not evoke any changes in the $\Delta\Psi_m$. In the neuroprotection assays, neither AMPH nor MPH prevented the toxicity induced by 6-OHDA or MPP⁺. AMPH and MPH only partially prevented the PQ-induced toxicity, but that needs to be further researched. Assessment of neurite formation, as well as their length and total cell number, or analyse if whether AMPH and MPH treatment in cells induce synaptic remodelling by identification of proteins such as synaptophysin and postsynaptic density 95 are required for a better disclosure of mechanisms. Thus, the different mechanisms of the toxins may have blurred the present results, but longer periods for some determinations, or using higher concentrations could reveal different results. In addition, as all the tested neurotoxins affect the $\Delta\Psi_m$, it would be interesting to assess the results of a pre-exposure of AMPH or MPH to the neurotoxins.

7 References

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