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Targeting the glycation defenses as a protective strategy for Parkinson's disease

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Mestrado de Biologia Molecular e Genética

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2017

"The plan is to fan this spark into a flame."

Lin-Manuel Miranda

Acknowledgements

I would first like to thank Professor Tiago Outeiro for giving me the opportunity to work in his lab and to provide me the necessary conditions for the elaboration of this dissertation. A big acknowledgment to our group, and those who have come and gone, for welcoming me and helping me in whatever I needed. Thank you to CEDOC for welcoming me and to provide an excellent environment to perform my thesis. I would also like to thank my co-advisor, Filomena Caeiro, for her availability and kind advice.

To Dr. Hugo Vicente Miranda, my thesis advisor, for all the guidance and for making me feel at ease working in the lab, and most of all, for always having an open door when I needed. Your support and criticism have been imperative for the development of this thesis. You have been much more than an advisor to me. Above all, you have become my friend and have inspired me beyond words. Thank you for always having a smile on your face and a joke up your sleeve, but mostly, for tormenting Ana with me. Thank you for making me a better scientist, and most of all, for believing in me. It has been a hard day's night, but I got by with a little help from my friend.

To Ana Chegão aka Avó Anita, there are no words to express how much you meant for my personal and professional growth. Your support has been unwavering right from the start, and I couldn't have done this without you. Your amazing energy has been vital for driving the well-oiled machine that is the "Morsas lab". From wooden spoons, to hospital rooms, from answering stupid questions to making sure there is never an empty food locker, you have always been there for me, and I will never forget it. I couldn't have asked for a better partner in crime! PS: You still smell bad. Gabs and Jojo, you have helped to make this experience much more than an academic degree. It is impossible to describe what you two girls mean to me. Jojo, the drooling queen, young and sweet, only seventeen, from Carnaval to Guns and Ships, from roadtrips to dramatic singing, you always make sure there is not a single tear left to cry from laughing so hard. Your contagious laugh is something that will stick with me for the rest of my days, and I will make sure to tell you every time I see a dead bird. Hoping we eventually become ghost friends and haunt Ana together. Thanks for making my trips to ChemiDoc easier. Gabigol, I didn't know that chocolate breaks could be so good until I met you. Thanks for making sure we get fat enough so we can't leave your side (we probably don't intend to), you won't ever be all by yourself again. Home is literally where you guys are (and where MCA is playing), Hakuna Labtata will live on. Morso, although you did not stay for too long, you made a big impact. I hope our Spice Morsas alter egos have big careers by now.

Rosa and Sara, you have made my life infinite times better. Rosa, thanks for upping my pun game, life is a million times easier when you have a telepathic kidney. Sara, thanks for all the advice and telling me that sometimes 'you just gotta punch science in the face', I will never forget it, thanks for being my mother away from home.

Marisa, Miguel, Carina, Ed, our dysfunctional little family is one I will always treasure, our dinners are the mother of all dinners, thanks for making sure I never look at a chicken the same way again.

Bea, my twin and evil princess, thanks for always being there for me, I hope your criminal ways don't come back to haunt you, because I'm hoping not having to visit you in jail for the rest of my life.

To my family, thank you for dealing with me for the past 23 years, it can't have been easy. Thank you for the support you have always given me in all my endeavours. Pedro, you are my MVP, I would be nothing without these last crazy 19 years. A special thank you to my mom, who always made sure I had everything I could wish for and more. I know it hasn't been easy being supermom, but you do it so well,

and I'm so thankful for it, you have been amazing every step of the way. You made me what I am today and I couldn't have asked for a better role model.

Resumo

O aumento da esperança média de vida tem-se traduzido no envelhecimento progressivo da população mundial e consequente aumento da incidência de doenças associadas ao envelhecimento, tais como as doenças neurodegenerativas de Alzheimer ou de Parkinson. A doença de Parkinson é a segunda doença neurodegenerativa mais comum, a seguir à doença de Alzheimer, e é caracterizada pela degeneração progressiva de neurónios dopaminérgicos na *substantia nigra pars compacta*. Uma das características mais comuns desta doença é a formação de corpos de Lewy, estruturas maioritariamente compostas por α-sinucleína agregada. A perda dos neurónios dopaminérgicos conduz à redução da produção de dopamina, um neurotransmissor essencial à sinalização do movimento voluntário. Tal facto é responsável por várias manifestações motoras apresentadas pelos doentes. Estas incluem tremores, bradicinesia e rigidez muscular. Os doentes de Parkinson também podem manifestar sintomas não motores como a psicose, depressão, hiposmia e cognição comprometida. A maioria dos casos de Parkinson tem origem desconhecida, sendo o envelhecimento o seu maior fator de risco. A diabetes tem vindo a ser sugerida como um fator de risco importante para o aparecimento/desenvolvimento desta doença. Nomeadamente, cerca de 80% dos doentes de Parkinson apresentam intolerância à glucose; proteínas modificadas por açúcares nos seus cérebros; e falhas nas defesas contra os açucares.

A hiperglicemia, consequência da diabetes, promove o aumento dos níveis de açúcares redutores no cérebro, tais como o metilglioxal. Este composto é um metabolito secundário, proveniente de diferentes vias metabólicas e, como outros açúcares redutores, pode reagir com proteínas numa reação denominada de glicação. A glicação é um processo que compreende uma série de reações entre açúcares redutores e grupos amina de proteínas, lípidos ou nucleótidos. Quando em reação com proteínas, formam como produto final os denominados produtos avançados de glicação (do inglês *advanced glycation end-products* – AGEs). A glicação pode promover diversas alterações a diferentes níveis celulares, tais como disfunção mitocondrial, alteração de degradação proteica, ou até promoção de processos de inflamação. No contexto de modelos de Parkinson, verificou-se que a glicação induz o aumento da agregação e da toxicidade da α-sinucleína, provocando efeitos negativos em diversos processos celulares. Em particular, a glicação da α-sinucleína induzida pelo metilglioxal, altera o seu *clearance*, e afeta o proteossoma, macro-autofagia e também a secreção, levando à sua acumulação. A glicação pelo metilglioxal conduz também à perda de neurónios dopaminérgicos na *substantia nigra* e no estriado em ratinhos, modelos da doença de Parkinson.

Enquanto que a formação do metilglioxal é inevitável, este pode ser catabolizado por diferentes vias, sendo o sistema dos glioxalases um dos mais eficientes. Para além de processos enzimáticos, existem também compostos capazes de sequestrar metilglioxal e impedir que este reaja com as proteínas. Existem também compostos que inibem o processo de glicação, ou que têm capacidade de remover os AGEs das proteínas. Assim, coloca-se por hipótese que os compostos que previnam a glicação possam ser benéficos na prevenção ou tratamento da agregação e citotoxicidade da α-sinucleína.

Tendo em conta que o metilglioxal assume um papel importante na doença de Parkinson, neste estudo foi avaliado o potencial terapêutico de diversos compostos supressores de glicação tais como a metformina, pioglitazona, sulforafane, resveratrol, carnosina e aminoguanidina. A atividade destes compostos foi avaliada pela sua capacidade em proteger dos efeitos deletérios induzidos pela glicação. Nomeadamente, em reduzir a citotoxicidade da α-sinucleína/metilglioxal; os níveis de α-sinucleína; a agregação de α-sinucleína; os níveis gerais de AGEs; bem como de potenciar o *clearance* de α-sinucleína. O potencial de proteção foi avaliado de forma preventiva (antes do insulto) ou restaurativa (após insulto com metilglioxal). Para este efeito, foi utilizado como modelo de estudo células H4

(neuroglioma) a sobre-expressar α-sinucleína. Os compostos a avaliar foram incubados na ausência e/ou presença metilglioxal. A citotoxicidade foi avaliada pela perda de integridade da membrana citoplasmática, medida pela actividade do enzima lactato desidrogenase libertado. Os níveis de α-sinucleína foram avaliados por Western blot. A solubilidade de proteínas em Triton X-100 permitiu estudar a solubilidade da α-sinucleína proveniente de um extrato nativo de células que expressam SynT, uma variante da α-sinucleína com maior tendência para agregar. Na presença deste detergente, as proteínas mais solúveis, após uma centrifugação a alta velocidade por um período longo de tempo, permanecem na fração solúvel, enquanto que as insolúveis ficam precipitadas na base do tubo de centrifugação (fração insolúvel). À fração insolúvel correspondem proteínas agregadas. A agregação da α-sinucleína foi também avaliada por imunocitoquímica. Ao hibridar a α-sinucleína com um anticorpo secundário fluorescente, é possível, recorrendo a um microscópio de fluorescência, visualizar a presença de agregados intracelulares. O "*clearance"* da α-sinucleína foi estudado pela monitorização dos níveis desta proteína por western blot, de extratos proteicos de células cuja síntese proteica *de novo* foi inibida (pelo uso de cicloheximida) em diferentes momentos no decurso do tempo.

Os resultados obtidos neste estudo revelaram que a metformina, a carnosina e a aminoguanidina, nas concentrações testadas, não apresentam citotoxicidade num contexto basal. O mesmo se verifica quando as células expressam a α-sinucleína e/ou quando a glicação é induzida pelo metilglioxal. A metformina, a carnosina e a aminoguanidina apresentaram capacidade de reduzir a citotoxicidade induzida pelo metilglioxal em células que sobre-expressam a α-sinucleína. Tal verificou-se num contexto preventivo bem como restaurativo. A metformina e a carnosina são igualmente capazes de prevenir o aumento dos níveis gerais de AGEs e de α-sinucleína/SynT, induzido pelo metilglioxal. No entanto, o mesmo não foi observado num contexto restaurativo, onde apenas a carnosina reduziu os níveis de AGEs em células que expressam a variante SynT, e por isso num contexto em que a agregação da α-sinucleína está potenciada. No que diz respeito ao perfil de solubilidade da α-sinucleína, a metformina solubilizou a proteína num contexto preventivo, o que sugere que tenha capacidade de prevenir a agregação da SynT. Por outro lado, a carnosina não parece modular a solubilidade da SynT. Num contexto restaurativo, nenhum dos compostos testados mostrou potencial para alterar a solubilidade da α-sinucleína. A agregação desta proteína foi também avaliada por imunocitoquímica. Uma vez que os compostos não apresentaram potencial restaurativo, a sua eficácia foi apenas avaliada num contexto preventivo. Os resultados preliminares revelam que tanto o tratamento com a metformina como com a carnosina resultam numa diminuição do número de células com agregados. Estes resultados estão em concordância com os observados nos testes de solubilidade. No que diz respeito ao *clearance* da α-sinucleína, os resultados preliminares sugerem que a metformina, não só previne contra os efeitos do metilglioxal (que impede o *clearance* da α-sinucleína) como potencia o *clearance* da α-sinucleína.

Em suma, os resultados obtidos neste estudo sugerem que a metformina e a carnosina apresentam elevado potencial de proteção contra a citotoxicidade da α-sinucleína (30% e 20%, respetivamente), principalmente num contexto de glicação. A sua eficácia é maior num contexto preventivo, em modelos que apresentam maior agregação da proteína, bem como em condições de glicação induzida pelo metilglioxal. Em contexto preventivo, tanto a metformina como a carnosina diminuem os níveis gerais de glicação, o que está em concordância com o facto destes compostos serem sequestradores de metilglioxal. A carnosina também apresentou capacidade de reduzir os níveis de α-sinucleína ou SynT em células glicadas com metilglioxal. Ainda num contexto preventivo, tanto a metformina como a carnosina preveniram a agregação da SynT. A metformina apresenta ainda potencial para aumentar o *clearance* da α-sinucleína. É importante referir que no que diz respeito aos estudos de agregação (imunocitoquímica), bem como os de *clearance* da α-sinucleína, os resultados apresentados nesta tese

são preliminares, e carecem por isso de confirmação com experiências adicionais. De futuro, pretende-se ainda testar o efeito da carnosina no *clearance* da α-sinucleína.

Com este estudo conclui-se que a metformina e a carnosina são compostos com elevado potencial de proteção/prevenção contra os efeitos citotóxicos da α-sinucleína, particularmente em condições de glicação induzida pelo metilglioxal. Sugere-se, por isso, que estes compostos são potenciais fármacos para o tratamento da doença de Parkinson.

Palavras-chave: doença de Parkinson; glicação; agregação proteica; terapêutica

Abstract

Parkinson's disease is the second most common neurodegenerative disorder, characterized by the loss of dopaminergic neurons in the *substantia nigra pars compacta*, leading to several features of the disease such as abnormal motor performance and, at later stages, cognitive impairment. The main hallmark of this disease is the presence of Lewy bodies, which are primarily composed of α-synuclein. Diabetes has been associated as a risk factor for the development of Parkinson's disease, and recently, glycation was suggested as the missing link between the two diseases. Reducing sugars, such as methylglyoxal, intervene in this reaction, leading to the formation of advance glycation end-products, which promote protein cross-linking. α-synuclein is an appealing target of glycation, since more than 10% of its residues are putative targets of this post-translational modification. Glycation is thought to play a key role in the aggregation of α-synuclein, as it promotes its aggregation and toxicity. As such, modulation of glycation defenses in Parkinson's disease can represent a new therapeutic approach to delay or prevent the disease progression.

This study aimed to evaluate the therapeutic potential of metformin, pioglitazone, sulforaphane, carnosine, and aminoguanidine, compounds already shown to protect against glycation toxic effects. To this purpose, the selected pharmacological compounds activity was tested, in a preventive or restorative paradigm, in cellular models of Parkinson's disease under methylglyoxal-induced glycation.

Regarding α-synuclein/methylglyoxal associated toxicity assessment, metformin and carnosine were the most active compounds. They were able to decrease toxicity both in a preventive and restorative manner. Moreover, these agents were also able to decrease the formation of advance glycation end-products in cells treated with methylglyoxal. Carnosine, in a preventive manner, decreased α-synuclein protein levels. On the other hand, metformin is able to maintain α-synuclein (SynT) solubility. Preliminary data suggests that metformin not only decreases the number of cells with α-synuclein aggregates, but also promotes its clearance. In contrast, under a restorative paradigm, neither metformin nor carnosine modulate α-synuclein levels.

In summary, metformin and carnosine have a protective potential against methylglyoxal-induced α-synuclein glycation in a cellular model of Parkinson's disease, making these a novel potential therapeutic approach to Parkinson's disease.

Key-words: Parkinson's disease, glycation, protein aggregation, therapeutics

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List of acronyms and abbreviations

6-OHDA	6-hydroxydopamine
AGEs	Advanced glycation end-products
ALDH	Aldehyde dehydrogenase
AMPK	Adenosine monophosphate-activated protein kinase
AR	Aldose reductase
aSyn	α-synuclein
ATP	Adenosine triphosphate
DHAP	Dihydroxyacetone phosphate
GAP	Glyceraldehyde 3-phosphate
GCS	γ-glutamylcysteine synthetase
Glo1	Lactoylglutathione methylglyoxal lyase
Glo2	Hydroxyacylglutathione hydrolase
GS	Glutathione synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
LB	Lewy body
LDH	Lactate dehydrogenase
MGO	Methylglyoxal
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NK-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor erythroid-related factor 2
PBS	Phosphate buffer saline
PD	Parkinson's disease
RAGE	Receptor for AGEs
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Synph 1	Synphilin-1
SynT	Aggregation-prone variant of α-synuclein
UPS	Ubiquitin-proteasome system
WT	Wild-type

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1 Background

1.1 Parkinson's disease

The incidence of neurodegenerative diseases has become more pronounced in the last decade due to the sharp increase in life expectancy achieved by modern medicine. This population ageing has increased the incidence of progressive neurodegenerative diseases such as Alzheimer's, Parkinson's (PD), and Huntington's disease, since ageing is their main risk factor. These diseases share a common denominator: formation of protein aggregates. Huntington's disease is characterized by the aggregation of the protein huntingtin. On the other hand, in Alzheimer's disease, two major types of protein aggregates may be found: extracellular neuritic plaques, mainly composed by amyloid-β peptide; and intracellular neurofibrillary tangles, which consist of tangled microtubule-associated protein tau [1]. The hallmark of PD is the formation of Lewy bodies (LBs), mainly composed by aggregated α-synuclein (aSyn) [2]. Although different areas of the brain are affected in these diseases, several symptoms may overlap. The clinical manifestations experienced by these patients are associated with the progressive neuronal loss, that could be due to the deposition of the proteins associated with the diseases.

PD was first described by James Parkinson in "*An Essay on the Shaking Palsy",* published in 1817, where he describes its pathognomonic features, and distinguishes PD from other diseases with similar clinical manifestations. He describes a diseased state of the *medulla spinalis* as a proximate cause of the disease, that extends to the *medulla oblongata* as the disease progresses [3]. Although Parkinson did not correctly describe the cause of the disease, he described the tremors as the main feature of this disease. The term 'Parkinson's disease' was later coined by French neurologist Jean-Martin Charcot [4], who also had a great impact in the description of the disease.

PD is the second most common neurodegenerative disease, following Alzheimer's disease. It is estimated that around 2% of the population over 80 years old present with the disease [5]. Females present a milder phenotype, lower incidence [6], and a later onset of the disease [7]. In Portugal, it is estimated that 0,24% of the population over 50 years old has PD [8].

1.1.1 Pathophysiology and Clinical Features

PD is characterized by the progressive degeneration of nigrostriatal dopaminergic neurons in the *substantia nigra pars compacta*, in the brain basal ganglia [9]. However, several other brain regions are affected, such as the amygdala and the hypothalamus [2].

The hallmark of PD is the accumulation of LBs and Lewy neurites, primarily composed of aSyn [2,10]. These are also composed by more than 76 components [11], including other proteins such as aSyn-interacting proteins (synphilin-1 and 1A) [12], ubiquitinated proteins [13] including ubiquitinated-aSyn [14] and even some organelles, such as mitochondria. aSyn belongs to a family of misfolding proteins [15], which are prone to aggregate. This process leads to the formation of fibrils that can be found in the brain and in the peripheral nervous system [2]. LBs do not necessarily promote neuronal death, and are hypothesized to be formed as a protective response to further neuronal damage, harvesting misfolded and dysfunctional proteins or organelles [11,16,17]. Both LBs and Lewy neurites are present in other pathologies such as dementia with Lewy bodies [18].

The loss of dopaminergic neurons leads to a dopamine deficiency and, consequently, triggers the main motor features associated with PD. These include resting tremor, bradykinesia, muscular rigidity, postural instability, and gait impairment. Non-motor features are also present in the disease, such as hyposmia, cognitive impairment, constipation, psychosis, depression, sleep disorders, autonomic

dysfunction, pain, and fatigue [2,9,10]. The onset of non-motor symptoms frequently precedes motor symptoms and can be a useful diagnostic tool [2,9].

1.1.2 Etiology

1.1.2.1 Genetic risk factors

Most PD cases are of unknown origin, however there are genetic mutations and genetic risk factors that are associated with the disease. The first gene associated with PD was the *SNCA* gene [19], which encodes for aSyn [2,9]. Mutations in this gene, although rare, have been implicated in monogenic forms of the disease [2]. Duplications and triplications of the *SNCA* gene have been reported. Triplications have an earlier onset and faster and more aggressive disease progression than *SNCA* duplications [20,21]. These findings suggest that the severity of the disease is aSyn dose-dependent [22].

The most frequent genetic cause of PD is the mutation of the *LRRK2* gene (*PARK8*), which encodes for leucine-rich kinase 2, and can account for 4-5% of hereditary PD, and 1% of sporadic PD [23,24]. When mutated, this gene confers an autosomal dominant form of the disease [2,9]. Mutations in *LRRK2* have similar characteristics to classic forms of PD, including the presence of LBs and Lewy neurites [25,26], although some cases may not present LBs.

Autosomal recessive forms of PD are associated with early onset of the disease, and implicate *Parkin*, *PINK1*, and *DJ-1*. Of these genes, mutations in the *Parkin* gene are the most common, responsible for 50% of hereditary cases [27] and around 15% of all patients under 45 years old [27,28]. *Parkin* accounts for most monogenic forms of early onset PD [29].

Parkin is an E3 ubiquitin-protein ligase [30] and is therefore involved in the ubiquitin-proteasome system (UPS). Thus, mutations in this gene lead to an impairment of protein degradation, potentiating misfolded protein accumulation, such as aSyn. This cytosolic protein also mediates the process of mitochondria autophagy (mitophagy), and is recruited to the mitochondria by PINK1, which is also associated with familial forms of PD [31]. PINK1 is a PTEN-induced putative kinase 1 and is localized in the mitochondria [32]. This protein is activated by alterations in the mitochondria membrane potential, and in turn recruits Parkin to regulate mitophagy [33].

Both *Parkin* and *PINK1* mutations lead to an early onset of PD, although the disease is of slower progression [27,28,34]. Patients with mutations in *Parkin* are also less likely to develop dementia [27]. The most notable difference between patients harboring the *Parkin* mutation and idiopathic PD is the absence of LBs and the restriction of neuronal loss to the *substantia nigra* and the *locus caeruleus* [27,28]. However, there is a very small percentage of cases that present LBs [35,36].

PD-associated *DJ-1* mutations are rare [37] and have similar phenotypes as the *Parkin* and *PINK1* mutations [38]. DJ-1 is a ubiquitous-expressed [39] multifunctional protein [38] with redox activity, that was shown to be involved in PD pathology. This protein, mainly present in the cytosol, is able to translocate to the mitochondria in an oxidation-dependent manner [40] to protect against oxidative stress. In vivo studies suggested that under basal conditions, *DJ-1* knockout has little to no impact on neurodegeneration [41–43]. However, once exposed to the PD toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), DJ-1-/- mice become susceptible to striatal denervation [44,45]. Studies involving other animal models such as zebrafish and *Drosophila* also report an increase in oxidative stress vulnerability in knockdown and knockout *DJ-1*, respectively [38].

Table.1.1 Most studied Monogenic forms of PD.
Gene nomenclature, protein and type of parkinsonism are presented.

Gene	Protein	Inheritance	Type of parkinsonism	Reference
SNCA	aSyn	Autosomal Dominant	Late onset/early onset PD	19
LRRK2	Leucine-rich repeat kinase 2	Autosomal Dominant	Late onset PD	9,46
Parkin	Parkin	Autosomal Recessive	Early onset PD	27,28,34
PINK1	PTEN-induced putative kinase 1	Autosomal Recessive	Early onset PD	27
DJ-1	DJ-1	Autosomal Recessive	Early onset PD	37,38

1.1.2.2 Non-genetic risk factors

Several non-genetic risk factors can account for the sporadic cases of PD, being ageing the major factor [2]. Upon ageing, there is a decrease in the efficiency of the UPS [47–50] and autophagy lysosome pathway [51,52], which are invaluable mechanisms through which proteins and organelles are degraded. The dysfunction of either of these pathways leads to an imbalance in protein degradation, an increase in oxidative stress due to mitophagy impairment, and, among other consequences, accumulation of misfolded proteins. Pathways and mechanisms that are altered upon ageing may increase the vulnerability of dopaminergic neurons, leading to their dysfunction, and eventual degeneration.

As previously mentioned, gender is also a risk factor for the disease, with the male-to-female ratio being 3:2 [2,6]. The prevalence of PD also seems to be affected by geography, as there is a higher prevalence in Europe, North America and South America, compared with African, Asia, and Arabic countries [2,5]. Patients with prior head injury are also at an increased risk to develop PD, while a decreased risk has been associated with tobacco smoking, coffee drinking, and alcohol consumption [2].

Diabetes has also been linked to PD as an increased diabetes prevalence was observed in PD patients [53–56], and has therefore been described as an important risk factor for the disease [55,56]. Both diseases share common dysfunctional pathways such as mitochondrial pathway, autophagy, and the UPS [57]. It is therefore understandable that there might be an association between the two diseases. Accordingly, studies have described that glucose tolerance was impaired in up to 80% of PD patients, and that blood sugar levels were higher than expected [58,59]. The increase of glucose levels associated with diabetes can also be correlated with an increase in methylglyoxal (MGO) [60], a potent reducing sugar capable of inducing aSyn aggregation [61,62]. A study conducted in endothelial cells and mice also shows that chronic hyperglycemia decreases proteasomal activity [63]. The impairment of the proteasome system is a well described characteristic of PD, and is thought to play a role in aSyn accumulation, thus helping to explain the association between the two diseases. Previous work has also demonstrated that a high-fat diet, that induces hyperglycemia, leads to an increase in dopamine depletion in the *substantia nigra* in 6-hydroxydopamine (6-OHDA) rat model of PD [64].

An increased risk for PD can also be attributed to toxins exposure. MPTP is a toxin frequently used to reproduce the pathological changes associated with PD in models of the disease, more specifically, it mimics the selective degeneration of dopaminergic neurons [65]. Rotenone is another compound used to mimic PD, as it selectively promotes de degeneration of nigrostriatal dopaminergic neurons in vivo, in addition to forming LB-like cytoplasmic inclusions containing ubiquitin and aSyn [66].

1.1.3 Treatment

Although PD is currently incurable, symptomatic treatments are available. Motor features are mostly treated by stimulating dopamine receptors, either through increasing dopamine levels with precursor drugs such as levodopa (L-DOPA), or with dopamine agonists [2,67]. However, several studies indicate that long-term levodopa treatment may increase oxidative stress in the cell, as an increase in free dopamine will lead to an increase in its degradation by monoamine oxidase and, consequently, an increase in hydrogen peroxide, a reactive oxygen species (ROS).

Deep brain stimulation is an established PD treatment also used to control the motor manifestations. This procedure consists in the electrical stimulation of areas of the brain associated with the tremors, similar to the activity of a pacemaker [68]. However, consistent stimulation may interfere with normal motor function [69], leading researchers to develop an intermittent more adaptive deep brain stimulation that outperforms standard continuous deep brain stimulation [70].

1.1.4 α-Synuclein

One of the key players in PD is aSyn. This soluble and heat-resistant [71] presynaptic protein, which aggregates in PD, is part of the synuclein family, which comprises α-, β-, and γ-synuclein. aSyn is the most common synuclein implicated in neurodegeneration [72], although β-synuclein has been shown to be neurotoxic for nigral dopaminergic neurons [73], and γ-synuclein led to motor neurons loss in the spinal cord of transgenic mice [74].

aSyn is a natively unfolded small protein of 140 aminoacids [75] that has three main regions: the N-terminal, the hydrophobic central region (NAC domain), and the C-terminal. Synucleins have a conserved N-terminal sequence, containing KTKEGV repeats that, in the case of aSyn, are proposed to be required for conformational change to α-helix after interacting with lipids and membranes [71]. This is speculated to promote aSyn folding and fibrillization [72]. This domain is suggested to work in synergy with the C-terminal to confer this protein a chaperon-like activity [76]. On the other hand, aSyn NAC domain has 12 aminoacids that differ from the other members of the synuclein family. This central hydrophobic region of the protein allows for a change of conformation to β-sheet structure, promoting the formation of oligomeric species [71,77]. Therefore, these aminoacids are essential for the protein's oligomerization and fibrillization [71,72,75,77]. Finally, the C-terminal region is negatively charged and has a disordered structure. It is hypothesized to contribute to the thermostability [72,76,78] and chaperon-like activity [76,79] of aSyn, acting as a solubilizing domain [79,80]. Intramolecular interactions between different regions of aSyn have been described and are proposed to stabilize the protein, inhibiting its oligomerization [71].

aSyn does not have a defined secondary level of structure [71,72,77]. Upon misfolding, this protein aggregates in a nucleation-dependent manner, generating several intermediate species [71]. *SNCA* mutations and post-translational modifications such as phosphorylation, ubiquitination, acetylation, glycation, among others, are known to modulate aSyn aggregation [71]. Oligomerized aSyn is accepted to be more toxic than aSyn aggregates [72,81–83]. Its accumulation is thought to promote the loss of dopaminergic neurons [72].

The function of aSyn is still unclear, however several putative roles have been identified. Studies showed that aSyn is required for vesicle trafficking, and regulation of synaptic function, neurotransmitter release, and modulation of proteasomal activity [72,75]. Overexpression of aSyn has been implicated in endoplasmic reticulum impairment, calcium homeostasis dysregulation, and abnormal mitochondria function [72,77,84,85].

1.1.4.1 Clearance of α-synuclein

In physiological conditions, proteins and organelles are degraded via the UPS or through the autophagy pathway, which involves the lysosome [86]. The UPS consists in the poly-ubiquitination of proteins which targets them for degradation by the 26S proteasome [87] in an ATP dependent manner [86,87], and is therefore affected by mitochondria related stress. Furthermore, this process may involve Parkin, a protein whose mutations are linked with the familial cases of PD [87]. Autophagy, on the other hand, can be split into three categories, depending on the cargo: macroautophagy, microautophagy, and chaperone-mediated autophagy [86]. While macroautophagy involves the engulfment of the target protein by the autophagosome, which fuses with the lysosome at later stages [88], microautophagy consists in the direct uptake of the target protein by the lysosome [89]. Chaperone-mediated autophagy degrades proteins containing a specific motif, and involves interaction with heat shock proteins and with the receptor LAMP2A, which internalizes the protein into the lysosome [88]. Finally, mitophagy is a specific type of autophagy intended for damaged mitochondria [86]. Its degradation generally involves the recruitment of cytosolic Parkin to the mitochondria and subsequent phosphorylation by PINK1 [90]. However, it was shown that clearance of damaged mitochondria may also be independent of Parkin recruitment [91]. The damaged organelle is then engulfed by the autophagosome and delivered to the lysosome for degradation [92].

aSyn is known to be degraded via proteasome, macroautophagy, or chaperone-mediated autophagy. However, the major degradation pathway is still not known, and it seems to depend on aSyn oligomerization status. [93,94]. In pathological conditions, such as PD, these systems may be impaired, leading to the accumulation of aSyn. Notably, proteasome function is impaired in sporadic PD patients [95,96]. Moreover, mutant aSyn showed a slower degradation rate compared to wild-type aSyn [97], and cause a direct inhibitory effect [94], as well as a downregulation of subunits of the proteasome [98,99]. Furthermore, mutant aSyn can also impair autophagy by interacting with chaperone-mediated autophagy receptor LAMP2A, inhibiting not only its degradation but also the degradation of other proteins [100].

1.2 Glycation

Diabetes is recently suggested as an important risk factor for PD. Studies have shown that some metabolic byproducts of glycolysis, known as glycation agents, potentiate aSyn aggregation and cytotoxicity [10,61,101]. These glycation agents participate in a process known as glycation. Glycation corresponds to the non-enzymatic reaction between the carbonyl group of a reducing sugar, like glucose, and the amine group of proteins, lipids, or nucleic acids, leading to the formation of a Schiff's base [72,101] (Figure 1.1). This base will then suffer Amadori rearrangements, and, through several reactions, will eventually lead to the formation of irreversible advanced glycation end-products (AGEs) [10,72,102] (Figure 1.1). Although glucose is a glycation agent and can directly modify proteins, it is one of the least reactive agents [10,102]. MGO is a byproduct of different metabolic pathways, mainly formed via the glucose metabolism, and is 20,000 times more reactive than glucose [10,102]. MGO is mainly formed through the non-enzymatic degradation of the phosphate group of the glycolytic triose phosphates: glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), and reacts with arginine, cysteine, and lysine residues of proteins [10,72,102]. This compound may also arise from the catabolism of threonine and acetone, or through lipid peroxidation [102] (Figure 1.3).

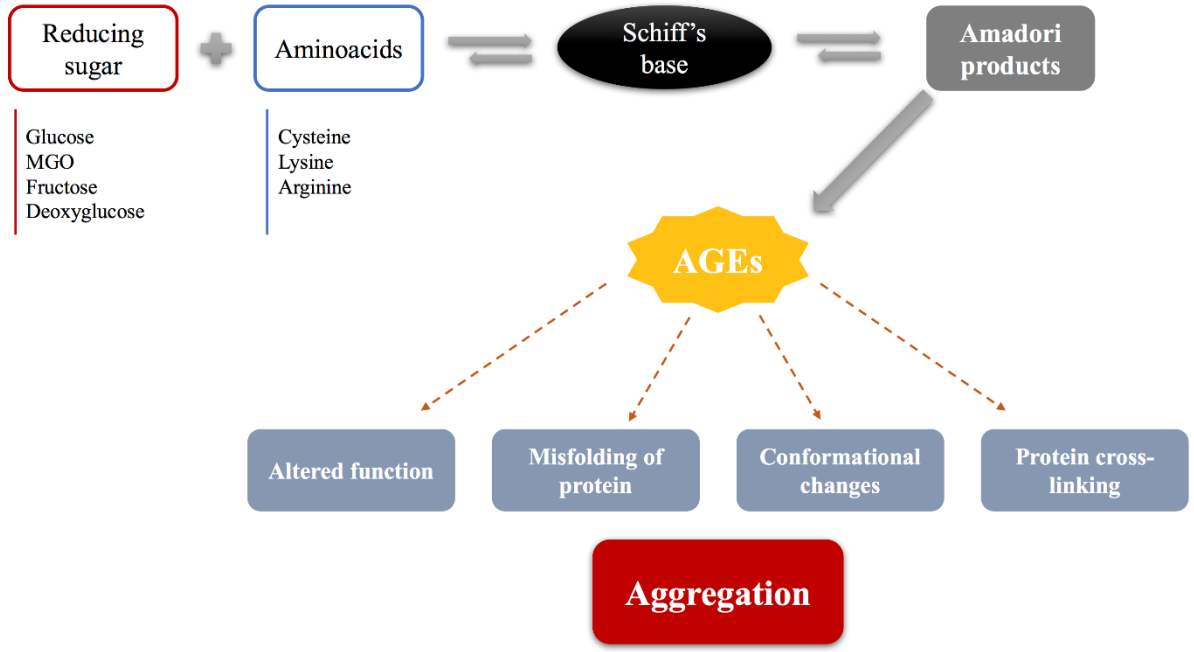

***Figure 1.1 Schematic representation of the* glycation reaction.**
Reducing sugars react with the side-chain amino-groups of aminoacids (lysine, arginine and cysteine) to form a Schiff's base, which then suffers Amadori rearrangements, generating advanced glycation end-products (AGEs). AGEs may alter protein structure and function ultimately contributing to the aggregation of proteins.

This process has severe consequences for the cell by altering cellular proteins function and structure, and dysregulating natural cell processes [102]. Furthermore, glycation can also occur in lipids, forming the advanced lipoxidation end products which can, for example, lead to the impairment of LDL clearance [103].

AGEs can bind to several receptors. The most well characterized is the receptor for AGE (RAGE), a multi-ligand receptor [104–106]. RAGE is a cell surface receptor that is involved in different responses such as inflammation, as it is part of the immunoglobulin superfamily [104,107]. RAGE is expressed on microglia, endothelial cells, and smooth muscle of the vasculature [105,106,108], among others, and has been reported to be increased in neurodegenerative diseases, such as dementia with Lewy Bodies, Alzheimer's disease, and in cases with early stages of parkinsonism [72,109–111]. The interaction between RAGE and AGEs leads to a cascade of responses that activates transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [105,112], which promotes inflammation (Figure 1.2). In turn, NF-kB further expresses RAGE[113], creating a vicious cycle [101] that contributes to sustained inflammation, eventually leading to cell death and neurodegeneration [72]. Furthermore, RAGE was also described to induce ROS formation [114,115].

1.2.1 Glycation in PD

Although glycation is mostly studied in a context of diabetes, it is important to recognize that it may also have a great impact in neurodegeneration. Studies have reported the occurrence of glycation in the brains of PD patients [61,111,116], as well as the presence of aSyn in Lewy bodies [78].

aSyn is a lysine rich protein [72,101], which makes it highly vulnerable to glycation. Glycation can affect different aspects of the protein, ranging from its cytotoxicity to its ability to aggregate. Several studies have reported glycation's effects on aSyn such as the formation of cross-links between proteins, and increase in its ability to aggregate [117] (Figure 1.2). Moreover, Lee et. al (2009) demonstrated that with an increasing concentration of reducing sugars such as MGO, aSyn oligomerization increased

concomitantly [62]. This phenomenon has also been confirmed by similar works [83,118]. Recently, Vicente Miranda and colleagues demonstrated that MGO induces aSyn aggregation and toxicity in yeast, mammalian cells and animal models of PD [61].

Uchiki and colleagues (2012) showed that the presence of MGO reduces intracellular protein degradation and that glycated proteins are less susceptible to degradation. Additionally, the authors showed that glycated ubiquitin decreases degradation, suggesting that proteins ubiquinated with glycated ubiquitin are less degraded than proteins bound to wild-type ubiquitin [119]. Moreover, since ubiquitin binds to the lysine residues of proteins to be targeted for degradation [120], and glycation also occurs in lysine residues, if aSyn is glycated, the lysine residues will not be available for ubiquitination, interfering with the protein's degradation. Recently, it was shown that glycated aSyn is less ubiquitinated, leading to the impairment of the proteasome and autophagy pathways [61](Figure 1.2). This ultimately leads to a deficient degradation of aSyn, resulting in its intracellular accumulation [61]. These authors also show that MGO-induced glycation of aSyn drives the loss of dopaminergic neurons in the *substantia nigra* and striatum of a mouse model of PD [61]. The effect of glycated aSyn was also investigated in a *Drosophila* model of PD which showed an impairment of its motor performance as well as a decrease in adult-stage survival [61].

Thus, glycated aSyn has a great impact not only on its ability to aggregate and its consequent cytotoxicity, but also in its clearance pathways (Figure 1.2), culminating in an aggravated PD phenotype. The resulting accumulation of this protein could account for the neuronal death observed in PD patient's brains.

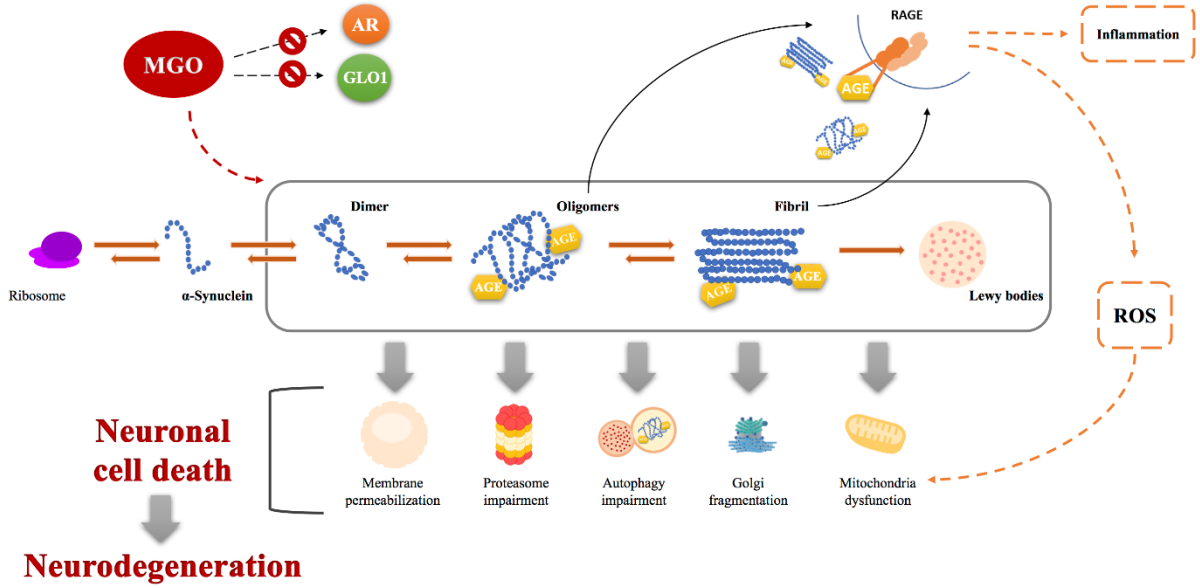

Figure 1.2 Schematic of glycation effects in PD.
MGO modifies aSyn causing its oligomerization and the generation of AGEs. Protein structural alterations have an impact on the function of several organelles, activation of inflammation pathways, and ROS generation. The resulting effects inevitably lead to neurodegeneration. Abbreviations: MGO: methylglyoxal, AR: aldose reductase, GLO1: glyoxalase-1, AGE: advanced glycation end-product, RAGE: receptor for AGE, ROS: reactive oxygen species.

1.2.2 Glycation defenses

MGO can be catabolized through different pathways, such as the glyoxalase system, aldehyde dehydrogenase, and the aldose reductase [10,102] (Figure 1.3). The glyoxalase system is the most efficient pathway of MGO degradation, and it is active in the cytoplasm of all mammalian cells [102]. This system comprises two important enzymes, lactoylglutathione methylglyoxal lyase (Glo1), and

hydroxyacylglutathione hydrolase (Glo2), in addition to reduced glutathione (GSH) as a co-factor [102] (Figure 1.3). Glo1 catalyzes the reaction between GSH and MGO forming *S*-D-lactoylglutathione, while Glo2, converts *S*-D-lactoylglutathione in D-lactate, restoring GSH [10,102](Figure 1.3).

GSH is one of the main protective agents against ROS, converting H_2O_2 to H_2O and generating oxidized glutathione (GSSG) via GSH peroxidase [121]. However, GSH levels decline with age [122–124]. In agreement, studies performed in aged rat brain reported the decrease of GSH and increase of GSSG levels, leading to a decreased GSH/GSSG ratio [123]. Furthermore, it was shown that γ-glutamylcysteine synthetase (GCS) which, along with glutathione synthetase (GS), generates GSH, has a decreased activity in aged rat brain [123]. GSH is also decreased in the *substantia nigra* of PD patients [125,126], which is consistent with the fact that age is the greatest risk factor for the disease. The dysfunction of the GSH pathway with ageing increases the cell's vulnerability to ROS and, consequently, oxidative stress, which is elevated in PD patients. This decrease in GSH levels impairs the glyoxalase system, increasing the risk for the accumulation of MGO which thereby potentiates the loss of dopaminergic neurons.

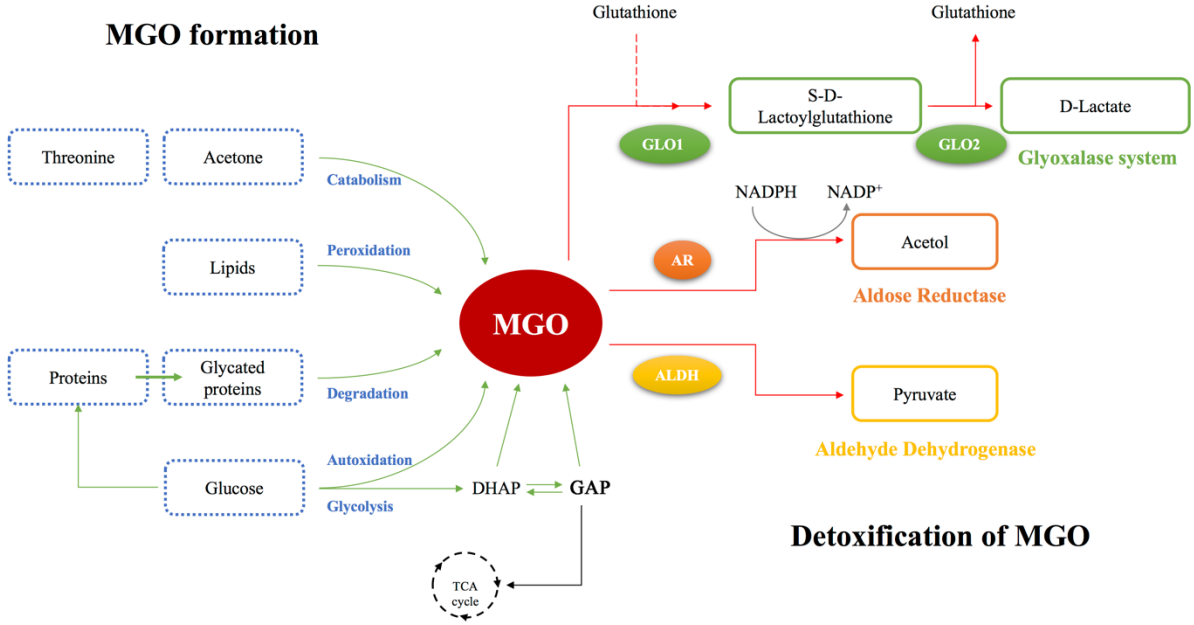

Figure 1.3 Schematic of methylglyoxal (MGO) metabolism.
Schematic representation of anabolic (green arrows) and catabolic (red arrows) pathways of MGO. Abbreviations: MGO: methylglyoxal, DHAP: dihydroxiacetone phosphate, GAP: glyceraldehyde 3-phosphate, TCA: tricarboxylic acid, GLO1: glyoxalase-1, GLO2: glyoxalase-2, AR: aldose reductase, ALDH: aldehyde dehydrogenase, NADPH: Reduced nicotinamide adenine dinucleotide phosphate, NADP+: Nicotinamide adenine dinucleotide phosphate.

Recently, DJ-1 was reported to have deglycase and methylglyoxalase activity, interacting with glycated proteins [127]. Richarme and colleagues suggest that, in contrast to the glyoxalase system, DJ-1 is able to degrade MGO in a GSH independent manner. Furthermore, it was also shown that DJ-1 can protect against the effects of glycated DNA[128].

The impairment of MGO catabolism brings severe consequences to the cell, as the accumulation of MGO leads to an increase in protein glycation and AGEs formation. The impairment of glycation defenses is especially deleterious in patients with diabetes, where the concomitant hyperglycemia leads to an increase of MGO levels [129–132].

1.2.3 Potential pharmacological compounds

Given that diabetes increases the risk for PD, it is reasonable that anti-diabetic compounds may have beneficial effects in PD. Potential pharmacological compounds such as MGO scavengers, AGEs

breakdown agents, or AGEs inhibitors [101] are likely to reduce glycation levels and, consequently, aSyn aggregation and toxicity [61]. MGO scavengers typically trap MGO molecules preventing its reaction with proteins, therefore suppressing glycation. On the other hand, AGEs breakdown agents have the ability to break-down protein cross-links promoted by AGEs, avoiding protein aggregation, whereas AGEs inhibitors halt glycation by interfering with the glycation process, reacting with its intermediates (Figure. 1.4).

Metformin, pioglitazone, sulforaphane, resveratrol, carnosine, aminoguanidine, tenilsetam, N-acetylcysteine, pyridoxamine, N-phenacylthiolozium bromide, and penicillamine, among several other compounds have already shown to inhibit or prevent glycation, therefore being promising therapeutic agents (Figure. 1.4).

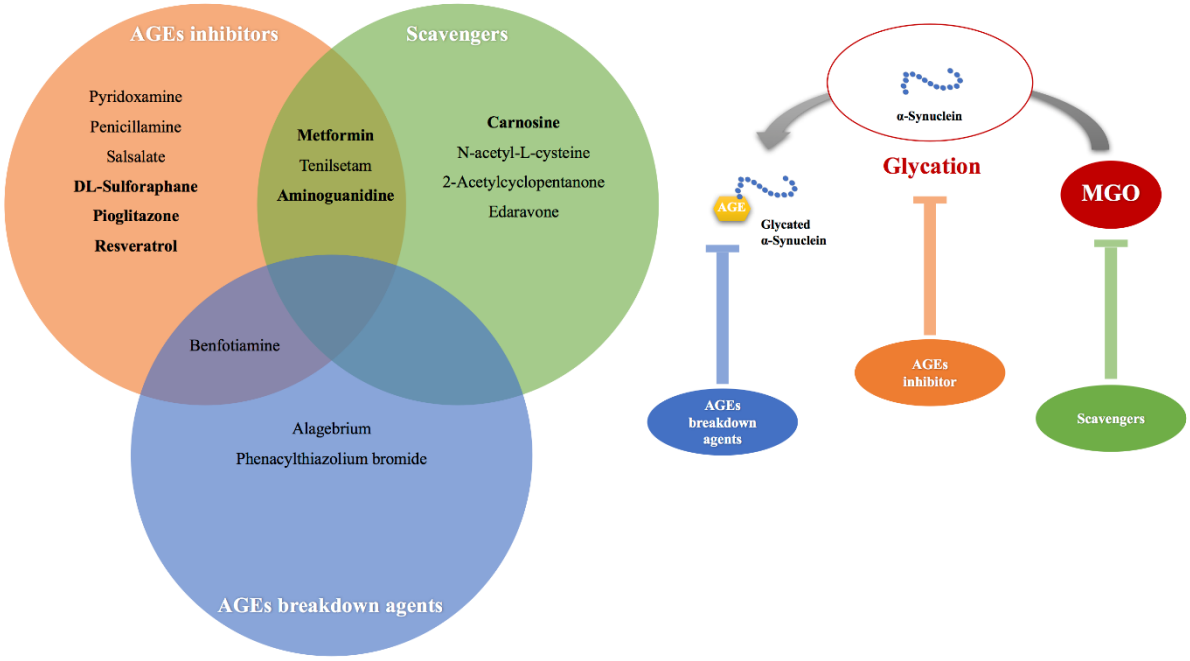

Figure 1.4 Potential anti-glycation pharmacological compounds.
Schematic representation of MGO scavengers, AGEs breakdown agents, and AGEs inhibitors, along with their proposed mechanism of action. AGEs breakdown agents act directly on AGEs, while AGEs inhibitors react with glycation intermediates, inhibiting the glycation process. MGO scavengers trap this compound, preventing glycation. The compounds selected for this study are in Bold. Abbreviations: AGEs: advanced glycation end-products, MGO: methylglyoxal.

1.2.3.1 Metformin

Metformin, a member of the biguanide family, is a mitochondrial complex I inhibitor and activator of adenosine monophosphate-activated protein kinase (AMPK), an important homeostasis regulator, possibly through glucose starvation [133]. Metformin is currently used for the treatment of type-2 diabetes [134–136]. This compound is known to lower blood glucose levels and enhance insulin sensitivity [134]. Metformin increases lifespan by mimicking a caloric restriction environment in mice [134] and yeast [137], and attenuate the expression of NF-κB, inhibiting chronic inflammation in mice [134]. In *Caenorhabditis elegans*, metformin promoted an increase of about 40% in median survival [138]. Metformin has been shown to be neuroprotective. Studies demonstrated that it prevents or attenuates MPTP-induced dopaminergic neurodegeneration [135,136], decreases aSyn expression, and increases autophagy and mitophagy through the activation of AMPK [136]. Furthermore, this compound is able to reduce neurotoxicity in aSyn overexpressing cells [139]. Moreover, this compound is known to be a MGO scavenger [140–144] and to reduce AGEs levels [141,142,145], making it a potential pharmacological agent against glycation.

1.2.3.2 Pioglitazone

Pioglitazone is a synthetic ligand that activates nuclear receptor peroxisome proliferator-activated receptor γ (a transcription factor) and increases insulin sensitization, being therefore useful in the treatment of type-2 diabetes mellitus [146,147]. Pioglitazone was shown to inhibit AGEs cross-link [148], block intracellular signaling of RAGE by down-regulating NF-kB activation, and counteract AGEs-induced pancreatic beta cell necrosis [149]. This compound can also inhibit tau phosphorylation and ameliorate Alzheimer's disease progression [147].

1.2.3.3 Sulforaphane

Sulforaphane is a natural compound found in vegetables such as broccoli and cauliflower, known to activate nuclear factor erythroid-related factor 2 (Nrf2), a transcription factor responsible for anti-oxidative stress response [150]. Several studies have shown its anti-proliferative [151] and anti-angiogenic properties [152,153] as well as its protective effect against oxidative stress [154] and hyperglycemia [155]. Moreover, it has been shown that the activation of Nrf2 inhibits the accumulation of MGO-modified proteins [156]. Recently, Angeloni and colleagues demonstrated that sulforaphane protects cells from necrotic and apoptotic cell death, increases Glo1 expression and activity along with GSH levels, and increases glucose uptake, being able to rescue from the cytotoxic phenotype induced by MGO [157]. Furthermore, this compound was shown to decrease AGE-induced RAGE expression [158,159] and ROS generation [159]. Moreover, it was demonstrated that sulforaphane increases DJ-1 expression, decreasing protein glycation levels [160]. Sulforaphane can also increase the nuclear translocation of Nrf2 [161], leading to the expression of genes associated with antioxidant response [162].

1.2.3.4 Resveratrol

Resveratrol is a natural occurring compound found in different plant species [163]. This compound activates sirtuin-1, a deacetylase found to stimulate oxidative stress response and cell survival [164,165]. Furthermore, resveratrol improves mitochondrial function via sirtuin-1 [166], and increases autophagic flux, promoting aSyn degradation[167]. This compound is also thought to modulate inflammation[168]. A study performed in neuroblastoma cells revealed that this compound was able to protect against dopamine-induced cytotoxicity and apoptosis [169]. Moreover, in another study conducted in neuroblastoma cells, resveratrol induced autophagy-dependent clearance of aSyn [170] via AMPK pathway. In a 6-OHDA-induced rat PD model, researchers also showed that resveratrol can attenuate neuronal damage in the *substantia nigra* [171]. Recently, it was observed that resveratrol had anti-glycating properties as it prevented the formation of AGEs in a dose-dependent manner [172]. Additionally, a different study suggests that resveratrol can modulate RAGE expression as it was able to decrease its expression in resveratrol-treated rats in contrast with a diabetic group, where these levels were elevated [173].

1.2.3.5 Carnosine

Carnosine is a naturally occurring dipeptide abundant in human muscle [174,175]. Several studies showed its ability to extend cell survival and division potential, to chelate metal ions, scavenge free radicals, as well as present antioxidant activity [176,177]. Carnosine was also proposed to play a role as neurotransmitter in the olfactory bulb [178]. Carnosine reacts with MGO interacting and protecting against MGO-induced protein modification [175,179]. Furthermore, it suppresses the formation of AGEs *in vivo*, protecting from lethal concentrations of glucose and MGO [180]. Carnosine also prevents cross-linking of β-amyloid, which aggregates in Alzheimer's disease [181].

1.2.3.6 Aminoguanidine

Aminoguanidine is a MGO scavenger and AGEs inhibitor. This compound extends yeast lifespan by 45-50%, reduces AGEs protein modifications, and increases mitochondria respiration [137]. Aminoguanidine significantly increases the survival of neuroblastoma cells challenged with MGO [182]. It also inhibits AGEs formation by reacting with MGO [182]. Recently, it was observed in cellular models that aminoguanidine, in addition to lowering MGO levels (20%), aSyn aggregation (20%), and toxicity (50%), it also reverted the impairment of aSyn clearance upon glycation [61]. Furthermore, this scavenger also improved *Drosophila* motor performance after 10 days treatment [61]. Aminoguanidine was evaluated in type 1 and type 2 diabetes mellitus. However, several side effects such as impaired liver function, and gastrointestinal disturbances, among others, were observed [183].

1.2.3.7 Tenilsetam

Tenilsetam is a MGO scavenger, shown to protect against MGO toxicity [182]. This compound inhibits the Maillard reaction and protects against AGEs cross-link [184]. A recent study revealed that tenilsetam could reduce intracellular levels of MGO by 20%, aSyn aggregation (20%) and toxicity (50%) [61]. Moreover, this compound improves aSyn clearance in a glycating conditions [61]. Similar to aminoguanidine, tenilsetam also improved *Drosophila* motor performance after 10 days treatment [61].

1.2.3.8 N-acetylcysteine

N-acetylcysteine is a derivative of the amino acid L-cysteine, and is known to possess antioxidant properties, mainly by restoring GSH [185]. A study involving three patients with PD showed that blood and brain glutathione levels increased transiently after N-acetylcysteine intravenous administration [186]. Furthermore, in a rotenone model of PD, treatment with N-acetylcysteine increased the percentage of dopaminergic neurons compared to untreated cells, conferring a protective effect [185]. Moreover, N-acetylcysteine may attenuate deleterious renal effects cause by chronic exposure to AGEs [187].

1.2.3.9 Pyridoxamine

Pyridoxamine is one of the natural occurring forms of vitamin B6 [188,189]. This compound has been proposed to be an AGE inhibitor and MGO scavenger [190–193], reacting with intermediates of the Maillard reaction [191]. Pyridoxamine was successfully able to reduce MGO levels in diabetic rats, not only when compared with non-treated diabetic rats, but also when compared to control ones [192]. Moreover, this compound can increase Glo1 activity in hyperglycemic conditions [192]. When compared with aminoguanidine, pyridoxamine proved to be a stronger inhibitor of post-Amadori AGE formation and ROS accumulation, although showing a weaker scavenging activity [190,191].

1.2.3.10 N-phenacylthiazolium bromide

N-phenacylthiazolium bromide is a thiazolium-based nucleophile that was shown to disrupt AGEs cross-links *in vitro* and *in vivo* [194]. N-phenacylthiazolium bromide treatment also leads to a decrease in vascular AGEs accumulation *in vivo* [195] and modulates inflammation through the downregulation of the RAGE receptor [196]. A study performed on human skeletal tissue showed that AGEs levels were decreased after N-phenacylthiazolium bromide treatment [197].

1.2.3.11 Penicillamine

Penicillamine was shown to be an AGE inhibitor [141,198]. This compound inhibits the formation of Amadori products by 56% and AGEs formation by 44% *in vitro* [198]. Furthermore, penicillamine prevents

glycation-induced cytotoxicity and GSH depletion by scavenging glyoxal compounds such as MGO [199]. Penicillamine can also improve cell proliferation by approximately 80% [200].

2 Objectives

PD significantly decreases the well-being of the affected patients, and it is currently untreatable. Recent findings suggested a link between PD and glycation, since glycated aSyn was detected in the brain of PD patients, and it promotes its aggregation and toxicity. Therefore, the main aim of this project was to investigate the protective ability of selected compounds in suppressing the deleterious effects of glycation in cellular models of PD. Specifically, this study aimed to evaluate the therapeutic potential of metformin, pioglitazone, sulforaphane, carnosine, and aminoguanidine, compounds already observed to modulate different aspects of the glycation process, in what regards to their cytotoxicity, their ability to modulate AGEs and aSyn/SynT expression, and their ability to suppress protein aggregation and/or promote aSyn clearance. Their protective properties were evaluated in cellular models of PD, namely H4 neuroglioma cells overexpressing aSyn/SynT, in the presence/absence of MGO-induced glycation.

3 Materials and Methods

3.1 MGO purification and measurement

MGO was produced as previously described in Vicente Miranda (2017) [61]. Briefly, MGO was prepared by sulfuric acid hydrolysis of 1,1-dimethyl acetal. Purified MGO was obtained by fractional distillation under reduced pressure and nitrogen bleed of the resulting mixture. MGO was fractioned through a vaporization process at 72 ºC. The first fraction was discarded, since it contains a mixture of MGO and methanol, whereas the second and third fractions were collected and stored at -80 ºC until further use.

MGO measurement was performed as described in Arai (2014) [201] by its derivatization with aminoguanidine. Briefly, purified MGO was diluted to around 50-100 µM in a solution with 1 mM aminoguanidine hydrochloride in 50 mM sodium phosphate buffer (pH 7.4), with incubation at 37 ºC for 4 hours. The resulting compound generated from the reaction between MGO and aminoguanidine, aminotriazine, was then assayed by spectrophotometry at 320 nm, from which the concentration of MGO is deduced ($\varepsilon 320 = 2411\ M^{-1}.cm^{-1}$).

3.2 Pharmacological compounds

Aminoguanidine hydrochloride (Sigma-Aldrich: 396494; St. Louis, MO, USA), Metformin hydrochloride (Sigma-Aldrich: PHR1084; St. Louis, MO, USA), and Carnosine (Santa Cruz Biotechnology: sc-202521; Dallas, TX, USA) were prepared in phosphate-buffered saline (PBS, Gibco). DL-Sulforaphane (Sigma-Aldrich: S4441; St. Louis, Missouri, USA), Pioglitazone (Santa Cruz Biotechnology: sc-204848; Dallas, TX, USA), and Resveratrol (Sigma-Aldrich: R5010; St. Louis, MO, USA) were prepared in DMSO.

3.3 Cell culture

H4 human neuroglioma cells (ATCC® HTB-148™; Manassas, VA, USA) were maintained at 37ºC in a humidified incubator with 5% CO_2; in 10 cm tissue culture dish (Techno Plastic Cultures AG, TPP;

Trasadingen, Switzerland) seeded at 1.000.000 cells in Opti-MEM® I Reduced Serum Medium (1x) (Gibco, Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific; Waltham, MA, USA).

3.3.1 Transfection

Cells were transfected with plasmids containing the gene sequence of human wild-type aSyn (aSyn WT) or were co-transfected with plasmids with the gene sequences for an aggregation-prone variant of aSyn (SynT) and synphilin-1 (Synph 1), using FuGENE® 6 Transfection Reagent (Promega; Madison, WI, USA), according to manufacturer's instructions. Briefly, 24 hours after cell seeding, the transfection mixture in a 1:3 ratio (DNA:Fugene) for one plasmid, or 1:5 ratio for two plasmids was prepared and added to cells.

3.3.2 Cell assays

3.3.2.1 Pharmacological compound cytotoxicity

For drugs' basal cytotoxicity evaluation and concentration range screening, H4 cells were seeded at 15,000 cells/well in 24-well plates (Techno Plastic Cultures AG, TPP; Trasadingen, Switzerland). After medium renewal, cells were treated with vehicle, PBS (Gibco), or aminoguanidine hydrochloride (150-450 µM), metformin hydrochloride (0.05-25 mM), carnosine (1-40 mM), DL-sulforaphane (0.5-10 µM), pioglitazone (2.5-30 µM) or resveratrol (0.5-30 µM) for 24 hours. The medium was renewed and the drug treatment was repeated, for an additional period of 24 hours. 48 hours post-transfection, cell toxicity was evaluated by lactate dehydrogenase (LDH) release (see 5).

3.3.2.2 Evaluation of pharmacological compounds' preventive potential

In order to assess the preventive potential of the drugs, H4 cells were seeded at 15,000 cells/well in 24-well plates (Techno Plastic Cultures AG, TPP; Trasadingen, Switzerland), 100,000 cells/well in 35 mm imaging dishes (Ibidi; Planegg, Germany), 170,000 cells/well in 6-well plates and at 300,000 cells in 6 cm plates (Techno Plastic Cultures AG, TPP; Trasadingen, Switzerland). 24 hours post-seeding, cells were transfected with empty vector or vector containing aSyn WT, or co-transfected with SynT and Synph 1. After 24 hours, the medium was renewed and cells were treated with vehicle (PBS), aminoguanidine hydrochloride (150-450 µM), metformin hydrochloride (0.05-25 mM), carnosine (1-40 mM), DL-sulforaphane (0.5-10 µM), pioglitazone (2.5-30 µM), or Resveratrol (0.5-30 µM) for 24 hours. The medium was renewed and the drug treatment was repeated, followed by a treatment with vehicle (PBS) or MGO (0.2 mM) for 24 hours. 48 hours post-initial drug treatment, cytotoxicity (LDH release), protein extraction for western blot (see 6.1 and 6.3), and aggregates analysis by immunocytochemistry (see 7) were performed.

3.3.2.3 Evaluation of pharmacological compounds' restorative potential

For drugs' restorative potential assessment, H4 cells were seeded at 20,000 cells/well in 24-well plates (Techno Plastic Cultures AG, TPP; Trasadingen, Switzerland), at 100,000 cells/well in 35 mm imaging dishes (Ibidi; Planegg, Germany), at 170,000 cells/well in 6-well plates, at 300,000 cells in 6 cm plates (Techno Plastic Cultures AG, TPP; Trasadingen, Switzerland). 24 hours post-seeding, cells were transfected with empty vector or vector containing aSyn WT, or co-transfected with SynT and Synph 1. After 24 hours, the medium was renewed and cells were treated with vehicle (PBS) or MGO (0.2 mM) for 18 hours. After that period, medium was renewed, and cells treated with vehicle, aminoguanidine hydrochloride (150-450 µM), metformin hydrochloride (0.05-25 mM), carnosine (1-40 mM), DL-

sulforaphane (0.5-10 µM), pioglitazone (2.5-30 µM), or resveratrol (0.5-30 µM). 24h post-treatment, medium was renewed and the drug treatment repeated 48 hours post-initial drug treatment, cytotoxicity (LDH release), protein extraction for western blot (see 6.1 and 6.3) or triton X-100 solubility (see 6.2 and 6.3), and aggregate analysis by immunocytochemistry (see 7) were performed.

3.4 LDH Cytotoxicity Assay

Cytotoxicity was measured using LDH kit (Clontech; Mountain View, CA, USA), according to manufacturer's instructions. LDH is an enzyme present in all cells that is released to the medium when cellular membrane integrity is lost, indicative of cell death. Briefly, conditioned medium from cells was collected and centrifuged at 750 rpm for 5 minutes (to discard cellular debris). Fresh medium containing 1% Triton®X 100 (Panreac; Barcelona, Spain) was added to cells, in order to completely lyse them. This conditioned media was collected, and used as 100% cell death control. Based on a colorimetric assay, the collected medium was then incubated with the reaction mixture to determine LDH activity and quantify cell death. The measured cytotoxicity was then calculated by the ratio between the measured absorbance and total cell death, which was then normalized to vehicle treated cells or MGO treated cells.

3.5 Protein Analysis

3.5.1 Extraction of total protein

Cells were lysed using NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with cOmplete™, Mini, EDTA-free Protease Inhibitor (Roche; Basel, Switzerland). Cells were scrapped and lysed by three cycles of freezing and thawing in liquid nitrogen. Proteins were separated from debris by centrifugation at 10,000 g, at 4 ºC, for 10 minutes. Quantification of total protein was performed using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA).

3.5.2 Triton-X 100 solubility assay

Cells were lysed using PBS supplemented with cOmplete™, Mini, EDTA-free Protease Inhibitor (Roche; Basel, Switzerland). After cell scrapping, lysates were sonicated 3 times for 30 seconds (amplitude of 10 microns) with 1 minute incubation on ice between each sonication step. Samples were then quantified with Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA). Triton®X 100 was added at a final concentration of 1% to 200 µg of protein extract, followed by an incubation, at 4ºC, for 30 minutes. Protein fractions were separated by centrifugation at 16,000 g, at 4˚C, for 1 hour. Soluble protein fraction was collected and the insoluble protein fraction pellet was resuspended in 40 µL of PBS supplemented with sodium dodecyl sulfate (SDS, 2% final concentration) and cOmplete™, Mini, EDTA-free Protease Inhibitor (Roche; Basel, Switzerland). Soluble fraction (10 µL), and insoluble fractions (15 µl), both supplemented with 2% SDS, were loaded and resolved by SDS-Page and immunoblotted as described below.

3.5.3 SDS-Page and Western Blotting

10 µg of total protein from cell extracts were separated by SDS-PAGE electrophoresis using a Tetra cell (Bio-Rad; Hercules, CA, USA), in 12% polyacrylamide separation gel and a 4% polyacrylamide stacking gel, applying a constant voltage of 120 V. Pre-stained standard proteins were also loaded onto the gel. Proteins were transferred to nitrocellulose membranes, using standard procedures with a Mini Trans-Blot system (Bio-Rad; Hercules, CA, USA). Membranes were incubated with blocking solution (3% bovine serum albumin) in 1x TBS (20 mM Tris, 136 mM NaCl, pH 7.6) at room temperature for 30 minutes. Membranes were incubated overnight at 4ºC with the primary antibody using given

concentrations (see 5.3.1) in blocking solution. Membranes were washed and incubated with secondary antibody in blocking solution for 1.5 hours. Detection procedures were performed according to ECL system (GE Healthcare, Life Sciences; Little Chalfont, UK), and the signal was detected using a ChemiDoc™ Imaging Systems (Bio-Rad, Hercules, CA, USA) with the most appropriate exposure time. Densitometry was performed using ImageJ - Image Processing and Analysis in Java [202]. When required, membranes were incubated with stripping solution (250 mM Glycine, 0.1 % of 10 % SDS, pH 2.0) for 45 minutes at room temperature with agitation, followed by 4 washing steps, twice with 1x TBS and twice with 1x TBS supplemented with 10% Tween 20 solution. Membranes were then incubated in blocking solution for 30 minutes, before reprobing with the required antibodies.

3.5.3.1 Antibodies

For glycation profile screening, membranes were incubated with the primary antibody Anti-Methylglyoxal monoclonal antibody (Cell Biolabs; San Diego, CA, USA) in a dilution of 1:1000 in blocking solution. For aSyn levels screening, the primary antibody used was Purified Mouse Anti-α-Synuclein antibody (BD Biosciences; San Jose, CA, USA) in a dilution of 1:1000 in blocking solution. β-actin was the housekeeping gene used as loading control for signal normalization, using the primary antibody Mouse Monoclonal anti-β-actin antibody (Ambion, Thermo Fisher Scientific; Waltham, MA, USA) in a dilution of 1:5000 in blocking solution. Secondary antibody used was ECL™ Anti-Mouse IgG, HRP-Linked antibody (Amersham™; Little Chalfont, UK) in a dilution of 1:5000 in blocking solution.

3.6 Immunocytochemistry

Immunocytochemistry assays were performed to evaluate aSyn aggregates formation in H4 cells co-transfected with plasmids containing SynT and Synph 1 gene sequences. These cells were treated with selected drugs in a glycation context (MGO insult) as described in section 4.2. Briefly, cells were washed with PBS and fixed and permeabilized in 100% ice-cold methanol, at -20 ºC, for 10 minutes. Cells were then incubated for 1 hour with blocking solution (1.5% normal goat serum in PBS) at room temperature, and immediately used or stored in PBS at 4ºC. Cells were incubated overnight at 4ºC with the primary antibody anti-α-Synuclein (Cell Signaling Technology, Danvers, MA, USA) in a dilution of 1:50 in blocking solution. Cells were then washed with PBS and incubated for 4 hours at room temperature with Alexa Fluor® 488 goat anti-rabbit conjugated secondary antibody (Invitrogen; Carlsbad, CA, USA) using a dilution of 1:1000 in blocking solution. Microscopy images were acquired in a Widefield fluorescent microscope Zeiss Axiovert 40 (Carl Zeiss MicroImaging) and in a point scanning confocal microscope Zeiss LSM 710 (Carl Zeiss MicroImaging).

3.7 Clearance assay

To assess aSyn clearance, H4 cells transfected with vector containing aSyn WT sequence were challenged with the selected pharmacological compound, MGO, and with cyloheximide (a *de novo* protein synthesis inhibitor). Briefly, cells seeded at 170,000 cells/well in 6-well plates (Techn-o Plastic Cultures AG, TPP; Trasadingen, Switzerland) were transfected with aSyn WT vector 24 hours post-seeding. 24 hours later, the medium was renewed and cells were treated with metformin (16 mM). After 24 hours, medium was renewed and cells were treated with metformin (16 mM) and MGO (0.2 mM), and challenged with cycloheximide (100 μM) for a period up to 12 hours. This treatment arrests *de novo* protein synthesis, which allows to monitor the clearance of aSyn.

3.8 Statistical analysis

Each assay was performed at least three times, unless stated otherwise, and all values are expressed as normalized means ± standard deviation. Statistical analysis was performed using GraphPad Prism version 7.0a. T-test or one-way ANOVA were used to compare differences among conditions, followed by Dunnett's multiple comparison test. Values of $p < 0.05$ were considered significant.

4 Results and Discussion

4.1 Cytoprotective activity of pharmacological compounds

4.1.1 Basal cytotoxicity of pharmacological compounds

First, the cytotoxicity of the selected pharmacological compounds under basal conditions was investigated. The assessment of these agents' cytotoxicity was performed according to Figure 4.1 A.

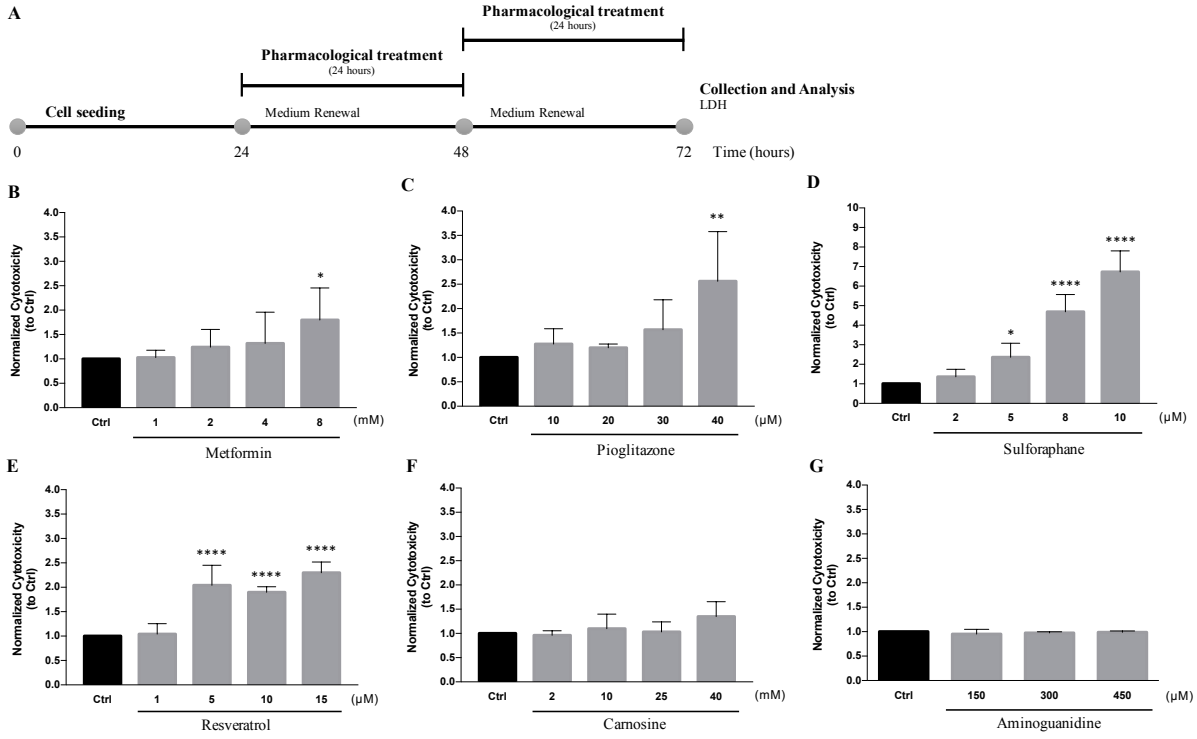

Figure 4.1 Pharmacological compounds' basal cytotoxicity.
(A) Schematic representation of pharmacological compounds basal cytotoxicity setup. Cytotoxicity was measured by LDH release and levels normalized to Ctrl. Toxicity of H4 WT cells treated with vehicle (PBS) (Ctrl), or with **(B)** metformin hydrochloride (1-8 mM), **(C)** pioglitazone hydrochloride (10-40 µM), **(D)** dl-sulforaphane (2-10 µM), **(E)** resveratrol (1-1 5µM), **(F)** carnosine (2-40 mM), or **(G)** aminoguanidine (150-450 µM), (n=3, at least). Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. One-way ANOVA, followed by Dunnett's multiple comparisons test.

Briefly, pharmacological treatment (48 hours) started 24 hours post-seeding and was re-enforced 24 hours later. Metformin showed little to no toxicity at low concentrations. However, at 8 mM, it was cytotoxic (Figure 4.1 B). Similarly, pioglitazone revealed little or no toxicity at low concentrations, being toxic at 40 µM (Figure 4.1 C). Sulforaphane was the most toxic compound under these conditions, exhibiting toxicity from 5 µM onwards, reaching 7-fold higher toxicity at 10 µM (Figure 4.1 D). Of all

concentrations tested for resveratrol, only 1 µM was not cytotoxic (Figure 4.1 E). Carnosine (Figure 4.1 F) and aminoguanidine (Figure 4.1 G) were not cytotoxic at all tested concentrations.

These findings suggest that from of all the compounds tested, metformin, carnosine, and aminoguanidine were the least cytotoxic. These assays allowed for the selection of the most appropriate concentrations to be used in cells overexpressing aSyn, where glycation will be induced by MGO.

4.1.2 Preventive activity of pharmacological compounds

4.1.2.1 Upon aSyn overexpression

To evaluate the preventive potential of pharmacological compounds, their cytotoxicity was assessed in H4 cells overexpressing aSyn. The experimental setup of this experiment is described in Figure 4.2 A.

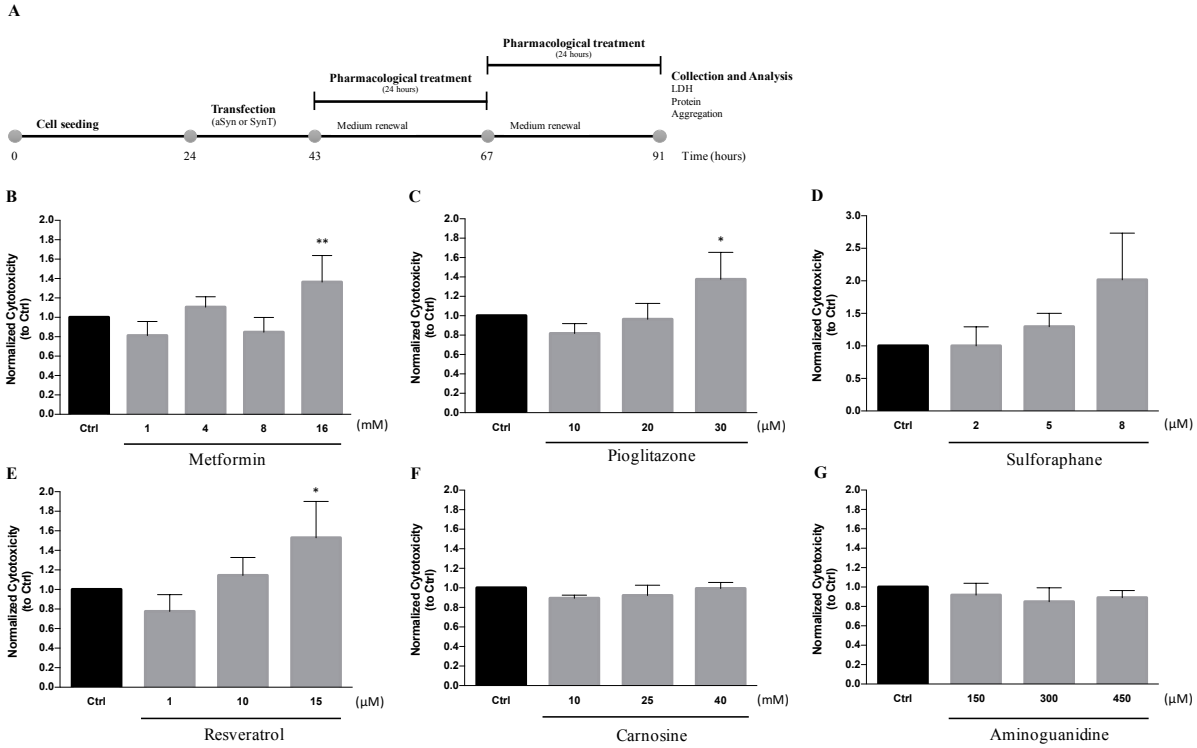

Figure 4.2 Pharmacological compounds potential to prevent from aSyn overexpression toxicity.
(A) Schematic representation of pharmacological compounds' preventive potential assay. Cytotoxicity measured by LDH release and normalized to non-treated cells (Ctrl). Toxicity of H4 cells transfected with aSyn and treated with vehicle (PBS) (Ctrl), or **(B)** metformin hydrochloride (1-16 mM), **(C)** pioglitazone hydrochloride (10-30 µM), ***(D)*** dl-sulforaphane (2-8 µM), **(E)** resveratrol (1-15 µM), **(F)** carnosine (10-40 mM), or **(G)** aminoguanidine (150-450 µM) (n=3, at least). Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. One-way ANOVA, followed by Dunnett's multiple comparisons test.

Briefly, the pharmacological treatment (48 hours) started 43 hours post-seeding and 19 hours post-aSyn overexpression and was re-enforced 24 hours later. Metformin showed a tendency to decrease cytotoxicity at 1 and 8 µM, although not statistically significant (Figure 4.2 B). At 16 µM, it induced cytotoxicity (Figure 4.2 B). Pioglitazone at 10 µM slightly decreased aSyn associated cytotoxicity, although not significantly. At 30 µM it was cytotoxic (Figure 4.2 C). With sulforaphane, no cytoprotection was observed (Figure 4.2 D). Resveratrol at 1 µM slightly decreased aSyn cytotoxicity, however not significantly (Figure 4.2 E). At 15 µM, it became significantly cytotoxic (Figure 4.2 E). Carnosine showed no effect at tested concentrations (Figure 4.2 F). Although aminoguanidine had a tendency to suppress aSyn cytotoxicity, data was not significant (Figure 4.2 G).

All tested compounds were not able to decrease aSyn associated cytotoxicity for the studied concentrations and timings, suggesting that their potential to suppress aSyn cytotoxicity is limited. Since these compounds are known glycation suppressors and/or MGO scavengers, it was hypothesized that they could show a preventive effect under glycation conditions.

4.1.2.2 Upon aSyn overexpression and MGO-induced glycating conditions

The preventive potential of these compounds was evaluated not only under aSyn overexpression, but also in a glycating environment. The experimental setup is described in Figure 4.3 A.

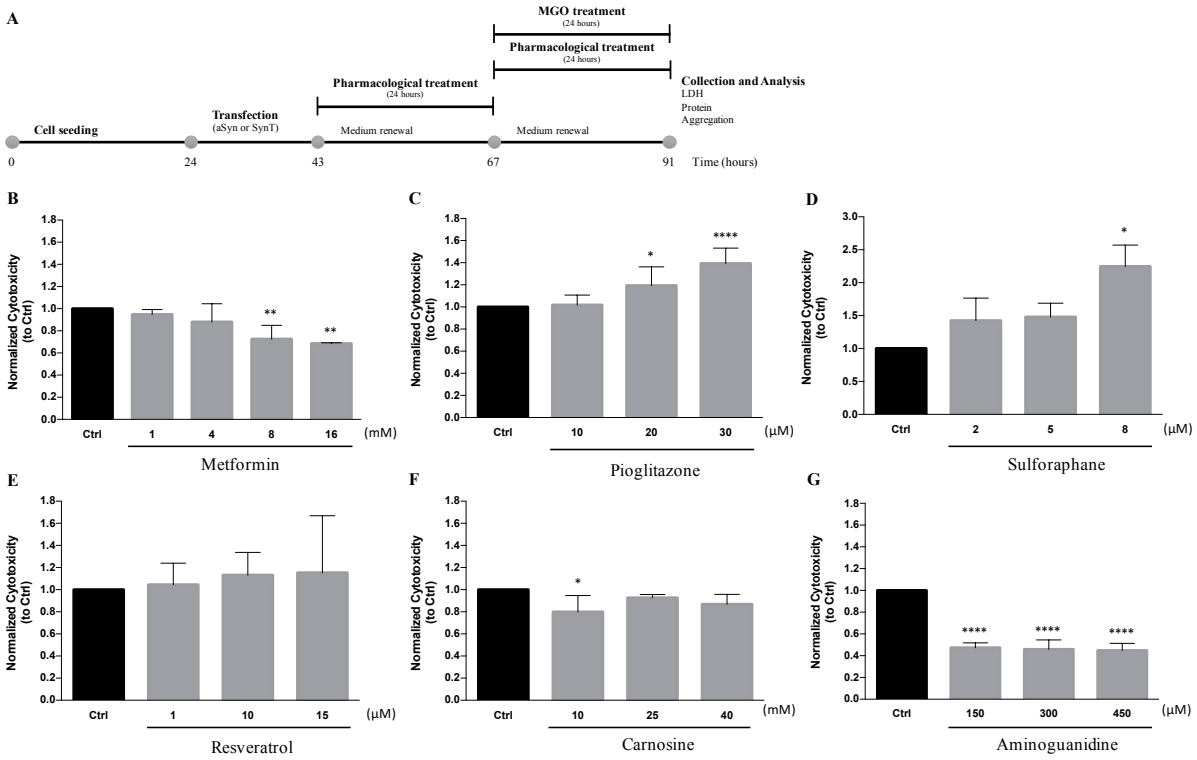

Figure 4.3 Pharmacological compounds' potential to prevent from aSyn overexpression toxicity under MGO-induced glycation conditions.
(A) Schematic representation of drugs' preventive potential assay. Cytotoxicity measured by LDH release and normalized to MGO-treated cells (Ctrl). Toxicity of aSyn expressing H4 cells treated with MGO (Ctrl) (0.2 mM), or treated with **(B)** metformin hydrochloride (1-16 mM), **(C)** pioglitazone hydrochloride (10-30 μM), **(D)** dl-sulforaphane (2-8 μM), **(E)** resveratrol (1-15 μM), **(F)** carnosine (10-40 mM), or **(G)** aminoguanidine (150-450 μM), followed by MGO treatment (n=3 at least). Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. One-way ANOVA, followed by Dunnett's multiple comparisons test.

Briefly, these experiments were performed as previously (Section 4.1.2.1), however cells were also challenged with MGO in the last 24 hours period before cell collection. Metformin significantly prevented aSyn/MGO associated cytotoxicity at 8 and 16 mM (~30% suppression) (Figure 4.3 B, Table 4.1). A concentration of 25 mM for metformin was also explored, however it did not further suppress the cytotoxicity (data not shown). Pioglitazone did not show preventive properties. In fact, aSyn and MGO cytotoxicity was aggravated in a concentration-dependent manner (Figure 4.3 C). Similar to pioglitazone, sulforaphane (Figure 4.3 D) and resveratrol (Figure 4.3 E) did not show preventive potential. Carnosine at 10 mM significantly prevented aSyn/MGO associated cytotoxicity (~20% suppression) (Figure 4.3 F, Table 4.1). The effect of carnosine at 1 and 5 mM was also investigated, however they did not further prevent cytotoxicity (data not shown). In agreement with previous findings[61], aminoguanidine markedly prevented MGO/aSyn associated toxicity by 60% (Figure 4.3 G, Table 4.1). The findings here presented suggest that metformin, carnosine, and aminoguanidine prevent

from aSyn associated toxicity in the presence of MGO. Since aminoguanidine was previously shown to reduce aSyn/MGO cytotoxicity [61], this compound was not further evaluated on its effects on protein levels. Metformin and carnosine were further explored, and their effects on AGEs and aSyn protein levels were evaluated.

4.1.3 Restorative activity of pharmacological compounds

4.1.3.1 Upon aSyn overexpression

The restorative potential against aSyn overexpression of the pharmacological compounds was also evaluated. The experimental setup of this assay is described in Figure 4.4 A.

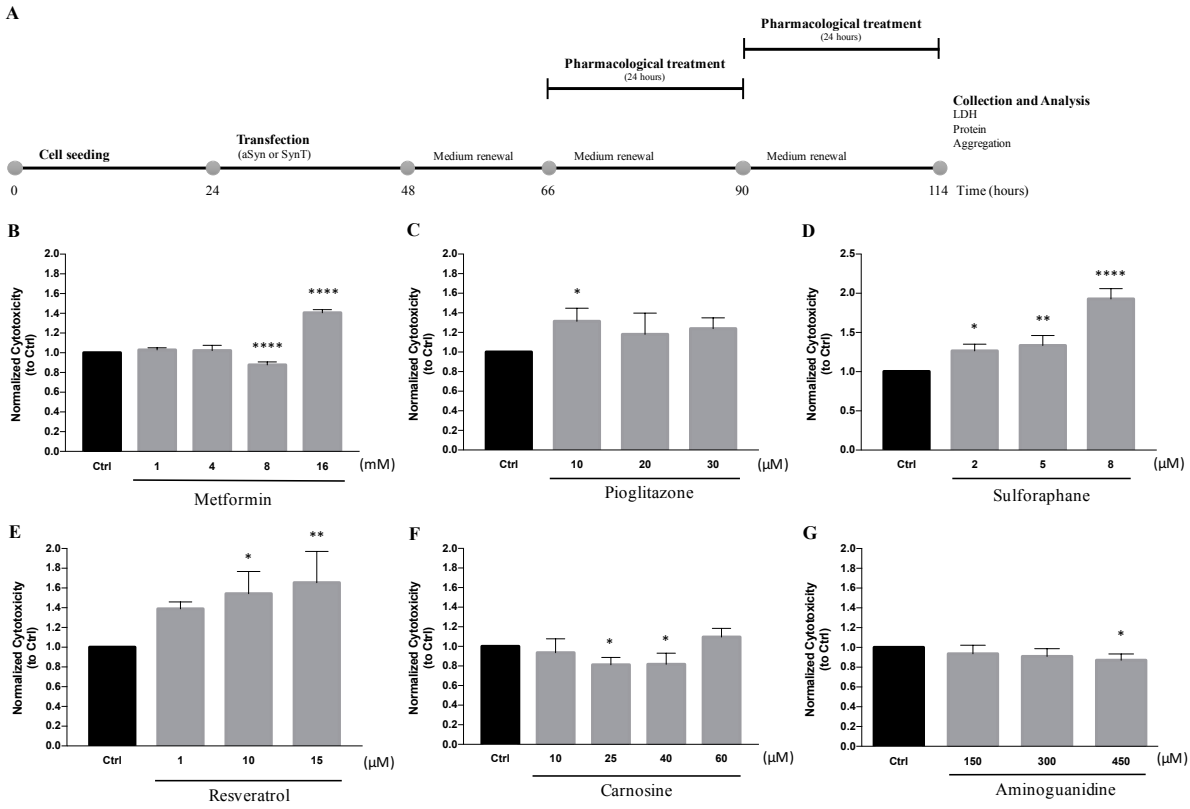

Figure 4.4 Pharmacological compounds' potential to restore from aSyn overexpression toxicity.
(A) Schematic representation of the pharmacological compounds' restorative potential assay. Cytotoxicity measured by LDH release and normalized to non-treated cells (Ctrl). Toxicity of H4 cells transfected with aSyn and treated with vehicle (PBS) (Ctrl), or **(B)** metformin hydrochloride (1-16 mM), **(C)** pioglitazone hydrochloride (10-30 μM), **(D)** dl-sulforaphane (2-8 μM), **(E)** resveratrol (1-15 μM), **(F)** carnosine (10-60 μM), or **(G)** aminoguanidine (150-450 μM), (n=3, at least). Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. One-way ANOVA, followed by Dunnett's multiple comparisons test.

In contrast to the previous conditions, the aim was to evaluate the restorative activity of the compounds in cells that were already affected by either aSyn or the combined action of aSyn/MGO (in 4.1.3.2). To that purpose, the timings of the pharmacological treatments were optimized, starting 66 hours post-seeding (in contrast to the 43 hours post-seeding performed previously). Metformin at 8 mM was able to restore from aSyn-induced cytotoxicity (~15% suppression), while at 16 mM it potentiated cytotoxicity (Figure 4.4 B). In contrast, neither pioglitazone (Figure 4.4 C), sulforaphane (Figure 4.4 D), nor resveratrol (Figure 4.4 E) showed restorative potential. In fact, at some concentrations they potentiated cytotoxicity. Carnosine at 25 and 40 mM was able to significantly decrease aSyn cytotoxicity (Figure 4.4 F) by ~20%. Aminoguanidine was also able to decrease aSyn associated toxicity at 450 μM (Figure 4.4 G, Table 4.1) (15%).

4.1.3.2 Upon aSyn overexpression and MGO-induced glycating conditions

Next, the restorative potential of the pharmacological compounds was investigated in cells overexpressing aSyn under glycation conditions. The experimental setup of this assay is described in Figure 4.5 A.

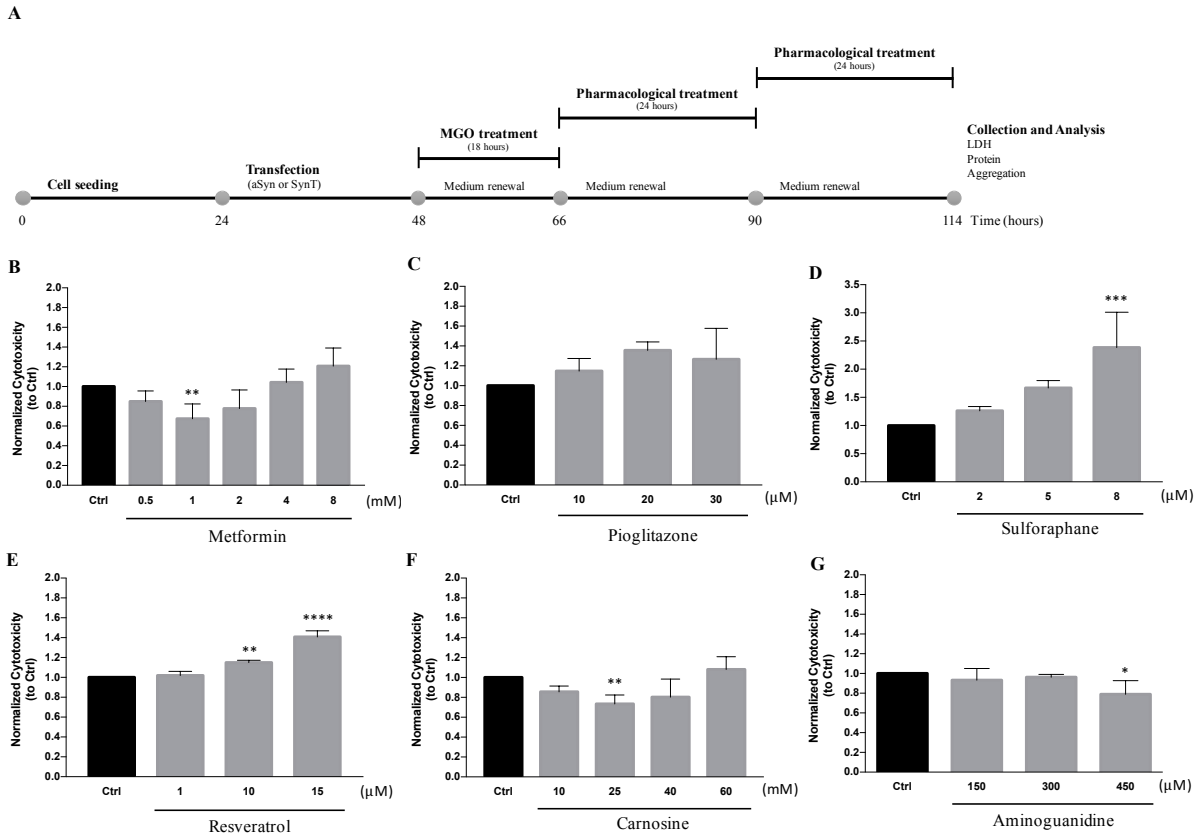

Figure 4.5 Pharmacological compounds' potential to restore from aSyn overexpression toxicity under MGO-induced glycation conditions.
(A) Schematic representation of drugs' restorative potential assay. Cytotoxicity measured by LDH release and normalized to Ctrl. Toxicity of H4 cells expressing aSyn treated with MGO (Ctrl), or treated with MGO followed by treatment with **(B)** metformin hydrochloride (0.5-8 mM), **(C)** pioglitazone hydrochloride (10-30 µM), **(D)** dl-sulforaphane (2-8 µM), **(E)** resveratrol (1-15 µM), **(F)** carnosine (10-60 mM), or **(G)** aminoguanidine (150-450 µM). Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. One-way ANOVA, followed by Dunnett's multiple comparisons test.

These assays were performed as previously (4.1.3.1), however cells were challenged with MGO 18 hours prior to the pharmacological treatment. In these conditions, metformin at 1 mM was able to successfully decrease cytotoxicity (~30%) (Figure 4.5 B, Table 4.1). In contrast, pioglitazone (Figure 4.5 C), sulforaphane (Figure 4.5 D) and resveratrol (Figure 4.5 E) were not able to decrease aSyn/MGO associated cytotoxicity. Carnosine at 25 mM also showed a ~30% suppression of cytotoxicity (Figure 4.5 F, Table 4.1). Aminoguanidine at 450 µM was able to decrease cytotoxicity (~20%) (Figure 4.5 G, Table 4.1). The results obtained for aminoguanidine were unexpected as it was previously described to reduce cytotoxicity by 50% in a preventive manner. These findings suggest that aminoguanidine protective activity is more efficient in a preventive manner. It is therefore hypothesized that although aminoguanidine is also an AGEs breakdown agent, it is more efficient as a MGO scavenger.

Table 4.1 Cytotoxicity protective potential of selected compounds.
Summary of cytotoxicity results regarding preventive and restorative potential of selected compounds under aSyn overexpression and MGO-induced glycation conditions.

Pharmacological compound	Preventive cytotoxicity decrease	Restorative cytotoxicity decrease
Metformin	30%	30%
Carnosine	20%	30%
Aminoguanidine	60%	20%

4.2 Protective effects of pharmacological compounds in the levels of glycation and aSyn/SynT

After evaluating the protective activity of the analyzed compounds, the levels of AGEs, aSyn, and SynT, the aggregation-prone variant of aSyn, were assessed. As previously mentioned, metformin and carnosine were the most active compounds. Therefore, their ability to modulate the levels of AGEs and aSyn at their most active concentrations was evaluated.

4.2.1 Preventive activity of pharmacological compounds on AGEs and aSyn/SynT levels

4.2.1.1 Upon overexpression of aSyn

First, the preventive activity of metformin and carnosine was evaluated. Impressively, metformin reduced the levels of AGEs (~40%) (Figure 4.6 A, Table 4.2). However, in comparison to MGO-treated cells, no changes in the levels of aSyn were observed (Figure 4.6 A). In contrast, carnosine only showed a tendency to decrease AGEs levels (not significant) (Figure 4.6 A). However, this compound was able to successfully decrease aSyn levels in MGO treated cells to the levels of non-MGO treated cells (~30% decrease) (Figure 4.6 A, Table 4.2).

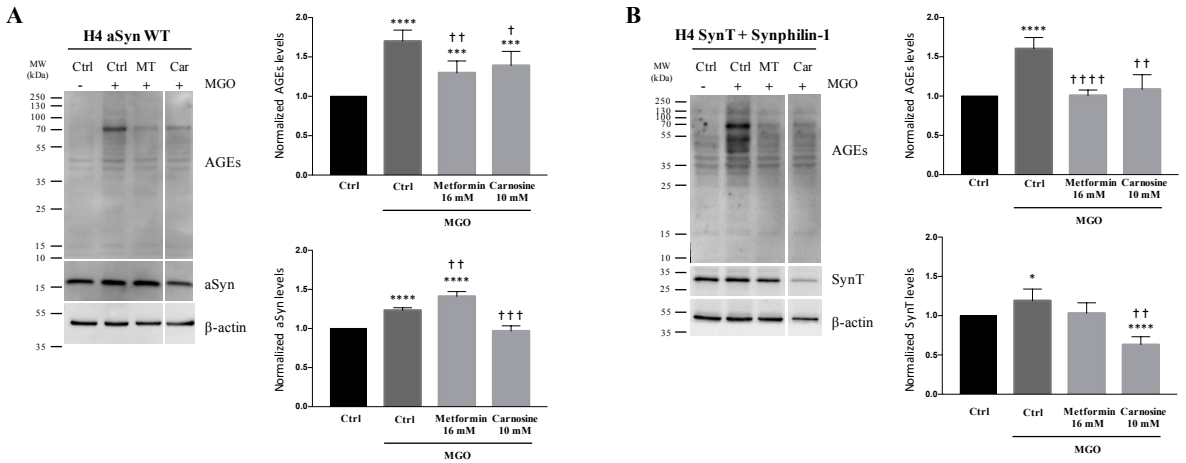

Figure 4.6 Preventive effects of metformin and carnosine in the levels of AGEs and aSyn/SynT.
(A) Cells were transfected with aSyn WT and treated in a preventive paradigm with vehicle (PBS) (Ctrl), metformin hydrochloride (16 mM), or carnosine (10 mM), followed by a MGO treatment (0.2 mM) (n=3, at least). **(B)** Cells co-expressing SynT and Synph 1 were treated with vehicle (PBS) (Ctrl), metformin hydrochloride (16 mM), or carnosine (10 mM), followed by MGO treatment (0.2 mM) (n=3, at least). Protein extracts were probed for AGEs, aSyn, and β-actin, for normalization. t-test was performed. Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. * comparison to Ctrl; † comparison to Ctrl MGO. MT: Metformin 16 mM, Car: Carnosine 10 mM.

The decrease of AGEs levels from MGO-treated cells by metformin was expected, as this compound is a well-known MGO scavenger and is currently used for the treatment of type-2 diabetes [134–136]. Furthermore, metformin was already shown to reduce the levels of AGEs in numerous studies [140–145]. However, since this compound was also described as neuroprotective [135,139], having the ability to decrease aSyn levels [136], we also expected that metformin treatment would decrease its levels, which

was not observed. The difference may be due to the evaluated cell type, which was different, as well as the experimental design, i.e., it used a different transfection agent, different vector for the expression of aSyn, and different metformin exposure times.

Carnosine was also described as a MGO scavenger, protecting against MGO-induced post-translational protein modifications [175,179]. The present findings show that this compound slightly decreases the levels of AGEs, although not significantly. Furthermore, they show that carnosine decreases aSyn levels by 30% (Table 4.2), corresponding to the levels present in non-MGO treated cells. These findings are in agreement with carnosine's antioxidant and neuroprotective ability, protecting against the aggregation of aSyn mediated by H_2O_2 and ceruloplasmin, a copper transporter [203]. Moreover, carnosine is a known MGO scavenger, therefore, its ability to decrease aSyn levels may be due to its successful scavenging of MGO. Carnosine could inhibit the formation of AGEs and potentially prevent aSyn aggregation. Glycation, as previously described [61], increases aSyn aggregation and impairs its correct clearance, leading to its accumulation [61]. On the other hand, carnosine could also be scavenging ROS. As mentioned previously, AGEs can bind to RAGE and lead to the formation of ROS [114,115]. These may have a direct impact on mitochondrial function, decreasing ATP production, which is fundamental for the proteasome activity [86,87]. Upon impairment of protein clearance, aSyn, especially when glycated, is susceptible to accumulate [61]. However, since the findings from the present work were evaluated in a preventive context, it is unlikely that carnosine would be acting on such a downstream level. However, this hypothesis should be evaluated in future experiments.

4.2.1.2 Upon overexpression of SynT

Next, the effects of these compounds in a context of increased aSyn aggregation were evaluated. Experimentally, this could be achieved by the overexpression of an aggregation-prone variant of aSyn – SynT [204]. Notably, metformin reduced the levels of AGEs to the ones present in non-glycated cells (~60%) (Figure 4.6 B, Table 4.2). As for the levels of SynT, it showed a non-significant tendency to decrease its levels (Figure 4.6 B). Carnosine presented a high preventive activity by suppressing both the levels of AGEs and SynT by 50% and 60%, respectively (Figure 4.6 B, Table 4.2).

In comparison to the previous findings in cells only expressing aSyn, both metformin and carnosine have a higher protective activity against MGO insult in an aggregation prone environment. They display a higher ability to decrease the levels of both AGEs and SynT.

Table 4.2. Preventive activity profile of pharmacological compounds in reducing the levels of AGEs and aSyn/SynT.
Summary of the preventive activity of metformin and carnosine in cells expressing aSyn or SynT.

Pharmacological compound	AGEs levels		Protein Levels	
	aSyn	SynT	aSyn	SynT
Metformin	- 40%	- 60%	No effect	No effect
Carnosine	- 30%	- 50%	- 30%	- 60%

4.2.2 Restorative activity of pharmacological compounds on the levels of AGEs and aSyn/SynT

4.2.2.1 Upon overexpression of aSyn

Metformin and carnosine's restorative activity on the levels of AGEs and aSyn were also evaluated. In contrast with what was observed in a preventive context, neither metformin nor carnosine (Figure 4.7 A) were able to significantly reduce the levels of AGEs or aSyn.

Under this experimental setup in cells expressing aSyn, MGO insult was not able to significantly raise AGEs levels (Figure 4.7 A). In the experimental setup for the preventive activity, MGO increased ~1.7-fold the levels of AGEs in both aSyn or SynT expressing cells (Figure 4.6). This could be explained by the fact that in a restorative experimental setup, cells are analyzed 66 hours post-MGO insult, in contrast to the 24 hours post-MGO insult in the preventive setup. This additional time in culture may be sufficient for cells to recover from overall levels of glycation. However, the levels of aSyn remain significantly increased (2-fold) (Figure 4.7 A) and are not altered by the compounds.

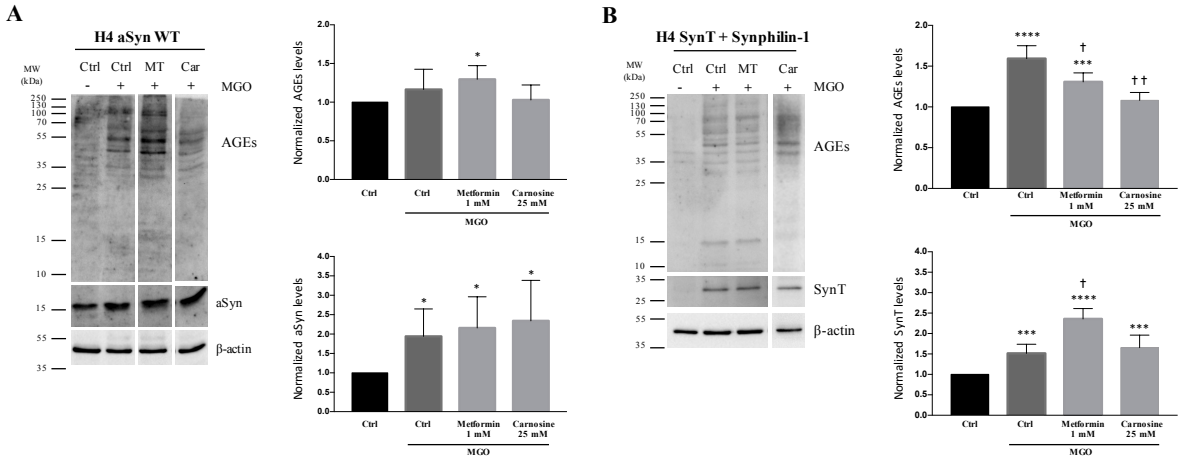

Figure 4.7 Restorative effects of metformin and carnosine in the levels of AGEs and aSyn/SynT.
(A) Cells were transfected with aSyn WT, and treated in a restorative paradigm with MGO (0.2 mM), followed by treatment with vehicle (PBS) (Ctrl), metformin hydrochloride (1 mM), or carnosine (25 mM) (n=3, at least). **(B)** Cells co-expressing SynT and Synph 1 were treated with MGO (0.2 mM), followed by treatment with vehicle (PBS) (Ctrl), metformin hydrochloride (1 mM), or carnosine (25 mM) (n=3, at least). Protein extracts were probed for AGEs, aSyn and β-actin, for normalization. t-test was performed. Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. * comparison to Ctrl; † comparison to Ctrl MGO. MT: Metformin 1 mM, Car: Carnosine 25 mM.

4.2.2.2 Upon overexpression of SynT

The levels of AGEs and SynT were also assessed in the aggregation paradigm. Metformin was able to decrease AGEs levels in 30% (Table 4.3), although the same was not observed for SynT levels (Figure 4.7 B). On the other hand, carnosine showed a significant decrease in AGEs levels (~50%) (Figure 4.7 B, Table 4.3), although not being able to modulate SynT levels (Figure 4.7 B). In contrast with aSyn expressing cells, in this experimental setup, MGO was able to increase ~1.6-fold the levels of AGEs in SynT expressing cells (Figure 4.7 B).

These findings suggest that both metformin and carnosine have a higher preventive activity in decreasing the levels of AGEs and aSyn.

Table 4.3. Restorative activity profile of pharmacological compounds in reducing the levels of AGEs and aSyn/SynT.
Summary of the restorative activity of metformin and carnosine in cells expressing aSyn or SynT.

Pharmacological compound	AGEs levels		Protein Levels	
	aSyn	SynT	aSyn	SynT
Metformin	No effect	- 30%	No effect	No effect
Carnosine	No effect	- 50%	No effect	No effect

4.3 Protective activity of pharmacological compounds in the aggregation profile of SynT

The data obtained in this study suggests that both metformin and carnosine are able to prevent the increase of the levels of AGEs and/or aSyn/SynT under MGO insult. Next, it was interrogated if these compounds are modulating aSyn aggregation status. To that purpose, Triton X-100 solubility assay and immunocytochemistry were performed in order to assess if these compounds are able to inhibit the aggregation of this protein. Although metformin and carnosine did not show restorative potential to suppress the levels of AGEs or aSyn/SynT, their restorative potential in modulating protein aggregation was evaluated.

4.3.1 Evaluation of SynT insolubility by Triton-X 100 solubility assay

To evaluate aSyn aggregation status, a Triton X-100 solubility assay was performed. Briefly, non-denatured protein extracts were solubilized in a given amount of Triton X-100 (1%). After a high-speed long centrifugation, soluble protein will remain in the supernatant, while insoluble protein will be retained at the bottom of the tube. Therefore, if the compounds are able to decrease aggregation, aSyn amount in the soluble fraction should be increased, while it should be decrease in the insoluble fraction. In fact, in a preventive paradigm, metformin (16 mM) prevented the aggregation of SynT by ~15% (Figure 4.8 A). In contrast, in a restorative paradigm, metformin (1 mM) did not restore SynT solubility and actually increased its insolubility (Figure 4.8 B). Carnosine, on the other hand, does not seem to modulate SynT aggregation (Figure 4.8).

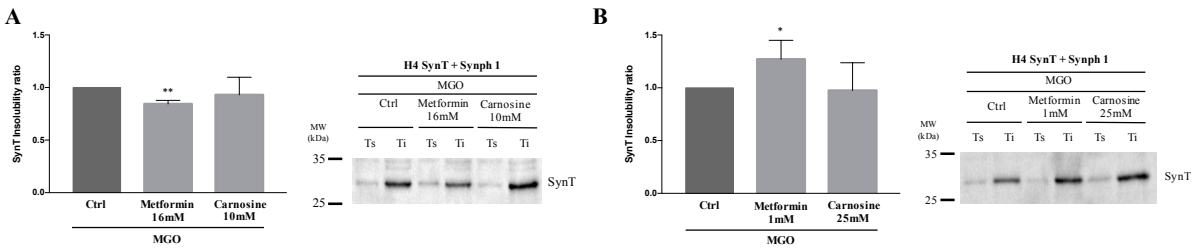

Figure 4.8 Metformin and Carnosine preventive and restorative effect on SynT insolubility.
(A) Cells were transfected with SynT and Synph 1 and treated in a preventive paradigm with vehicle (PBS) (Ctrl), metformin hydrochloride (16 mM), or carnosine (10 mM), followed by a MGO treatment (0.2 mM) (n=3, at least). **(B)** Cells overexpressing SynT and Synph 1 were treated in a restorative paradigm with MGO (Ctrl) (0.2 mM), followed by treatment with vehicle (PBS) (Ctrl), metformin hydrochloride (1 mM), or carnosine (25 mM) (n=3, at least). The ratio between insoluble and soluble fraction are presented as SynT insolubility. Triton X-100 soluble and insoluble (TS and TI) fractions were probed for aSyn. t-test was performed. Data in all panels are average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4.3.2 Evaluation of SynT aggregation by immunocytochemistry

The status of SynT aggregation in the preventive paradigm was also assessed by immunocytochemistry. Briefly, cells co-expressing SynT and Synph 1 were challenged with metformin or carnosine, followed by MGO insult, and probed for aSyn. One should take caution that the data presented is preliminary (n=1).

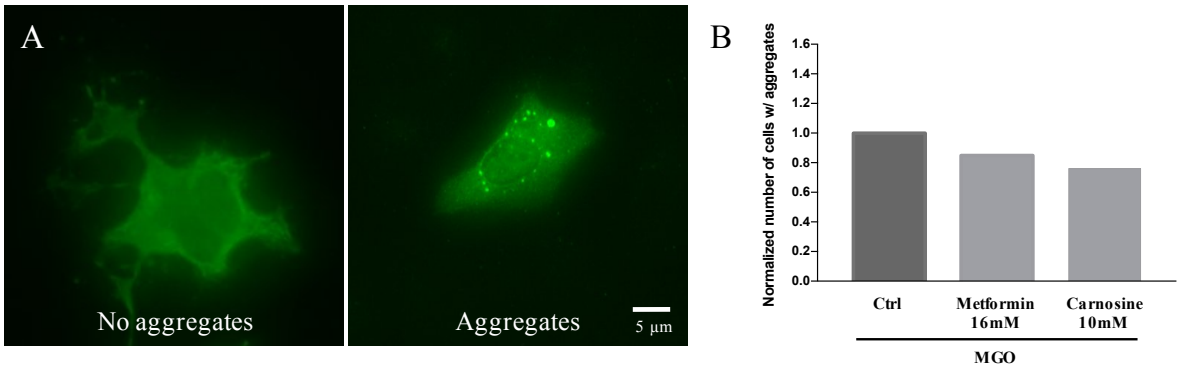

Figure 4.9 Metformin and Carnosine preventive effects on SynT aggregation.
H4 cells co-expressing SynT and Synph 1 were treated in a preventive paradigm with PBS (Ctrl), metformin hydrochloride (16mM) or carnosine (10mM), followed by a MGO treatment (0.2mM). **(A)** Representative images of H4 cells with or without aSyn aggregates. **(B)** The normalized number of cells exhibiting SynT aggregates (to Ctrl) is presented (n=1). Scale bar = 5 µm.

Data obtained by Vicente Miranda and colleagues demonstrated that a MGO-treatment increases the percentage of cells with aggregates (~1.6-fold) [61]. In the present study, cells were also challenged with MGO and preventively treated with the selected active compounds. The preliminary data suggests that metformin or carnosine prevent the formation of aSyn aggregates, since a decreased number of cells displaying SynT aggregates was observed (Figure 4.9). Previous data already indicates that metformin and carnosine are able to decrease not only aSyn/MGO associated cytotoxicity (Figure 4.3), but also AGEs (Figure 4.6 A, B), and aSyn/SynT levels in the case of carnosine (Figure 4.6 A, B). Furthermore, metformin promoted SynT solubility (Figure 4.8A), suggesting it decreases SynT aggregation, in comparison to MGO-treated cells. Thus, the findings presented in Figure 4.9 D are in agreement with the ones obtained in the Triton X-100 solubility assay.

Since this procedure was performed only once, these results should be further explored. aSyn aggregation will also be assessed in a restorative context.

4.4 Preventive activity of Pharmacological compounds in aSyn clearance

Previous findings indicated that MGO-induced glycation impairs the clearance of aSyn [61]. It was previously observed that metformin is preventing overall glycation (Figure 4.6), therefore it is expected that this agent is also able to prevent aSyn glycation, and consequently, stimulate its normal clearance. Thus, despite not observing a decrease in the total levels of aSyn with a metformin treatment, the effects of this compound in modulating aSyn clearance were investigated. To that purpose, a time-chase experiment was performed. Briefly, *de novo* protein synthesis was arrested using cycloheximide and aSyn protein levels were followed as illustrated in Figure 4.10 A. The following presented data is preliminary, since this assay was performed only once.

In agreement with Vicente Miranda and colleagues [61], MGO-glycation impairs aSyn clearance (Figure 4.10 B, C). Notably, metformin is able to prevent MGO effects, promoting aSyn clearance even in comparison to non-MGO treated cells (Figure 4.10 B, C).

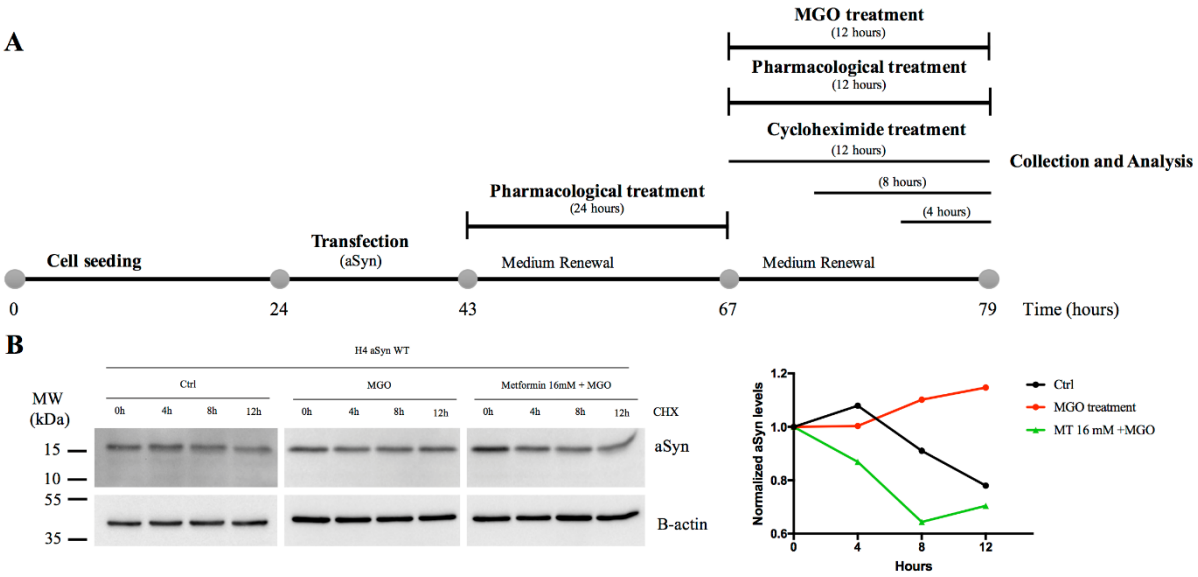

Figure 4.10 Modulation of aSyn clearance by metformin in a MGO-induced glycation context.
(A) Schematic representation of pharmacological compounds preventive potential on the modulation of protein clearance assay. **(B)** H4 cells overexpressing aSyn treated with vehicle (PBS) (Ctrl), or metformin hydrochloride (16mM), followed by MGO insult, and challenged with cycloheximide for the last 0, 4 and 12 hours. Protein extracts were probed for aSyn and β-actin, for normalization (n=1). Normalized aSyn levels are presented (Ctrl, black MGO, red; MGO + metformin hydrochloride, green).

Metformin prevented aSyn/MGO associated cytotoxicity, likely through glycation prevention, since it was observed that it is able to decrease the levels of AGEs levels, in comparison to MGO-treated cells (Figure 4.6 A). These preliminary results suggest that metformin could be promoting aSyn clearance, most likely due to its anti-glycation beneficial effects. It can then be hypothesized that metformin could be preventing aSyn glycation, allowing its successful clearance. Furthermore, metformin is a known AMPK activator [136], which potentiates autophagy [133]. It is plausible that this compound could also potentiate protein autophagic clearance, as observed in the preliminary data shown in Figure 4.10. Surprisingly, the levels of aSyn/SynT are not modulated by metformin (Figure 4.6), although SynT levels do show a non-significant tendency to decrease (Figure 4.6B). One should expect that the total levels of SynT would be lower in comparison to MGO-treated cells. Nevertheless, metformin promoted SynT solubility (Figure 4.8 A). It is plausible that aSyn synthesis should be elevated upon metformin treatment. However, the produced aSyn should be non-toxic, more soluble and with a faster degradation rate. Since these findings are preliminary, this data needs to be further confirmed. mRNA levels and the activity of carnosine should also be evaluated.

5 Concluding remarks

Recent advances in modern medicine are increasing the lifetime expectancy of the world population. However, with ageing, the incidence of ageing-related disorders, such as neurodegenerative diseases, is increasing, representing a significant burden for today's society. PD is the second most common neurodegenerative disorder, and it is characterized by severe motor features, along with cognitive impairment due to the loss of dopaminergic neurons in the *substantia nigra pars compacta.*

Protein glycation was recently suggested to play an important role in several neurodegenerative disorders, such as PD. Upon glycation, protein's structure and function may be altered and, in the case of aSyn, it impairs its clearance and potentiates its aggregation and cytotoxicity [61]. Recently, several

evidences establish a link between diabetes and PD. Glycation is one of the main consequences of hyperglycemia, a critical condition of diabetes, and was described to play a major role in PD, as one of the culprits of aSyn aggregation. MGO is the most potent glycation agent in living cells, promoting the formation of AGEs. In turn, these may potentiate aggregation.

Since glycation could be the missing link underlying the molecular pathogenesis of PD, strategies aimed at increasing glycation defenses might represent novel therapeutic approaches for this disorder. The main purpose of this work was to evaluate the pharmacological potential of several compounds in suppressing the deleterious effects of glycation in cellular models of PD, either in a preventive or restorative manner. The compounds for this study were selected according to their evidenced neuroprotective properties or ability to interfere with AGEs metabolism, by scavenging MGO or by inhibiting glycation.

Metformin and carnosine presented the highest therapeutic potential, both under preventive or restorative paradigm. These findings are in agreement with their pharmacological activity, since they are described as MGO scavengers. Therefore, one could hypothesize that by suppressing MGO, they would prevent the formation of AGEs. On the other hand, the fact that metformin is an AGEs inhibitor and carnosine antioxidant ability might explain their efficacy in a restorative paradigm.

The glycation profile analysis showed that these compounds prevented/restored from increased levels of AGEs, in the aSyn/SynT PD cellular models. Moreover, carnosine also modulates aSyn/SynT protein levels in a preventive manner. Impressively, both compounds were most efficient in modulating AGEs and aSyn levels when the aggregation-prone variant of aSyn, SynT, was expressed. This suggests that metformin and carnosine could be important therapeutic tools in more advanced stages of the disease, where aSyn aggregation already occurred.

Moreover, metformin improved the solubilisation of aSyn and preliminary data suggests that aSyn aggregation is decreased and clearance is potentiated.

Under a restorative paradigm, neither metformin nor carnosine modulated aSyn/SynT levels or SynT insolubility. This may be due to the experimental setup chosen for this analysis. Cells were maintained in culture for a long period of time after MGO-insult, which may be sufficient for them to recover. Therefore, further experiments are required to understand the effects of these compounds in a restorative paradigm.

Although overall glycation was suppressed by metformin and carnosine, it is still not known if they are able to specifically modulate aSyn glycation levels. To that purpose, aSyn immunoprecipitation assays should be performed, and the levels of glycated aSyn measured.

Compounds such as pioglitazone, sulforaphane and resveratrol showed either no cytoprotective effects, or in fact they exacerbated the cytotoxicity induced by aSyn, in the presence or absence of MGO. These findings are surprising, since pioglitazone is a known AGEs cross-link inhibitor, and was expected to prevent aSyn/MGO associated cytotoxicity. Sulforaphane was also expected to reduce cytotoxicity, since it increases Glo1 expression and GSH levels, possibly promoting MGO degradation through the glyoxalase pathway. However, the basal cytotoxicity of this compound was very high, meaning that its inherent toxicity in this model might be hindering any therapeutic potential. Resveratrol is able to enhance autophagy-dependent aSyn degradation, and was expected to be cytoprotective. However, no protective activity was observed.

In future studies, it will be important to perform additional clearance and aggregation assays in the presence of metformin and carnosine, to better understand their protective effects on aSyn. Moreover,

it will be important to evaluate how they are able to modulate aSyn clearance mechanisms, with major focus on autophagy and the proteasome-dependent protein degradation systems. For validation of their therapeutic potential, it would be of great interest to investigate these compounds activity *in vivo*, in a *Drosophila* or a mouse model of PD. Their activity in other aspects of the disease, such as motor performance and cognitive impairment, should also be evaluated. Ultimately, for the case of metformin, in case it is proven to display a high therapeutic potential, its usage for the treatment of PD could be easily translated, since metformin is already approved by the FDA as an anti-diabetic medication.

In conclusion, metformin and carnosine protect against MGO-induced glycation in cellular models of PD (Figure 5.1). Therefore, they may represent a novel pharmacological approach for the treatment of PD, and could also be extended for the treatment of other protein-aggregating diseases.

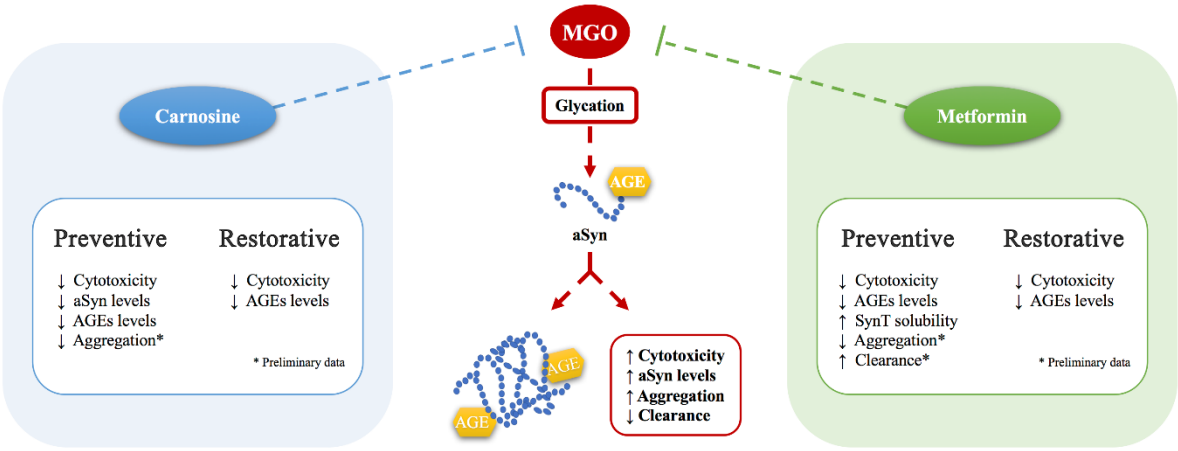

Figure 5.1 Schematic representation of the protective effects of Metformin and Carnosine in PD.
Metformin and carnosine protect against MGO-induced glycation. Carnosine is able to protect from the increase in cytotoxicity, aSyn and AGEs levels, and aSyn aggregation induced by MGO glycation. Metformin can protect from the increase in cytotoxicity, AGEs levels, aggregation, and the decrease in protein clearance promoted by MGO-induced glycation. It is also able to increase SynT solubility. This is possibly due to their MGO-scavenging activity, preventing AGEs formation and their toxic effects on different cellular mechanisms. Abbreviations: MGO: methylglyoxal, AGEs: advanced glycation end-products, aSyn: α-synuclein.

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