Universidade do Minho
Escola de Ciências

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Novel synthetic compounds with pharmacological potential: protection against oxidative stress

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Thank you all…through darkness, you gave me hope…

Novel synthetic compounds with pharmacological potential: protection against oxidative stress

Abstract

Oxidative stress has been described as an imbalance in the equilibrium between oxidants and antioxidants in favour of the former, and has been commonly associated with several pathologies, including neurodegenerative and cardiovascular diseases.

As part of a broader research project aiming the development of drugs useful for the improvement of human health, we studied the pharmacological potential of four novel synthetic nitrogen compounds (FMA4, FMA7, FMA762 and FMA796), on different situations involving oxidative stress.

Experiments using cell-free systems, by the use of DPPH discolouration and 2-deoxy-D-ribose degradation assays, indicated their high antiradical activity, which revealed to be superior for FMA762 and FMA796. The free radical scavenging properties of the nitrogen compounds were confirmed by observing their protective effects on intracellular ROS/RNS formation and on lipid peroxidation, both induced to PC12 cells, with the oxidant pair ascorbate/iron. Their relatively good liposolubility seemed to contribute to their protective role, since it enables them to cross the lipid bilayer and act intracellularly. Moreover, the protective effects of the nitrogen compounds seemed to be mediated by their ability to scavenge free radicals, since they were unable to revert the *t*-BHP-induced decrease in the activity of the natural antioxidant defence systems, such as GSH/GSSG levels, SOD and GPx.

Since oxidative DNA damage is regarded as the type of damage most likely to occur in neuronal cells, we studied the effects of the compounds on the protection and repair of this kind of damage. Simultaneously, a similar study with the polyphenols, luteolin, quercetin and rosmarinic acid, was performed. Using the Comet assay, we observed a protective effect for all the four nitrogen compounds (superior for FMA762 and FMA796) against *t*-BHP-induced strand-breaks formation, as well as an increase in the repair of this kind of damage. In this regard, although the nitrogen compounds showed a smaller protection relatively to luteolin and quercetin, they showed no genotoxicity for longer incubation periods (as described for quercetin) and presented higher increases in strand-breaks repair capacity, which were similar to rosmarinic acid. In addition, at least FMA796 and rosmarinic acid increased the repair of oxidised bases induced with the photosensitiser

compound Ro 19-8022. Furthermore, using the *in vitro* base excision repair assay, we demonstrated that both nitrogen compounds and rosmarinic acid led to an increase in the activity of DNA repair enzymes. Whereas in the case of rosmarinic acid this increase seemed to be mediated by a regulation at the gene expression level, namely of *OGG1*, the positive action of the nitrogen compounds might be attributed to their ROS scavenging activity.

Oxidative stress has also been reported to be involved in several cardiovascular diseases, including atherosclerosis and ischemia/reperfusion injury. So, we evaluated the protective role of the nitrogen compounds on *t*-BHP-induced oxidative injury and apoptosis in H9c2 cells. The synthetic compounds, namely FMA762 and FMA796, decreased *t*-BHP-induced cell death, as measured both by the SRB assay and by quantification of cells showing nuclear chromatin condensation (Hoechst 33342 staining). In addition, evidence is shown for their involvement in the regulation of intracellular signalling mechanisms leading to apoptotic cell death, namely those mediated by mitochondria. In fact they were able to overcome *t*-BHP-induced cellular and mitochondrial morphological changes (triple labelling with TMRM, calcein-AM and Hoechst probes), loss of mitochondrial membrane potential, increased expression of the pro-apoptotic proteins p53, Bax and AIF, and activation of caspases 3 and 9. Moreover, the results obtained indicate that the compounds' ROS scavenging ability plays a crucial role in this protection profile, as a decreased intracellular ROS formation was observed in their presence.

Altogether, the results herein presented reveal the antioxidant potential of novel synthetic compounds, demonstrating their strong ability to act on oxidative stress-mediated injury in two different cell models. In some aspects they also prove to have advantages relatively to some polyphenols from natural origin. In this way, the nitrogen compounds may represent a major breakthrough, as active principles of drugs with pharmacological potential, useful in the treatment of disorders involving oxidative stress, including neurodegenerative and cardiovascular diseases.

Novos compostos sintéticos com potencial farmacológico: protecção contra stresse oxidativo

Resumo

O stresse oxidativo tem sido descrito como um desequilíbrio entre espécies oxidantes e antioxidantes em favor dos oxidantes, e tem sido comummente associado a diversas patologias, incluindo doenças neurodegenerativas e cardiovasculares.

No âmbito de um projecto mais alargado com vista ao desenvolvimento de compostos úteis para o melhoramento da saúde humana testou-se, neste trabalho o potencial farmacológico de quatro compostos azotados sintetizados *de novo* (FMA4, FMA7, FMA762 e FMA796), em situações envolvendo stresse oxidativo.

Experiências efectuadas em sistemas livres de células, usando os testes de descoloração do radical DPPH e da degradação da 2-deoxi-D-ribose, indicaram a sua elevada actividade anti-radicalar, superior para o FMA762 e FMA796. As propriedades de "scavenging" de radicais livres dos compostos azotados foram confirmadas através da observação dos seus efeitos protectores na formação de ROS/RNS intracelulares e na peroxidação lipídica, ambas induzidas pelo par oxidante ascorbat/ferro em células PC12. Os valores de lipossolubilidade encontrados parecem contribuir para o seu papel protector, uma vez que são indicativos que os compostos atravessam a membrana lipídica, sendo capazes de actuar intracelularmente. Além disso, os efeitos protectores dos compostos pareceram ser mediados pela sua capacidade de complexação de radicais livres, uma vez que os mesmos foram incapazes de reverter a diminuição na actividade dos sistemas naturais de defesa antioxidante, tais como os níveis de GSH/GSSG, SOD e GPx, induzida por *t*-BHP.

Uma vez que os danos oxidativos no DNA são vistos como o tipo de dano mais provável de ocorrer em células neuronais, estudaram-se os efeitos dos compostos na protecção e reparação deste tipo de dano. Simultaneamente, realizou-se um estudo similar com os polifenóis luteolina, quercetina e ácido rosmarínico. Usando o ensaio de Cometa, observou-se um efeito protector para todos os quatro compostos (superior para o FMA762 e FMA796) na formação de quebras de DNA induzidas por *t*-BHP, assim como um aumento na reparação deste tipo de dano. Embora os compostos azotados tenham apresentado uma menor protecção relativamente à luteolina e à quercetina, eles não demonstraram genotoxicidade para períodos de

incubação mais longos (como descrito para a quercetina) e apresentaram maior capacidade de reparação de quebras no DNA, de forma semelhante ao ácido rosmarínico. Além disso, pelo menos o FMA796 e o ácido rosmarínico levaram ao aumento da reparação de bases oxidadas induzidas com o composto fotosensibilizador Ro 19-80222. Por seu turno, usando o ensaio *in vitro* de reparação de excisão de bases demonstrou-se também que ambos os compostos azotados e o ácido rosmarínico levam a um aumento da actividade das enzimas de reparação de DNA. Enquanto que para o ác. rosmarínico esse aumento parece ser mediado por uma regulação a nível da expressão de genes, nomeadamente o *OGG1*, os efeitos benéficos dos compostos azotados poderão ser atribuídos apenas à sua capacidade de "scavenging" de ROS.

O envolvimento do stresse oxidativo em doenças cardiovasculares, incluindo aterosclerose e isquémia/reperfusão, tem sido igualmente descrito. Sendo assim, avaliou-se o papel protector dos compostos azotados nos danos oxidativos e em apoptose induzida por *t*-BHP em células H9c2. Os compostos sintéticos, nomeadamente o FMA762 e FMA796, diminuíram a morte celular induzida por *t*-BHP, tal como determinado pelo ensaio de SRB e pela quantificação de células com condensação de cromatina (marcação nuclear com Hoechst 33342). Além disso, obtivemos evidência para o seu envolvimento na regulação de mecanismos de sinalização intracelular conducentes a apoptose, particularmente os mediados pela mitocôndria. De facto, os compostos mostraram capacidade para reverter alterações morfológicas a nível celular e mitocondrial (marcação tripla com TMRM, calceína-AM e Hoechst), perda de potencial de membrana mitocondrial, aumento de expressão das proteínas pró-apoptóticas p53, Bax e AIF, e activação das caspases 3 e 9, eventos estes induzidos por *t*-BHP. Além disso, os resultados obtidos são indicativos de que a capacidade de "scavenging" de ROS parece desempenhar um papel crucial no perfil de protecção dos compostos, uma vez que na sua presença se observou uma diminuição na formação de ROS intracelulares.

No seu conjunto, os resultados aqui apresentados revelam o potencial antioxidante dos novos compostos sintéticos, demonstrando a sua forte capacidade para actuar em danos mediados por stresse oxidativo em dois diferentes modelos celulares. Nalguns aspectos os compostos sintéticos provaram ter vantagens relativamente a alguns polifenóis de origem natural. Deste modo, os novos compostos nitrogenados poderão representar uma enorme inovação como princípios activos de fármacos úteis no tratamento de várias patologias envolvendo stresse oxidativo, incluindo doenças neurodegenerativas e cardiovasculares.

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Abbreviations

8-oxo-Gua – 8-oxo-7,8-dihydroguanine
AIF – Apoptosis-inducing factor
AP – Abasic (apurinic/apyrimidinic)
APE1 - Apurinic/apyrimidinic endonuclease
BER – Base excision repair
BSA – Bovine serum albumin
CCCP – carbonyl cyanide *m*-chloro phenyl hydrazone
DCF – 2'-7'-dichlorofluorescein
$\Delta\psi$ – transmembrane electric potential
DMSO – Dimethylsulphoxide
DPPH – 1,1-diphenyl-2-picrylhydrazyl
FPG - Formamidopyrimidine DNA glycosylase
GSH – Glutathione (reduced form)
GSH-Px – Glutathione peroxidase
GSSG – Glutathione (oxidised form)
IC_{50} – Concentration needed to reduce the initial response in 50 %
LDH – Lactate dehydrogenase
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH – Nicotinamide adenine dinucleotide (reduced form)
NBT – NitroBlue Tetrazolium
$^{\bullet}NO$ – Nitric oxide radical
NOS – Nitric oxide synthase
OGG1 - 8-oxoganine DNA glycosylase 1
$^{\bullet}OH$ – Hydroxyl radical
$O_2^{\bullet -}$ – Superoxide radical
PBS – Phosphate buffer saline
PMS – Phenazine methosulfate
RNS – Reactive nitrogen species
ROS – Reactive oxygen species
SNP – Sodium nitroprusside
SOD – Superoxide dismutase
SRB – Sulforhodamine B
TBARS – Thiobarbituric acid reactive substances
t-BHP – *tert*-Butyl hydroperoxide
TMRM – Tetramethyl rodhamine methyl ester

“Living with the risk of oxidative stress is a price that aerobic organisms must pay for more efficient bioenergetics.”

V. P. Skulachev

CHAPTER: 1

GENERAL INTRODUCTION

1.1. Oxidative stress

There is a general agreement among the scientific community that significant amounts of oxygen (O_2) first appeared in the Earth's atmosphere about 2.5 billion years ago. However, the toxicity of oxygen led the existing anaerobic organisms to adapt to the new environment by developing defence mechanisms, in order to protect themselves and survive (Wilcox *et al.*, 2004). Though some of them chose to live in an oxygen-free micro-environment, others became capable of performing both respiration and fermentation (facultative anaerobes). Only later on there was the development of new species that used respiration in an exclusive way, the aerobic organisms (Halliwell and Gutteridge, 1999; Behl, 1999; Limón-Pacheco and Gonsebatt, 2008).

Initially, O_2 toxicity was thought to be due to the inactivation of enzymes, mainly the thiol group of cysteine residues. Later, toxicity was also attributed to the effects of hydrogen peroxide (H_2O_2). Ultimately, molecular biology techniques established that the toxic effects of O_2 are directly linked to its partially reduced forms, the reactive oxygen species (ROS), acting on cellular components (Cemeli *et al.*, 2008). More than 50 years ago, Denham Harman, in his "Free Radical Theory of Aging", described these reactive species as being responsible for the most part of cellular damage and as playing a key role in the aging process (*in* Valko *et al.*, 2007). The discovery of the enzyme superoxide dismutase (SOD) in 1969 by McCord and Fridovich (McCord and Fridovich, 1969) definitively convinced the rest of the scientific community of the importance of free radicals in living systems. This led to further investigation in this field, which resulted in the finding that these free radicals also intervene in the regulation of intracellular signalling. So, as living systems evolved, they have not only adapted to a coexistence with free radicals but have developed various defence mechanisms to protect themselves against the toxicity of oxygen (*in* Droge, 2002; Valko *et al.*, 2007).

In normal physiological conditions, the production of free radicals and other reactive species is maintained in an equilibrium state by this antioxidant defence system (Halliwell and Gutteridge, 1999). However, when an imbalance occurs between oxidants and antioxidants in favour of the oxidants, we come to a situation defined as oxidative stress (Sies, 1997; Nordberg and Arner, 2001). This can result from two different factors: a decrease in antioxidants, due to mutations affecting antioxidant enzymes or depletion of antioxidants along with other essential diet

constituents; or an excessive production of oxygen and nitrogen reactive species, for example, by exposure to high levels of O_2, by the presence of toxins that are metabolised to produce ROS, among others (Halliwell and Gutteridge, 1999).

Oxidative stress can cause damage to all biomolecules and ultimately lead to cell death, being implicated in the etiology of several pathologies, such as atherosclerosis (Uemura *et al.*, 2002), neurodegenerative diseases (Halliwell, 2001; Jang *et al.*, 2004), and ischemia-reperfusion injury (Aikawa *et al.*, 1997; He *et al.*, 2007).

1.1.1. Formation of reactive species

Intracellular accumulation of reactive oxygen (ROS) and nitrogen (RNS) species can be triggered by both exogenous and/or endogenous factors (Fleury *et al.*, 2002), as summarised in Figure 1.1.

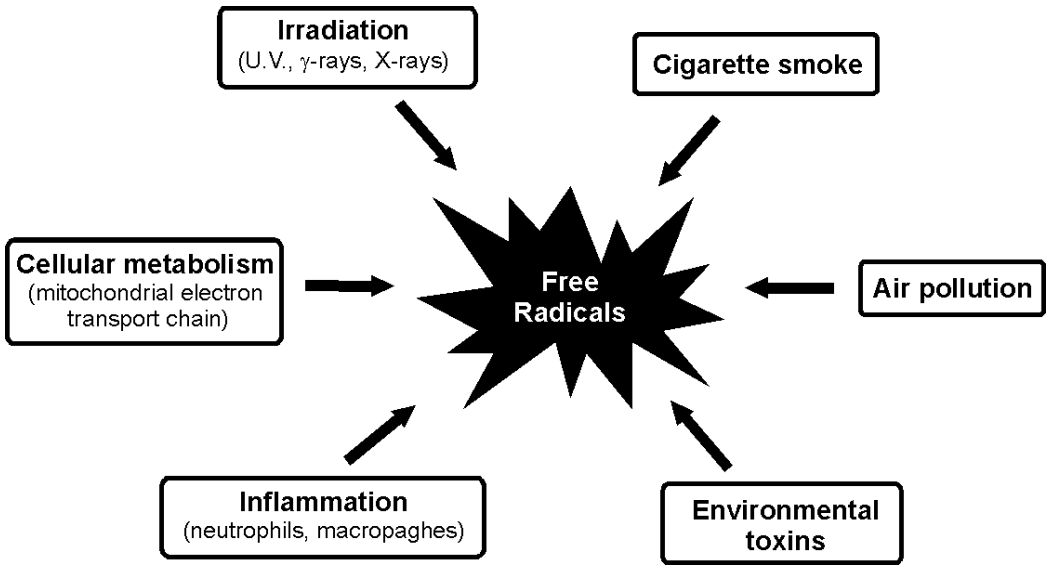


Figure 1.1 – Summary of exogenous and endogenous sources of free radicals. Adapted from Stadtman (2004) and Wilcox *et al.* (2004).

Mitochondria reduce to water over 95% of all the oxygen used by the cell, mostly due to the presence of an electron transport chain (ETC). However, a small percentage of the total oxygen escapes resulting in the formation of superoxide radicals, and subsequently other reactive species, like hydrogen peroxide and hydroxyl radicals (Cadenas and Davies, 2000; Somayajulu *et al.*, 2005). In fact,

these organelles are packed with several redox carriers that can potentially leak single electrons to oxygen and convert it into superoxide anion, which, by its turn, leads to the subsequential formation of other ROS. Given the moderate redox potential of the superoxide/dioxygen ($O_2^{\bullet -}$ / O_2) couple ($E_{1/2}$ = - 0.16 V), the reaction of one-electron reduction of oxygen is thermodynamically favourable for numerous mitochondrial oxidoreductases (*in* Andreyev *et al.*, 2005). Considering that superoxide is effectively removed from the reaction by some detoxifying enzymes, like superoxide dismutase, and the possibility of highly reduced state of many redox carriers, the reaction becomes virtually irreversible. So, which of the electron carriers do become the sites of ROS production is kinetically controlled (Turrens, 2003; Andreyev *et al.*, 2005). Some examples of these carriers include the cytochrome *b*5 reductase and the monoamine oxidases (located in the outer mitochondrial membrane), α-glycerophosphate dehydrogenase (outer surface of the inner mitochondrial membrane), succinate dehydrogenase (complex II, situated at the inner surface of the inner membrane), aconitase (in the mitochondrial matrix) and the α-ketoglutarate dehydrogenase complex (associated with the matrix side of the inner membrane). Nevertheless, the majority of ROS are produced in complexes I and III of the mitochondrial respiratory chain (Jezek and Hlavata, 2005). Best understood is the Complex III (ubiquinol-cytochrome *c* reductase) contribution to superoxide generation. Within this complex, the transfer of electrons from ubiquinol (UQH_2) to cytochrome *c* proceeds through a set of reactions known as the Q-cycle. However, during this process, the one-electron reduction of oxygen by the ubisemiquinone anion radical ($UQ^{\bullet -}$), an unstable intermediary of the Q-cycle, may lead to the formation of superoxide, in both the inner and outer sides of the inner mitochondrial membrane (Andreyev *et al.*, 2005; Jezek and Hlavata, 2005).

Complex I (NADH dehydrogenase, also named NADH-ubiquinone oxidoreductase), appears to be the primary source of superoxide formation in the brain, under normal conditions (Barja, 1999; Turrens, 2003). It oxidises NADH using ubiquinone as an electron acceptor in a reversible reaction coupled with a proton pump generating transmembrane potential. To date, the increase in ROS production in this complex has been observed in three different situations: during normally functioning respiratory chain, in the presence of rotenone (an inhibitor that blocks the transfer of electrons from complex I to ubiquinone) and during reverse electron transfer (a set of reactions in the respiratory chain that allow electrons to be transferred against the gradient of redox potentials of electron carriers, from reduced

ubiquinone to NAD^+, instead of oxygen. The main sites of ROS production in mitochondria are summarised in Figure 1.2.

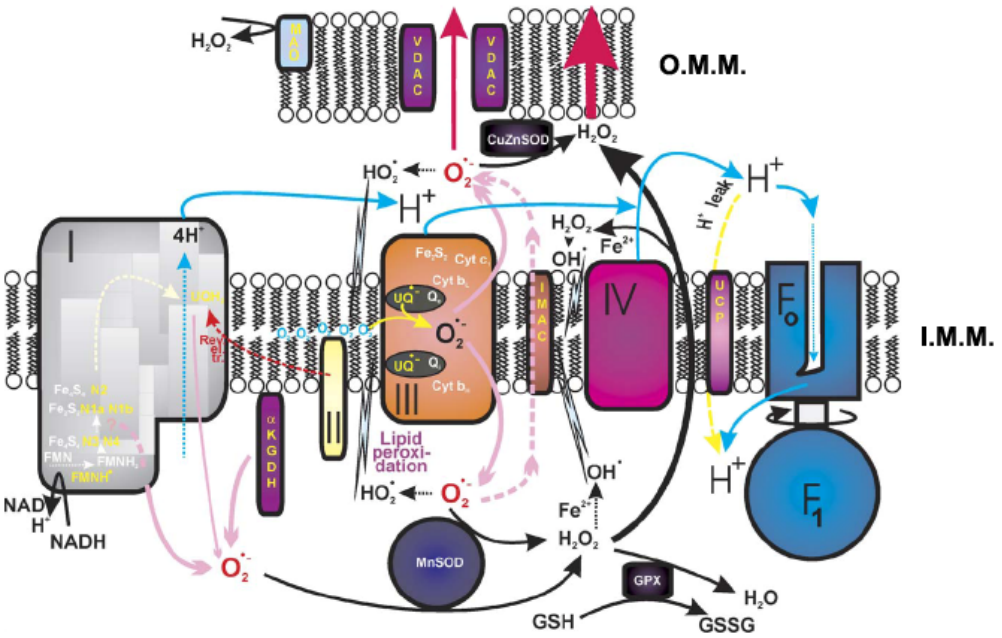


Figure 1.2 – Major sources of ROS in mitochondria and their respective fluxes. The majority of ROS production occurs at complexes I and III. Many of these species are removed by antioxidant enzymes present in the matrix, such as superoxide dismutase (MnSOD and CuZnSOD) and glutathione peroxidase (GPx). However, the main contribution of mitochondria to cytosolic ROS is due to the hydrogen peroxide that escapes degradation by GPx in the matrix, and residual $O_2^{\bullet -}$, which can enter the cytosol via the porin VDAC. O.M.M. – outer mitochondrial membrane; I.M.M. – inner mitochondrial membrane. Adapted from Jezek and Hlavata (2005).

Besides mitochondria, there are other endogenous sources of ROS. For example xanthine oxidase, a widely distributed enzyme in the tissues of mammals, during the catalisation of the reaction of hypoxanthine to xanthine and xanthine to uric acid, leads to the reduction of molecular oxygen, forming the superoxide anion in the first step and hydrogen peroxide in the second one (Valko *et al.*, 2004). Additional sources of cellular ROS are inflammatory cell activation (neutrophils, eosinophils and in particular macrophages) (Conner and Grisham, 1996), cytochrome P450 metabolism (namely following the breakdown or uncoupling of the P450 catalytic cycle) (Moon *et al.*, 2006) and peroxisomes, which mainly lead to the production of H_2O_2 (Gupta *et al.*, 1997).

1.1.2. Chemistry and biochemistry of ROS/RNS

The most common intracellular forms of reactive oxygen and nitrogen species include radical ($O_2^{\bullet -}$, $^{\bullet}OH$, $^{\bullet}NO$, among others), as well as non-radical (O_2, $ONOO^-$, H_2O_2, HOCl, O_3) moieties, that can be deleterious to cells (Butterfield *et al.*, 1999).

Free radicals can be defined as molecules or molecular fragments, capable of independent existence, which contain one or more unpaired electrons. The presence of these unpaired electrons confers them a considerable degree of reactivity, since they need another electron to fill the orbital and become stable (Wilcox *et al.*, 2004; Valko *et al.*, 2006). Free radicals are often reactive species, although the opposite is not always true. Hydrogen peroxide (H_2O_2), for example, although considered a reactive species, is not regarded as a free radical since it has no unpaired electrons in its structure (Nordberg and Arner, 2001). On the other hand, molecular oxygen (dioxygen, O_2) has a unique electronic configuration and is considered a reactive species. In the ground state, it is sometimes viewed as a bi-radical, with two parallel unpaired electrons in antibonding π* orbitals, thus forming a triplet state molecule (Valko *et al.*, 2004).

The addition of one electron to molecular oxygen forms the superoxide anion radical ($O_2^{\bullet -}$), which, despite being a free radical, is not highly reactive, since it lacks the ability to cross lipid membranes. In this way, it stays enclosed in the compartment where it is generated (Nordberg and Arner, 2001). It is considered the "primary" ROS, from which "secondary" ROS can be generated (Valko *et al.*, 2006), and arises mainly through metabolic processes. Among them is the electron transport chain in the mitochondria, as previously described, but it can also be produced by flavoenzymes (*e. g.* xanthine oxidase), lipoxygenases and ciclooxygenases. In addition, the activity of the NADPH-dependent oxidase of phagocytic cells, a membrane-associated enzyme complex, constitutes an example of a high production of superoxide anion. In the presence of superoxide dismutase, two molecules of this radical can easily be transformed to more effective species: hydrogen peroxide and molecular oxygen (Nordberg and Arner, 2001; Spiteller, 2001).

Singlet oxygen is also a very reactive ROS that induces various genotoxic, carcinogenic, and mutagenic effects through its action on polyunsaturated fatty acids (PUFAs) and DNA (Cui *et al.*, 2004).

Hydrogen peroxide (H_2O_2) is not a free radical. Nonetheless, is highly important, much because of its ability to penetrate biological membranes. It plays a radical

forming role as an intermediate in the production of more reactive ROS molecules including HOCl (hypochlorous acid) by the action of myeloperoxidase, an enzyme present in the phagosomes of neutrophils and, most importantly, formation of the hydroxyl radical (Reaction 2) via oxidation of transition metals (Nordberg and Arner, 2001; Willcox *et al.*, 2004). Additionally, it can be directly produced by several enzymes, such as xanthine oxidase and glycollate oxidase (Halliwell, 2001). Once produced by the above mentioned mechanisms, H_2O_2 is removed by at least three antioxidant enzyme systems, namely catalases, glutathione peroxidases, and peroxiredoxins (Nordberg and Arner, 2001).

The hydroxyl radical ($^{\bullet}OH$) is highly reactive, being able to attack and damage all biomolecules: carbohydrates, lipids, proteins and DNA (Halliwell, 2001). Since it has a half-life in aqueous solution of less than 1 nanosecond, when produced *in vivo*, this radical reacts close to its site of formation. Production of $^{\bullet}OH$ close to DNA could lead to this radical reacting with DNA bases or the deoxyribosyl backbone of DNA to produce damaged bases or strand breaks (Valko *et al.*, 2006). It can be generated through several mechanisms, including ionising radiation and photolytic decomposition of alkylhydroperoxides, but the majority of the hydroxyl radicals produced *in vivo* comes from the metal catalysed (mainly involving iron and copper) breakdown of hydrogen peroxide, according to the Fenton reaction (Reaction 1.1) (Nordberg and Arner, 2001; Valko *et al.*, 2004):

$$H_2O_2 + Cu^{+}/\ Fe^{2+} \rightarrow {}^{\bullet}OH + OH^{-} + Cu^{2+}/Fe^{3+} \qquad \text{(Reaction 1.1)}$$

Superoxide also plays an important role in connection with Reaction 1.1 by recycling the metal ions (Reaction 1.2):

$$Cu^{2+}/Fe^{3+} + O_2^{\bullet -} \rightarrow Cu^{+}/\ Fe^{2+} + O_2 \qquad \text{(Reaction 1.2)}$$

The sum of Reactions 1 and 2 is the Haber-Weiss reaction (Reaction 1.3):

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe/Cu} {}^{\bullet}OH + OH^{-} + O_2 \qquad \text{(Reaction 1.3)}$$

Transition metals thus play an important role in the formation of various free radicals, as is the case of hydroxyl radicals. The redox state of the cell is largely linked to an iron (and sometimes copper) redox couple and is maintained within strict physiological limits. In fact, iron, for example, is required by the human body

for the synthesis of several enzymes and its adequate supply during early life is essential for normal brain development (Halliwell, 2001; Mwanjewe *et al.*, 2001). However, its ability to transfer single electrons as it oscillates between the ferrous and ferric states makes it a powerful catalyst of free radical reactions (Halliwell and Gutteridge, 1999). These transition metals can be released from proteins like ferritin and the [4Fe-4S] center of different enzymes of the dehydratase-lyase family, by reactions with the superoxide anion (Levi *et al.*, 1996; Nordberg and Arner, 2001).

Nitric oxide ($^{\bullet}NO$) is synthesised from L-arginine by three isoforms of nitric oxide synthase (NOS), neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) (Beal, 2002).

Like superoxide anion, $^{\bullet}NO$ does not readily react with most biomolecules, despite its unpaired electron. On the other hand, it easily reacts with other free radicals (*e.g.* peroxyl and alkyl radicals), generating mainly less reactive molecules (Rubbo *et al.*, 2000), thus in fact functioning as a free radical scavenger (Nordberg and Arner, 2001). In addition, nitric oxide seems to be also involved in neurotransmission, regulation of vascular relaxation and in inflammatory processes (Beal, 2002; Antunes *et al.*, 2005). However, it can react with the superoxide anion, yielding peroxynitrite ($ONOO^-$). This compound has the capacity to act in a hydroxyl radical-like manner to induce lipid and protein oxidation, readily reacts with CO_2 to form nitroso peroxocarboxylate ($ONOOCO_2^-$), can become protonated as peroxonitrous acid (ONOOH) and undergo homolysis to form either $^{\bullet}OH$ and $^{\bullet}NO_2$, or be rearranged to nitrate (NO_3) (Radi *et al.*, 2001). In addition, peroxynitrite is known to play a key role in neuronal damage associated with excitotoxicity (Barañano and Snyder, 2001).

Other reactive species derived from oxygen that can be formed in living systems include peroxyl radicals ($ROO^{\bullet}$). The simplest peroxyl radical is $HOO^{\bullet}$, which is the protonated form of superoxide and is usually termed either hydroperoxyl radical or perhydroxyl radical. Its involvement in the initiation of lipid peroxidation has already been demonstrated (Valko *et al.*, 2007).

1.1.3. Physiological functions of reactive species

Reactive species are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems (Valko *et al.*, 2006). These species are maintained at low, but measurable, concentrations in the cells, through a balance between their rates of production and their rates of removal by

antioxidants. Thus, each cell is characterised by a particular concentration of electrons (redox state) stored in many cellular constituents, and the redox state of a cell and its oscillation determines cellular functioning. A temporary shift of the intracellular redox state towards more oxidising conditions results in a temporary imbalance that represents the physiological basis for redox regulation (Schafer and Buettner, 2001).

A great number of physiological functions are controlled by redox-responsive signalling pathways (Droge, 2002). Several evidences suggest that ROS participate in the defence against intrusion of foreign bodies (Babior *et al.*, 2003). Activated neutrophils and macrophages produce large quantities of ROS via the phagocytic isoform of NAD(P)H oxidase, in order to kill the pathogens. This massive production of ROS during an inflammatory event is called “oxidative burst” and plays an important role as the first line of defence against environmental pathogens (Nordberg and Arner, 2001; Valko *et al.*, 2007). At a smaller scale, some types of non-phagocytic cells, like fibroblasts, vascular smooth muscle cells, cardiac myocytes and endothelial cells, are also known to produce ROS by NAD(P)H oxidase to regulate intracellular signalling cascades. Thus, ROS play an important role in the regulation of cardiac and vascular cell functioning (Griendling *et al.*, 2000).

Nitric oxide plays several regulatory functions. In fact, its own production by iNOS, the only isoform of NOS that is not constitutively expressed, is regulated at the transcriptional and post-transcriptional levels by signalling pathways involving redox-dependent transcription factor NF-κB or mitogen-activated protein kinases (MAPKs). In addition, $^{\bullet}NO$, in combination with H_2O_2, leads to the activation of the enzyme soluble guanylate cyclase (sGC), which catalyses the formation of cyclic guanosine monophosphate (cGMP) that, by its turn, is used as an intracellular amplifier and second messenger in a variety of physiological responses, such as modulation of protein kinases, ion channels, smooth muscle tone and inhibition of platelet adhesion (Droge, 2002; Martinez-Ruiz and Lamas, 2004).

In higher organisms, oxygen homeostasis is maintained by a tight regulation of the red blood cell mass and respiratory ventilation. It has been proposed that changes in oxygen concentration are sensed independently by several different ROS-producing proteins, including a *b*-type cytochrome. Other studies also suggested that a change in the rate of mitochondrial ROS may play a role in this oxygen sensing by the carotid bodies, which are sensory organs that detect alterations in arterial blood oxygen. Other responses to changes in oxygen pressure

include the regulated production of certain hormones (*e.g.* erythropoietin) controlled by the transcription hypoxia inducible factor-1 (HIF-1) (Zhu and Bunn, 1999; Semenza, 2000).

ROS also seem to be involved in cell adhesion, a mechanism that plays an important role in embryogenesis, cell growth, differentiation, wound repair, among other processes. The expression of cell adhesion molecules is stimulated by bacterial lipopolysaccharides and by various cytokines such as TNF, interleukin-1a, and interleukin-1b. The adherence of leukocytes to endothelial cells is also induced by ROS. Moreover, the oxidant-induced adherence of neutrophils is inhibited by hydroxyl radical scavengers or iron chelators, suggesting that the induction of adherence may be mediated by hydroxyl radicals generated from hydrogen peroxide within the cell (Sellak *et al.*, 1994; Droge, 2002).

Reactive oxygen and nitrogen species can directly affect the conformation and/or activities of all sulfhydryl-containing molecules, such as proteins or glutathione (GSH), by oxidation of their thiol moiety. This type of redox regulation affects many proteins important for signal transduction and carcinogenesis, such as protein kinase C, Ca^{2+}-ATPase, collagenase and tyrosine kinases, among many other enzymes and membrane receptors (Dalton *et al.*, 1999). In addition, ROS/RNS are known to trigger apoptotic cell death, by causing Bcl-2 (a protein located in the outer membranes of mitochondria) to activate a related protein, Bax, which by its turn leads to the release of cytochrome *c* from mitochondria (Cai and Jones, 1999; Fleury *et al.*, 2002; Montiel-Duarte *et al.*, 2004; Nunes *et al.*, 2008). This release then results in the activation of several other proteins, in a process that will be discussed further on in this chapter (section 1.4.3.).

1.2. Antioxidants as protectors of oxidative stress

The excess of ROS is generally inactivated by endogenous and/or exogenous antioxidant molecules (Cui *et al.*, 2004).

Antioxidant is a term widely used, but difficult to define clearly. According to some authors, a generic definition should be associated with the notion of the ROS/RNS that have to be neutralised, as well as the target of damage that is measured (Halliwell and Gutteridge, 1999; Azzi *et al.*, 2004). In fact, it is quite natural that an antioxidant gives protection in one system, but fails to protect, and sometimes even cause damage, in others (Halliwell *et al.*, 1995). Taken this into account, an antioxidant could be defined as “any substance that, when present at

low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate" (Sies, 1997; Halliwell and Gutteridge, 1999). However, some authors have stated that the concept of an antioxidant *in vitro* should not be extended to cells, organs, animals or populations until the evidence has been obtained, since a molecule demonstrated to have antioxidant properties *in vitro* might have additional properties in a more complex system (Azzi *et al.*, 2004).

A compound might exert antioxidant actions *in vivo* by inhibiting the generation of ROS, by directly scavenging free radicals or by removing or lowering the local concentrations of metal ions, which catalyse oxidation. Additionally, it might also act by enhancing the endogenous antioxidant defences (*e.g.* by upregulating the expression of the genes encoding for the cells' natural antioxidant enzymes) (Halliwell *et al.*, 1995; Cui *et al.*, 2004). Some examples of antioxidants are discussed below.

1.2.1. Cellular antioxidant defences

The balance between the physiological production of ROS and their detoxification is maintained in cells by effective enzymatic and non-enzymatic antioxidant systems (Behl and Moosmann, 2002), as summarised in Figure 1.3.

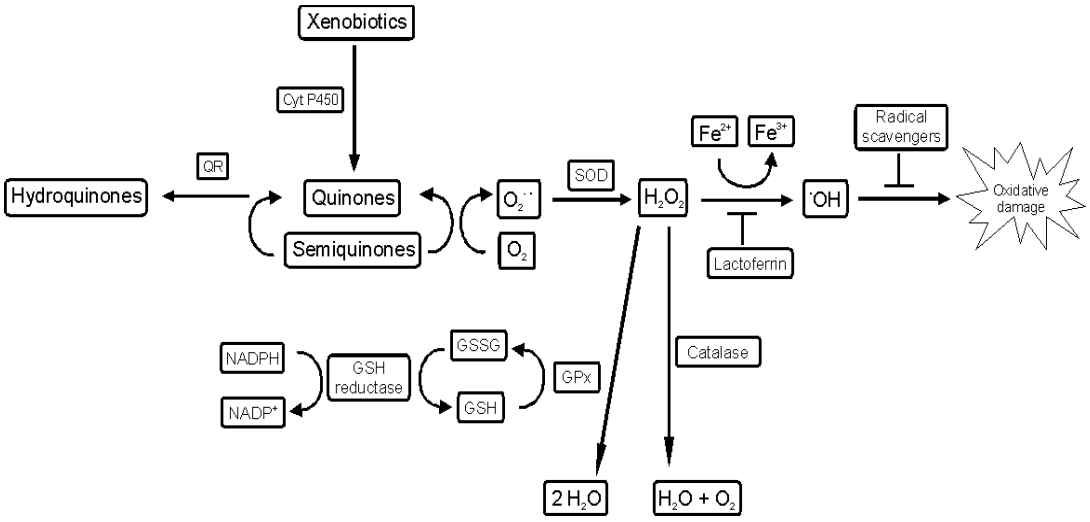


Figure 1.3 – Formation of reactive oxygen species and some of the endogenous antioxidant defence mechanisms. Abbreviations: SOD - superoxide dismutase; GSH – reduced glutathione; GSSG - oxidised glutathione; GPx - glutathione peroxidase; QR – quinone reductase. Adapted from Butts *et al.* (2008).

As a first line of defence, preventive antioxidants act by binding to and sequestering promoters of oxidation and transition metal ions, like iron and copper, which contain unpaired electrons and strongly accelerate the formation of free radicals. Some examples of preventive antioxidants are transferrin, lactoferrin, ceruloplasmin, haptoglobins, hemopexin and albumin (Cui *et al.*, 2004; Bitto *et al.*, 2007). This class of preventive antioxidants also comprises the enzymatic antioxidant defences, like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These enzymes act on specific ROS following their formation and degrade them to less harmful products (Willcox *et al.*, 2004; Cui *et al.*, 2004).

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase. This enzyme, whose activity was firstly proven in 1969 (McCord and Fridovich, 1969), catalyses the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. The H_2O_2 formed in this reaction is then destroyed by the action of catalases and glutathione peroxidises. In humans, there are three forms of SOD: the cytosolic Cu/Zn-SOD, which contains copper and zinc in its active centre, the mitochondrial Mn-SOD (that contains manganese in the active site) and the extracellular SOD (EC-SOD) (Buettner *et al.*, 2006).

Catalase is a heme-containing enzyme, mainly localised in the peroxisomes of mammalian cells, which decomposes hydrogen peroxide to water and molecular oxygen. Thus, it lowers the risk of $^{\bullet}OH$ formation from H_2O_2 via the Fenton reaction (Reaction 1, in page 9). In addition, catalase is involved in the detoxification of other substrates that work as H^+ donors, such as phenols and alcohols (Matés, 2000; Nordberg and Arner, 2001).

The selenium-containing peroxidases, of which glutathione peroxidase may be considered the most important example, catalyse the reduction of a variety of hydroperoxides (H_2O_2 or ROOH) in the presence of the reduced form of glutathione (GSH) (Matés, 2000). This enzyme (for which at least four different mammalian forms are known), oxidises two molecules of GSH to GSSG (the oxidised form of glutathione), which can subsequently be reduced to GSH again by glutathione reductase (Nordberg and Arner, 2001).

In addition to these enzymatic defences, cells possess low molecular mass agents that also exert an antioxidant protection, by removing or degrading ROS to less harmful products, acting as a second line of defence. These include, among others, molecules like glutathione, thioredoxin and coenzyme Q (Halliwell and Gutteridge, 1999; Cui *et al.*, 2004).

The tripeptide glutathione exists either in the reduced (GSH) or oxidised (GSSG) form and is ubiquitously present in all cells (distributed between the nucleus, endoplasmic reticulum and mitochondria). Its main functions include: restoration of oxidised protein sulphydryls; detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds either directly (via GPx-catalysed reactions) or indirectly (through glutathione S-transferases-catalysed conjugation reactions); transport of amino acids through the plasma membrane; direct scavenging of hydroxyl radical and singlet oxygen; regeneration of some important antioxidants, like vitamins C and E, to their active forms; and protection of brain tissue from oxidative stress (Nordberg and Arner, 2001; Drake *et al.*, 2003; Masella *et al.*, 2005; Valko *et al.*, 2006). Along with GSH, thioredoxin, a small and multifunctional disulphide-containing polypeptide, is also important for the maintenance of cellular thiol homeostasis, contributing to the total antioxidant protection (Lillig *et al.*, 2004; Baty *et al.*, 2005). It mainly functions as a general protein disulfide reductant, but is also able to provide control over several transcription factors that affect cell proliferation and death (Nordberg and Arner, 2001; Dobra and Hjerpe, 2008).

Coenzyme Q (CoQ), or ubiquinone, is a ubiquitous electron and proton carrier, playing an important role in the mitochondrial electron-transport chain. Its reduced form, ubiquinol, can function as an antioxidant, preventing lipid peroxidation by reduction of peroxyl radicals and is also able to interact with α-tocopheroxyl radical, thus regenerating endogenous vitamin E within the lipid membrane (Shapiro and Saliou, 2001).

Other cellular low molecular mass agents, including bilirubin (Barañano and Snyder, 2001), lipoic acid (Vanasco *et al.*, 2008), melatonin (Korkmaz *et al.*, 2009) and uric acid (Cherubini *et al.*, 2005), have been reported to help cells in the removal of free radicals.

Since the processes of prevention and removal of reactive species are not completely effective, damage still accumulates in biomolecules (Sies, 1997). So, adding to the several types of antioxidants mentioned above, cells protect their critical structures by other mechanisms, such as repair and *de novo* enzymes. These act as the last line of defence, by repairing or eliminating damaged molecules, reconstituting them, and even by clearing the toxic and waste products (Niki, 2000; Cui *et al.*, 2004). This type of antioxidants includes heat shock proteins, DNA repair enzymes, proteases, lipases and transferases (Wilcox *et al.*, 2004).

1.2.2. Antioxidant protection by compounds derived from the diet

Several compounds present in plants and vegetables have been suggested to have the ability of reacting with free radicals, without generating further radicals. Other compounds become oxidised after scavenging ROS, and thus need to be regenerated for further use (Masella *et al.*, 2005).

Vitamins are considered to be antioxidants of major biological importance. Ascorbic acid (vitamin C) is a water-soluble antioxidant, commonly found in relatively high amounts in fruits and vegetables (Arrigoni and de Tullio, 2002). It effectively scavenges several types of ROS/RNS *in vitro*, resulting in the formation of an ascorbyl radical, which can be further oxidised to dehydroascorbate. This molecule can then be regenerated, through the cellular reducing molecules of the cell, to ascorbate. In addition, ascorbic acid can regenerate other small antioxidant molecules from their respective radical species (such as α-tocopherol or GSH). Moreover, it can function as a cofactor for many enzymes, by maintaining the metal ions (such as iron and copper) present in the active centre of those enzymes, in a reduced state. However, the reduction of iron and copper by ascorbate can also result in a pro-oxidant effect, since these ions in the reduced form can be used to fuel the Fenton reaction (Carr and Frei, 1999; Shapiro and Saliou, 2001).

Vitamin E is a lipophilic vitamin, synthesised only by plants, that exists in eight different forms, of which α-tocopherol is the most active. It is the major membrane-bound antioxidant employed by the cell, whose main function resides in protecting cells against lipid peroxidation (Shapiro and Saliou, 2001; Munteanu *et al.*, 2004). As mentioned above, during the antioxidant reaction, α-tocopherol is converted to a α-tocopheryl radical, which is then reduced to the original α-tocopherol form by ascorbic acid (Pryor, 2000).

Vitamin A and its equivalent in animals, retinol, are also considered important antioxidants. It is present in many foods and can be formed from β-carotene transformation (Robichova *et al.*, 2004; Romieu, 2005). Vitamin B comprises a family of chemically distinct water-soluble vitamins that play important roles in cell metabolism, although only some of them show antioxidant properties. Vitamin B_1 (thiamine), for example, has been shown to offer protection in different situations where high levels of oxidative stress were involved (Mehta *et al.*, 2008). In the same way, high levels of vitamins B_2 (riboflavin) and B_{12} (cobalamin) have also been reported to protect against oxidative injury (Depeint *et al.*, 2006).

Carotenoids, of which β-carotene is probably the most studied, are pigments responsible for the colour of many fruits and vegetables. These substances have been recognised as the most potent quenchers of singlet oxygen (Cemeli *et al.*, 2008) and have been described to help in the prevention of cancer, heart disease and stroke (Valko *et al.*, 2004; Tamimi *et al.*, 2005; Riso *et al.*, 2006). Other examples of carotenoids with protective effects on oxidative conditions are lycopene (a bright red carotenoid found in red fruits) and xanthophylls (*e.g.* lutein and zeaxanthin) (Santocono *et al.*, 2007; Rao and Rao, 2007).

(Poly)phenolic compounds are secondary plant metabolites possessing one or more aromatic rings with one or more hydroxyl groups within their structure (Ferguson, 2001). These comprise a wide variety of molecules, divided into several classes (like flavonoids, phenolic acids, stilbenes and others), commonly found in fruits, vegetables, wine, tea, coffee and some cocoa products like dark chocolate (Manach *et al.*, 2004; Mozaffarieh *et al.*, 2008). Many studies have reported a wide range of biological activities for these compounds, including anti-inflammatory (Ruiz and Haller, 2006), anti-carcinogenic (Moon *et al.*, 2006), anti-allergic (Hendriks *et al.*, 2004) and anti-hepatotoxic (Lima *et al.*, 2006), which have been described to be mostly due to their antioxidant activity and also their ability to activate endogenous defence systems (Di Carlo *et al.*, 1999; Serafini *et al.*, 2000; Masella *et al.*, 2005).

The interaction of polyphenols with biological systems is sometimes difficult to understand. For example, the metabolisation of phenolic compounds *in vivo* may give rise to compounds that lose the original antioxidant activity (Masella *et al.*, 2005). Moreover, it should be taken into account that, *in vivo*, polyphenols and their metabolites are found at lower concentrations in plasma or tissues than those of other antioxidants (ascorbic acid and α-tocopherol, for example), since polyphenols may need to undergo structure modifications (*e.g.* hydrolysis) in order to be absorbed (Manach *et al.*, 2004; Williamson and Manach, 2005).

Despite the numerous beneficial effects mentioned above for these natural compounds, it should be noted that pro-oxidant effects have been described for many of them, resulting in the induction of apoptosis, cell death and the blocking of cell proliferation (Elbling *et al.*, 2005; Lambert *et al.*, 2005). For example, a high intake of vitamin C has been associated to an increased risk of disease (Ahkang *et al.*, 1998; Lee *et al.*, 2004). In the same way, α-tocopherol has been reported to be involved in the progression of low-density lipoprotein (LDL) oxidation and atherosclerosis, due to its pro-oxidant effects (Schneider, 2005). Despite being considered an antioxidant, clinical trials regarding the use of β-carotene for cancer

prevention, have demonstrated harmful effects for this compound, as reported by the Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group, in 1994, and also by Omenn *et al.* (1996). Some other compounds (polyphenols) have also presented these opposite effects (Leung *et al.*, 2005; Elbling *et al.*, 2005; Boots *et al.*, 2007).

1.2.3. New synthetic molecules with pharmacological potential

The above mentioned drawbacks associated to the use of some natural compounds are in part responsible for the undergoing development of novel synthetic compounds, conceived to protect cells against oxidative injury. In fact, the number of reports identifying new synthetic antioxidants has been growing rapidly (Cemeli *et al.*, 2008).

Many studies have described the synthesis of analogues of existing natural molecules known to possess antioxidant properties, which were modified in order to improve features like their stability and cellular uptake, but maintaining the functionality of the original structure (Halliwell, 2001). In this way, synthetic molecules that can mimic antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase have been reported as potential new drugs able to reduce damage caused by oxidative stress (Lanza and Vecchio, 2008). For example, salens, which comprise a class of compounds containing manganese (III) complexes, and metalloporphyrins, a class consisting of a porphyrin combined to a metal ion (usually iron or manganese), have been indicated as mimetics of superoxide dismutase activity (Trova *et al.*, 2003; Batinic-Haberle *et al.*, 2006; Day, 2009). Moreover, compounds like ebselen (a selenium-based compound), as well as diselenide and ditelluride compounds (which contain selenium, sulphur or tellurium in their structures) have been reported to possess glutathione peroxidase-like activity (Day, 2009). In the same perspective, Ehrlich and colleagues (2007) have recently reported the design and synthesis of a group of GSH analogues, which were named UPF peptides, that showed a higher hydroxyl radical scavenging ability and enhanced antiradical efficiency against the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical, when compared to GSH, without influencing cell viability. Structures of molecules produced by plants, with known antioxidant activity, like polyphenols (Siddaiah *et al.*, 2007; Bitto *et al.*, 2007) or chalcones (Vogel *et al.*, 2008) have also been modified in order to increase their biological activity and stability (Cotelle *et al.*, 1996; Lebeau *et al.*, 2000).

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyl hydroquinone (*t*-BHQ) are synthetic antioxidants, commonly used as food additives to prevent food spoilage, which are able to block lipid peroxidation. However, probably due to their phenolic nature (presence of OH groups), these compounds have shown contradictory effects (Suh *et al.*, 2005; Cemeli *et al.*, 2008). In addition, other synthetic compounds, like dimethyl sulfoxide (DMSO), lazaroids (21-aminosteroids drugs structurally related to glucocorticoids), salicylates (anti-inflammatory), pyrrolidine dithiocarbamate (PDTC) and dimethylurea have been found to scavenge free radicals and have also been used in therapeutic approaches (Macdonald *et al.*, 2003; Cui *et al.*, 2004).

In this work, novel nitrogenated structures, composed of an amidine unit linked to a phenol ring, were studied. Their synthesis, which has been recently described (Areias, 2006), was performed in the Group of Organic Synthesis of the Department of Chemistry of the University of Minho, headed by Prof. F. Proença. The introduction of their structures in a database created for this purpose by a group of russian researchers (personal information provided by Prof. F. Proença), identified them as molecules with pharmacological potential for disorders involving oxidative stress.

The choice to synthesise these kind of structures was based on different aspects: 1) in living organisms exist several compounds containing nitrogen atoms in their structure (*e. g.* serotonin, purinic and pyrimidinic DNA bases, catecholamines, among others), which are known to easily interact with active centres responsible for different functions in those same organisms (Areias, 2006); 2) the presence of hydroxyl groups within a phenol ring has been reported to be responsible for the antioxidant properties of a compound, since those groups are able to donate an hydrogen (Rice-Evans *et al.*, 1996; Cotelle *et al.*, 1996; Lien *et al.*, 1999); and 3) it has also been acknowledged that molecules incorporating conjugated systems with nitrogen atoms can stabilise free radicals (Wentrup, 1984). The combination of these factors was expected to enhance their ability to scavenge free radicals.

More details on the compounds in study will be provided further ahead.

1.3. Oxidative damage to biomolecules

Due to their high reactivity, ROS are prone to cause damage and are thereby potentially toxic, mutagenic and carcinogenic. Their targets include all biomolecules, namely lipids, proteins and nucleic acids (Kohen and Nyska, 2002).

1.3.1. Proteins

Proteins can function as important targets for attack by ROS (Kohen and Nyska, 2002). Direct oxidation of the side chains of all amino acid residues of proteins (especially proline, arginine, lysine and threonine) by ROS yields reactive carbonyl derivatives, particularly via metal-catalysed oxidation (Stadtman, 2004; Dalle-Donne *et al.*, 2006). Protein carbonyl derivatives can also be generated from the cleavage of peptide bonds by the α-amidation pathway or by oxidation of glutamyl residues. In addition, carbonyl groups may be introduced into proteins by secondary reaction of the primary amino group of lysine residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of reducing sugars or their oxidation products with lysine residues of proteins (glycation/ glycoxidation reactions), eventually leading to the formation of advanced glycation end-products (AGEs). Finally, carbonyl groups may be formed by adduction of byproducts of lipid peroxidation. These include malondialdehyde, which reacts with lysine residues and α,β-unsaturated aldehydes (4-hydroxy-2-nonenal and acrolein), which can undergo Michael-addition reactions at their C=C double bond with the sulfhydryl group of cysteine, the ε-amino group of lysine or the imidazole group of histidine residues, forming advanced lipoxidation end-products (ALEs) (Beal, 2002; Dalle-Donne *et al.*, 2006).

The introduction of carbonyl derivatives may alter the conformation, and/or even cause fragmentation, of the polypeptide chain, thus determining the partial or total inactivation of proteins. In addition, it can result in the loss of enzymatic activity, increased proteolytic degradation, altered cellular functions such as energy production, interference with the creation of membrane potentials and changes in the type and level of cellular proteins (Fagan *et al.*, 1999; Kohen and Nyska, 2002; Dalle-Donne *et al.*, 2006).

Protein carbonyl content is the most widely used marker for the measurement of protein oxidation. Highly sensitive assays for the detection of protein carbonyls

involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which results in the formation of a stable 2,4-dinitrophenyl(DNP)hydrazone product. Stable DNP adduct can then be detected spectrophotometrically, a technique that can be coupled to protein fractionation by High-Performance Liquid Chromatography (HPLC). Alternatively, in recent years, identification of carbonylated proteins has been performed by immunoblotting analysis, with the use of specific antibodies anti-DNP (Dalle-Donne *et al.*, 2003).

Protein damage is likely to be repairable and is a known non-lethal event for a cell. However, oxidation of proteins is associated with a number of age-related diseases including (but not limited to) Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, amyotrophic lateral sclerosis, and ageing (Stadtman, 2004). Additionally, two examples of human pathologies in which pathophysiological aspects of protein carbonylation have been extensively investigated are adult (or acute) respiratory distress syndrome (ARDS) and inflammatory bowel diseases (IBDs) (Dalle-Donne *et al.*, 2003).

1.3.2. Lipids

Polyunsaturated fatty acids (PUFAs), because of their multiple double bonds, are also extremely sensitive to oxidation by free radical attack. In addition, PUFAs can be formed enzymatically by the action of lipoxygenases (Niki *et al.*, 2005). Arachidonic and linoleic acids are the main PUFAs in the mammalian membranes and are able to undergo both enzymatic and non-enzymatic lipid peroxidation. However, since linoleic acid is much more abundant than arachidonic acid, most of lipid peroxidation products derive from the former (Spiteller, 2001).

Briefly, the overall process of lipid peroxidation consists of three main stages: initiation, propagation and termination. Lipid peroxidation may be induced when a radical species (*e. g.* hydroxyl radicals generated via Fenton reaction) is sufficiently reactive to remove a hydrogen atom from the polyunsaturated lipid (LH), leading to the formation of a lipid radical ($L^{\bullet}$), which results in the formation of lipid hydroperoxide (LOOH) species (Fig. 1.5). Thus, many molecules of lipids may be oxidised to lipid hydroperoxides for every initiation event. Termination reactions occur when two radical species combine to form non-radical final products or when substrate is depleted (Girotti, 1998; Lehuédé *et al.*, 1999).

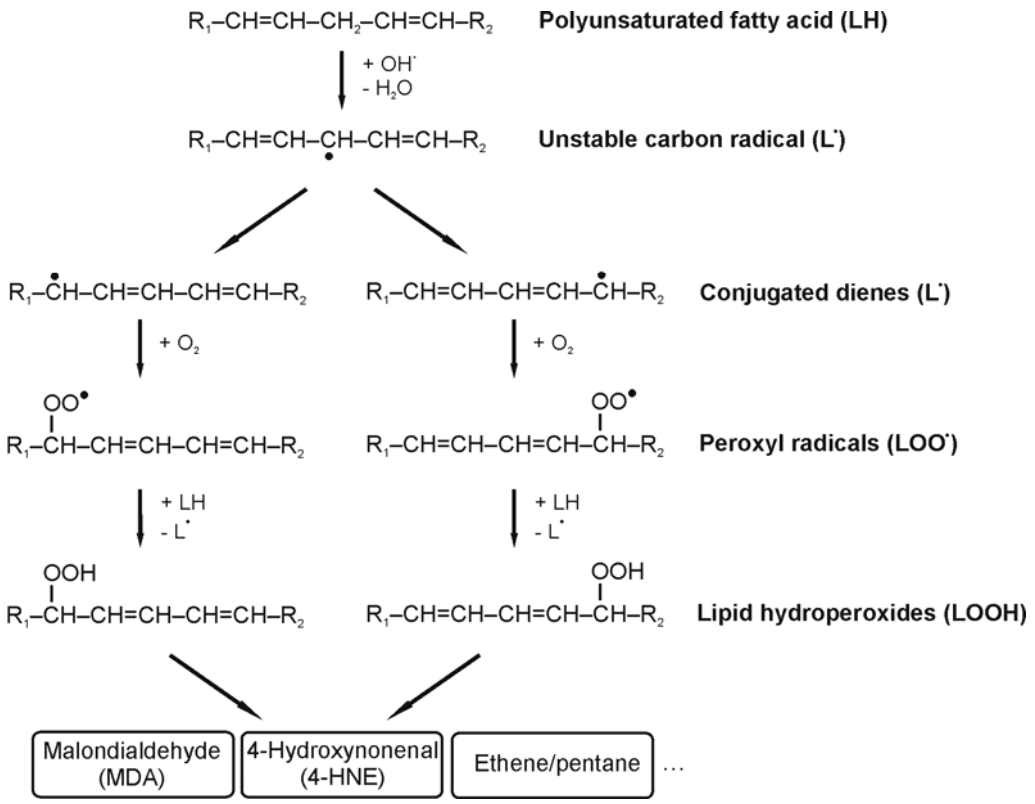


Figure 1.5 – Basic reactions occurring during lipid peroxidation. Reactive species (*e.g.* hydroxyl radicals) abstract an hydrogen atom from a polyunsaturated fatty acid, yielding a lipid radical ($L^{\bullet}$) that may undergo some molecular rearrangements. Oxygen uptake by these radicals propagates the reaction via peroxyl radicals ($LOO^{\bullet}$), which leads to the formation of lipid hydroperoxides (LOOH). These can then combine and generate the final products of lipid peroxidation (*e.g.* MDA 4-HNE, acrolein, ethane/pentane, among others). Adapted from Spiteller, 2001.

Once formed, lipid hydroperoxides can degrade rapidly into a variety of breakdown products, such as malondialdehyde (MDA), C_3–C_{10} straight chain aldehydes and α,β-unsaturated aldehydes, including 4-hydroxy-2-nonenal (4-HNE) and acrolein (Meagher and Fitzgerald, 2000; Lovell and Markesbery, 2007). In addition, lipid peroxidation also leads to production of isoprostanes and neuroprostanes. Although these molecules do not show toxicity, they serve as excellent markers of arachidonic and docosahexaenoic acid peroxidation in brain and cerebrospinal fluid (Lovell and Markesbery, 2007).

Estimates of lipid peroxidation can be obtained by measuring the formation of lipid hydroperoxides and their breakdown products, as well as by quantifying the disappearance of PUFAs. The most widely used method to assess oxidative damage to lipids is the thiobarbituric acid reactive substances (TBARS) assay,

which is thought to reflect the production of MDA (McCall and Frei, 1999; Meagher and Fitzgerald, 2000).

Some byproducts of lipid hydroperoxidation have been reported to possess cytotoxic, mutagenic and genotoxic properties (McCall and Frei, 1999). For example, MDA, 4-HNE and acrolein can damage DNA either by reacting directly with DNA bases or by generating more reactive bifunctional intermediates, which form exocyclic DNA adducts with a five-membered ring (etheno adducts) or a six-membered ring (propane adducts) attached to DNA bases. These adducts can then induce base pair substitution mutations (Nair *et al.*, 2007). Hydroxynonenal and acrolein are neurotoxic and can, among other things, inhibit enzymes critical for neuron survival (Lovell and Markesbery, 2007). Moreover, lipid peroxidation seems to be also involved in atherosclerotic processes. The oxidation of low density lipoproteins (LDL) results in their uptake by phagocytes in the subendothelial space via their scavenger receptor. These phagocytic cells then accumulate in the subendothelial space, where they stimulate formation of atherosclerotic plaques (Young and McEneny, 2001).

1.3.3. DNA

Damage to nuclear DNA has been proposed to occur through two different mechanisms: oxidative modification and DNA fragmentation mediated by endonucleases (an irreversible feature of programmed cell death). While the latter is supposed to take place during the late stage of cell death, oxidative DNA damage is believed to stand for an early event (Cui *et al.*, 2000; Nagayama *et al.*, 2000). Mitochondrial DNA has, by its turn, been reported to be even more susceptible to oxidation than nuclear DNA. The greater accumulation of damage may be related to: mitochondria being the main producers of ROS in the cells; repair capacity of mitochondrial DNA being limited (proteins responsible for DNA repair are expressed in lower levels in mitochondria); and mitochondrial DNA not being protected by histones (Inoue *et al.*, 2003; Martin, 2008).

The attack of ROS and RNS is responsible for introducing several modifications to DNA, which include: oxidised bases, the most studied being 8-oxo-7,8-dihydroguanine (8-Oxo-Gua), a product derived from the oxidation of a guanine by ROS; modifications to the sugar moiety of DNA, which may result in base loss – abasic (apurinic/apyrimidinic) sites – and/or strand breakage (single- and double-strand breaks); DNA-DNA intra-strand adducts and DNA-protein cross-links (Cadet

et al., 1999; Powell *et al.*, 2005). The formation of some of these lesions is summarised in Fig. 1.6. In addition, exposure of cells to UV radiation can result in dimerisation reactions between adjacent pyrimidine bases, yielding cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (Clingen *et al.*, 1995; Douki *et al.*, 2003).

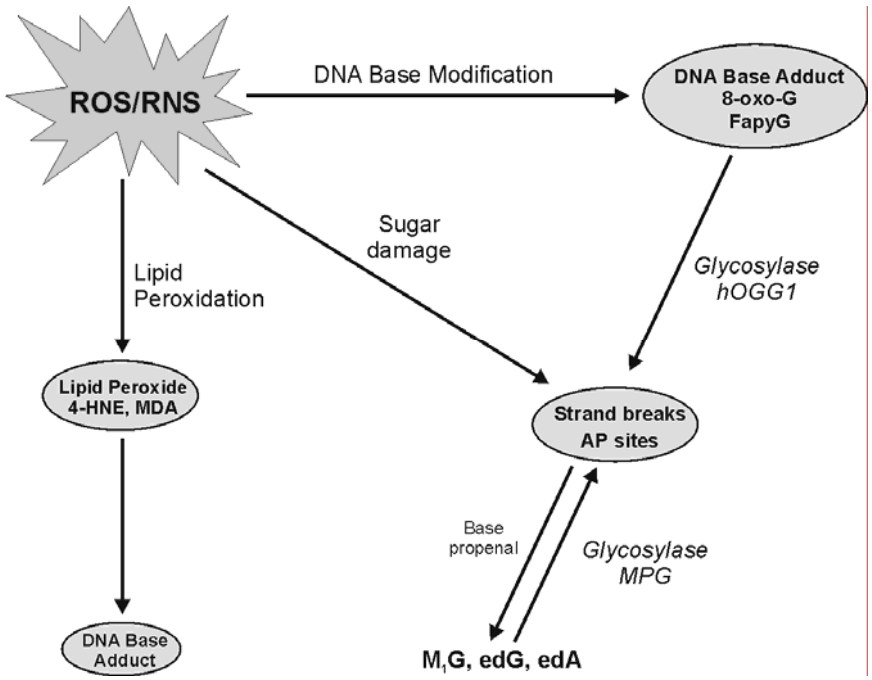


Figure 1.6 – Types of DNA damage induced by reactive oxygen species. Abbreviations: 4-HNE: 4-hydroxy-2-nonenal; 8-oxo-G: 8-oxo-7,8-dihydroguanine; edG: etheno-deoxyguanosine; edA: etheno-deoxyadenosine; FapyG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine; hOGG1: human 8-oxoguanine DNA glycosylase 1; M_1G: pyrimidopurinone; MDA: malondialdehyde; MPG: *N*-methylpurine DNA glycosylase. Adapted from Powell *et al.* (2005).

DNA lesions, if left either un- or mis-repaired, may interfere with DNA-dependent processes (such as transcription, replication, recombination and chromosome segregation), leading to mutations, chromosomal instability and even cell death (Kruman, 2004). Therefore, cells are equipped with specific and efficient repair mechanisms, which are able to repair the modifications introduced in DNA (Evans *et al.*, 2004). During these repair processes, cell cycle may be arrested at any checkpoint, which allows a time window to repair damaged DNA (Kruman, 2004; de Waard *et al.*, 2008). In addition, it should also be noted that oxidative stress leads to an upregulation of the expression of many repair enzymes, which may result in an enhancement of enzymatic repair mechanisms (Valko *et al.*, 2006; Fishel *et al.*, 2007).

DNA double-strand breaks are repaired via two different mechanisms: homologous recombination (HR), in which the sister chromatid is used as a template to copy the missing information into the broken locus (Helleday, 2003), and non-homologous end joining (NHEJ), which consists in the fusion of the two broken ends with little or no regard for sequence homology, a process that may result in mutations (Seluanov *et al.*, 2004). The mismatch repair (MMR) pathway plays an important role in the post-replicative process, by repairing replicative and recombinatorial errors that may result in mispaired bases (mismatches) (Neri *et al.*, 2005; Gorbunova *et al.*, 2007). The nucleotide excision repair (NER) pathway recognises bulky adducts, as well as covalent linkages between adjacent pyrimidines resulting from exposure to UV radiation, and removes short DNA oligonucleotides containing a damaged base; it can be classified into global genome repair (GG-NER), which removes DNA damage from the entire genome, and transcription-coupled repair (TC-NER), which specifically removes damage from the transcribed strand of active genes, thereby releasing transcription arrest that occurs at the lesions sites (Hanawalt, 2002; de Waard *et al.*, 2008). However, oxidatively damaged DNA is mainly removed by the base excision repair (BER) pathway, in which oxidised bases are eliminated by different DNA glycosylases, leaving abasic sites (Frosina, 2006; Coppedè *et al.*, 2007).

Base excision repair consists of four main steps (base removal, apurinic/apyrimidinic site incision, synthesis and ligation), and can be divided in two sub-pathways, the short patch or single nucleotide replacing pathway and the long patch pathway (Figure 1.7), which involves the incorporation of up to 13 nucleotides (Rao, 2007; Martin, 2008).

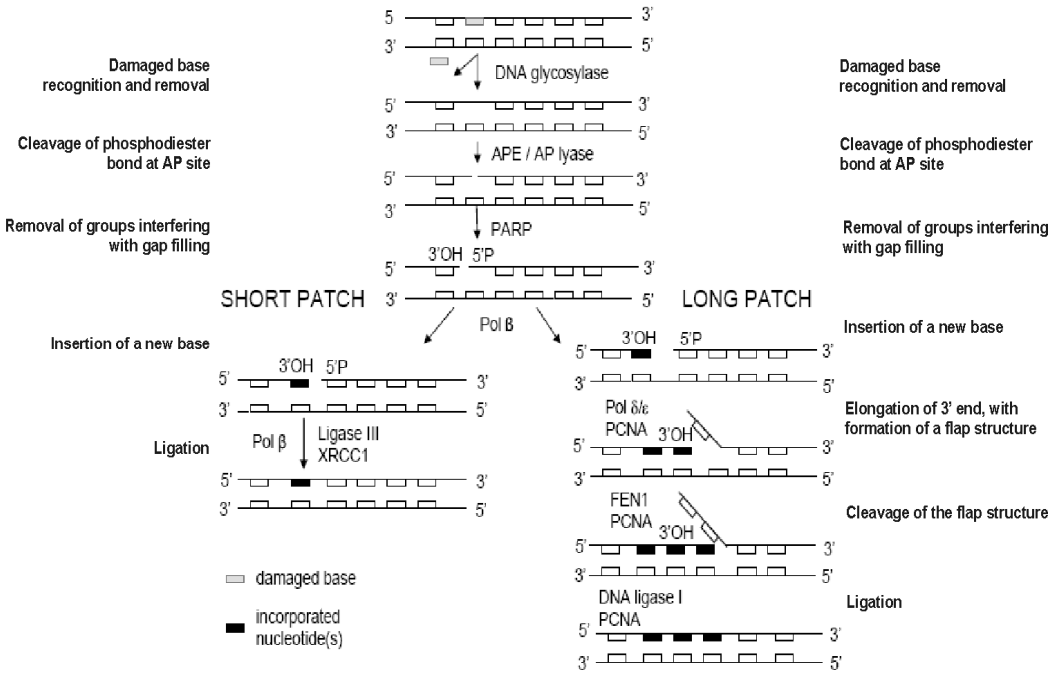


Figure 1.7 – Base excision repair pathways. First, a damaged base is recognised and removed by DNA glycosylases, yielding an apurinic/apyrimidinic (AP) site, which is then cleaved by an endonuclease (APE), forming a strand break. Then, after some chemical groups that may interfere with gap filling and ligation are removed, different polymerases (depending whether the short patch or the long patch pathway is active) fill the gap in the DNA strand. Finally, DNA ligase inserts one (SHORT PATCH) or more (LONG PATCH) new bases. Other proteins, like poly (ADP-ribose) polymerase (PARP), XRCC1, proliferating cell nuclear antigen (PCNA) and FEN1 aid in the regulation of these pathways. The bacterial enzymes FPG and Endo III, used in the Comet assay, act on the first step of this pathways (damaged bases recognition and removal). Adapted from Tudek *et al.* (2006).

BER follows mainly through the short patch pathway. In the first step of this pathway, a damaged base is recognised and removed from the deoxyribose phosphate moiety (Rao, 2007). Two classes of DNA glycosylases are involved in this step: Class I (bifunctional or complex), which includes, for example, 8-oxoganine DNA glycosylase 1 (OGG1), an enzyme that removes 8-oxoguanine and nicks the DNA backbone; and Class II (monofunctional or simple), including *N*-methylpurine DNA glycosylase (MPG) and uracil DNA glycosylase (UDG), which remove alkylated DNA bases or uracil, respectively, but without nicking the DNA backbone (Collins and Gaivão, 2007; Fishel *et al.*, 2007). Due to the catalytic inefficiency of OGG1's lyase activity, this enzyme remains stuck to the site of incision, limiting the overall rate of repair (Dodson and Lloyd, 2002; Frosina, 2006).

So, following the glycosylase reaction, APE1 (apurinic/apyrimidinic endonuclease, also called APEX, HAP1 and Ref-1), easily displaces the glycosylase and cleaves the 5' terminus of the apurinic/apyrimidinic (AP) site, resulting in the loss of a base and a single-strand break with 5′-phosphate and 3′-OH termini (step 2) (Collins and Gaivão, 2007; Martin, 2008). Additionally to its role in BER, APE has also been implicated in the redox activation of transcription factors, such as p53 (Fantini *et al.*, 2008). In the third step of BER, the AP site that is generated by the APE1 repair activity is removed by deoxyribosephosphate hydrolase (dRPase) activity provided predominantly by DNA β-polymerase (β-pol). This is then followed by the insertion of a new base by β-pol and ligation by DNA ligase I (step 4) (Fishel *et al.*, 2007).

The long patch pathway is catalysed by β-pol or other polymerases like δ/ε-pol, which have a proof-reading activity associated to them. Repair follows this pathway when the terminal sugar phosphate formed after the APE1 incision (step 2) develops a complex structure that cannot be acted upon by the dRPase activity of β-pol (*e.g.* oxidised abasic site). In this case, the repair synthesis still continues, but in a strand displacement manner (Rao, 2007).

It should be noted that despite the presence of all these DNA repair systems, DNA damage still occurs. In fact, DNA is continuously attacked by reactive species, an event that can be attenuated by antioxidant defences. Whereas some of the damage induced by ROS attack to DNA can induce the signalling pathways leading to apoptotic cell death, the majority results in the oxidation of DNA. However, DNA repair enzymes help to maintain an equilibrium state between damage formation and repair, by repairing most of the oxidised DNA (Collins and Gaivão, 2007). This implies that, in normal physiological conditions, oxidative damage is maintained at a tolerable level in terms of genetic stability (Tomasetti *et al.*, 2001; Collins and Gaivão, 2007). Nevertheless, the oxidised DNA that does not get repaired can become mutated, which may then result in further damage to cells. A scheme demonstrating this equilibrium state is shown in Figure 1.8.

There are several methods that allow the measurement of oxidative DNA damage. However, determining the background levels of the most common product of DNA damage, 8-oxo-Gua, has brought some difficulties, since damage can arise from the preparation of samples. With the purpose of normalizing the procedures to measure this kind of damage, the European Standards Committee on Oxidative DNA Damage (ESCODD) was formed (ESCODD, 2003; Gedik and Collins, 2004). Although some chromatographic methods, like Gas Cromatography (GC) and High Performance Liquid Cromatography (HPLC), usually coupled to mass spectrometry

(MS) techniques, have been employed, they have shown inflated values, probably due to erroneous oxidation during DNA isolation. So, a set of enzymatic methods have been described as the most suitable. These methods make use of the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG) to convert 8-oxoGua to apurinic sites, which can then be measured by the Comet assay, alkaline unwinding or alkaline elution (ESCODD, 2003; Powell *et al.*, 2005).

The comet assay is a simple, fast and sensitive method that detects DNA strand breaks and abasic sites, although it can detect oxidised bases via the use of repair enzymes, such as FPG or endonuclease III (Endo III), which nick DNA at oxidised purines and pyrimidines, respectively, at the initial step of the BER pathway (Fig. 1.7). Moreover, this assay reduced the risk of occurring oxidation during sample preparation (Loft and Moller, 2006).

Oxidative DNA damage can result in the arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which have been implicated in cancer and neurodegenerative diseases, besides being associated to the normal process of aging (Bjelland and Seeberg, 2003; Cooke *et al.*, 2003).

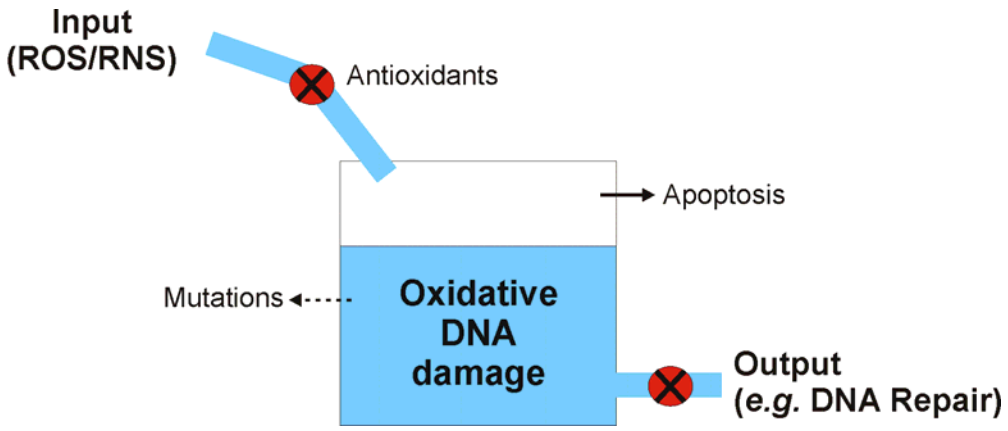


Figure 1.8 – Balance between oxidative DNA damage input and output. Oxidative DNA damage can be regarded as a dynamic steady state: an equilibrium is maintained between input of damage (*i.e.* endogenous and/or exogenous free radicals attack), which can be attenuated by antioxidant defences, and output from this damage (*i.e.* DNA repair). A change in input or output can cause an increase or decrease in the level of damage, until a new equilibrium is established. Adapted from Collins and Gaivão (2007).

1.4. Cell death

The exposure to oxidative stress may ultimately result in cell death, as a consequence of severe damage caused to biomolecules by reactive oxygen and nitrogen species (Nordberg and Arner, 2001). Cell death can occur by two main mechanisms: necrosis and programmed cell death (PCD) (Boujrad *et al.*, 2007).

Necrosis is usually referred as an "accidental", or uncontrolled, form of cell death (Henriquez *et al.*, 2008). During this process, there is a rapid swelling of the cell, leading to the loss of membrane integrity and consequent release of the cells' contents, which is known to evoke an inflammatory response. In this way, cell death becomes a passive consequence of irreparable damage, hence the term "accidental" (de Bruin and Medema, 2008; Henriquez *et al.*, 2008).

Apoptosis was initially considered as the only form of programmed cell death. However, more recently, a variety of cell behaviours that may lead to active forms of cell death have been observed. These include the typical apoptotic cell death, autophagic death, mitotic catastrophe, oncosis, anoikis, excitotoxicity, Wallerian degeneration, cornification and paraptosis. In this way, and using nuclear morphology as a distinction criterion, programmed cell death has been divided into three possible classifications: classical apoptosis, autophagic cell death and necrosis-like PCD (Lorenzo and Susin, 2007; Boujrad *et al.*, 2007; Kroemer *et al.*, 2008).

Type I PCD or classical apoptosis has been described as an active process by which dying cells are removed in a safe, non-inflammatory manner (Leist and Jaattela, 2001). It is a tightly regulated process, involved in many vital functions, including tissue development, carcinogenesis, immune response and control of the balance between proliferation and differentiation (Cai and Jones, 1999; Gil *et al.*, 2003a; Krantic *et al.*, 2007). At the morphological and biochemical levels, it is characterised by shrinkage of the cell, membrane surface blebbing, oligonucleossomal DNA fragmentation and the breakdown of the cell into various membrane-bound fragments, called apoptotic bodies. Along with these events, occurs the activation of specific cysteine proteases, named caspases, as well as the loss of membrane phospholipid asymmetry, which results in the externalisation of phosphatidylserine (Matés *et al.*, 1999).

Type II PCD or autophagic cell death is mainly characterised by the presence of double- or multiple-membrane vacuoles, mitochondrial dilation, as well as enlargement of the endoplasmic reticulum (ER) and Golgi apparatus (Lorenzo and

Susin, 2007). The vacuoles formed during this process, which are also called autophagosomes, engulf portions of cytoplasm and organelles (like mitochondria and ER), then fuse with lysosomes, where the intravacuolar content is disintegrated by lysosomal enzymes (Kourtis and Tavernarakis, 2008). Nevertheless, it should be noted that “autophagic cell death” is a process that differs from autophagy. Indeed, autophagy is a dynamic process of protein degradation, used mainly to provide an alternative source of nutrients, which is usually associated to cell death, with either necrotic or apoptotic phenotype (Boujrad *et al.*, 2007; de Bruin and Medema, 2008). The mammalian protein kinase TOR (which stands for “target of rapamycin”) plays a major role in the regulation of the autophagic process, by inhibiting this pathway. Downstream of this protein, several other proteins encoded by *Atg* genes intervene in the execution of the autophagic process (Kourtis and Tavernarakis, 2008; Henriquez *et al.*, 2008). The identification of these genes and the observation that their inactivation protects from this type of death, supports the evidence of this PCD as a specific type of death.

Recently, an active form of necrosis (type III PCD) was found to occur, not only under pathological conditions, but also under normal physiological conditions (Boujrad *et al.*, 2007). It lacks caspase and lysosomal involvement and is mainly characterised by an early swelling of intracellular organelles, followed by loss of plasma membrane integrity (although nuclear disintegration is retarded). This type of PCD can also be subdivided into two sub-types: IIIA or “non-lysosomal disintegration”, characterised by nuclear disintegration, and IIIB or “cytoplasmic degeneration”, which displays karyolysis (complete dissolution of the chromatin of a dying cell). Moreover, programmed necrosis can occur by the induction of the tumour necrosis factor (TNF) or Fas ligand, via their respective death receptors, and gives an idea that necrosis is in fact a process that cells can control (Lorenzo and Susin, 2007; Boujrad *et al.*, 2007; Henriquez *et al.*, 2008).

A summarised classification of cell death phenotypes is presented in Figure 1.9.

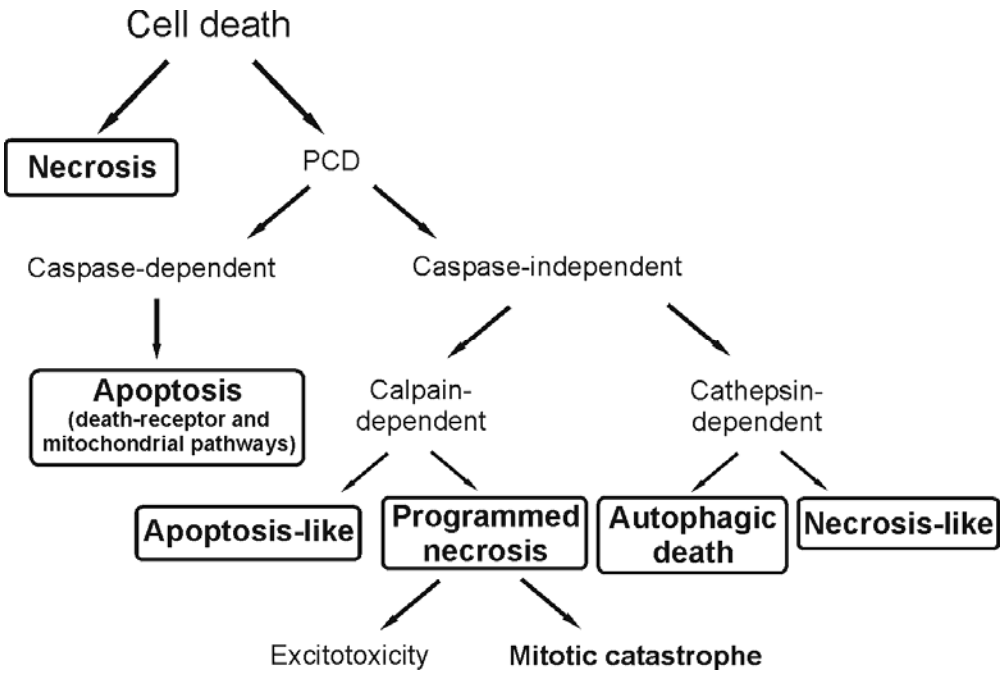


Figure 1.9 – Classification of different cell death outcomes. This classification regroups cell death phenotypes according to the family of proteases involved, and introduces programmed necrosis as a distinct PCD type. Secondary activation of other classes of proteases (*e.g.* cathepsin activation after calpain activation in apoptosis-like PCD) has not been taken into account. In addition, some cell death phenotypes, such as paraptosis, are not included in this classification. Adapted from Boujrad *et al.*, (2007).

1.4.1. Programmed cell death pathways

Apoptosis, the most common form of programmed cell death, is a complex process involving both pro- and anti-apoptotic proteins, which can be initiated by two different signalling pathways: the death receptor (extrinsic) pathway and the stress- or mitochondria-mediated (intrinsic) pathway (Dong *et al.*, 2005; de Bruin and Medema, 2008). A set of highly conserved cysteine-dependent aspartate-specific proteases, named caspases, are regarded as the central executioners of apoptosis. These caspases use a cysteine residue as the catalytic nucleophile and share a specificity for cleaving their substrates after aspartic acid residues in target proteins (Hengartner, 2000; Chowdhury *et al.*, 2008).

The main intracellular signalling pathways leading to apoptotic cell death are summarised in Figure 1.10.

The death receptor or extrinsic pathway is activated by the binding of an extracellular ligand, such as Fas (also known as CD95 or APO-1) ligand, tumour necrosis factor (TNF) or TNF-related apoptosis induced ligand (TRAIL), to their

specific death receptors present in the cell membrane (Beere, 2005; Reeve *et al.*, 2007). This results in the recruitment of procaspase-8 and subsequent activation of caspase-8, which then leads to the activation, either directly or indirectly, of downstream caspases, like procaspase-3 (Cain *et al.*, 2002; Beere, 2005) (Fig. 1.10).

As shown in the same figure (Fig. 1.10), the mitochondrial or intrinsic pathway can be initiated by the translocation of several proapoptotic proteins from the Bcl-2 family (*e.g.* Bax, Bid or Bad) to the mitochondria (Zimmermann *et al.*, 2001). These proteins induce the permeabilisation of the mitochondrial outer membrane (MOMP), which has been considered as a point of no return in cell death (Bouchier-Hayes *et al.*, 2005; Dong *et al.*, 2005). As a consequence of an increase in its permeability, mitochondria release several proapoptotic factors normally present in the intermembrane space, which include cytochrome *c*, the serine protease HtrA2/Omi, the Apoptosis Inducing Factor (AIF), Smac/DIABLO (which stands for second mitochondria-derived activator of caspases/Direct IAP Binding Protein With Low pI) and endonuclease G (Hengartner, 2000; Zimmermann *et al.*, 2001; Krantic *et al.*, 2007).

The released cytochrome *c* can bind to procaspase-9 and Apaf-1 (from apoptotic protease-activating factor 1) in the presence of deoxyadenosine triphosphate (dATP)/adenosine triphosphate (ATP). This complex, termed the apoptosome, results in the activation of caspase-9, which is mainly activated through this mitochondrial pathway (Gil *et al.*, 2003a; Schafer and Kornbluth, 2006).

The intrinsic and extrinsic pathways can be interconnected by the action of Bid, a protein from the Bcl-2 family (see figure 1.10). This protein is normally found in the cytosol, where it can be cleaved by caspase-8. After cleavage, the carboxylic terminus of Bid is translocated to mitochondria, leading, directly or indirectly (by interaction with Bax) to the release of cytochrome *c* (Tang *et al.*, 2000; Beere, 2005).

Both activated caspase-8 (as well as caspases 2 and 10, also mediators of the death-receptor pathway) and the activated caspase-9 (from the mitochondrial pathway) converge to the downstream activation of effector caspases, such as procaspases 3, 6 and 7 (Krantic *et al.*, 2007). These caspases activate a DNase, which is responsible for the fragmentation of oligonucleosomal DNA (Ferri *et al.*, 2000; Krantic *et al.*, 2007). In addition, other enzymes and/or substrates, like poly-(ADP-ribose) polymerase (PARP), fodrin, p75, actin, among others, are activated in the process, culminating in the display of several of the phenotypic characteristics of

apoptotic cell death, including loss of mitochondrial membrane potential, cell blebbing and redistribution of lipids in the outer plasma membrane (Beere, 2005).

Alternatively to the caspase-dependent pathways, the characteristic features of apoptosis can be induced by a caspase-independent way, mediated by the apoptosis inducing factor (AIF). This is a mitochondrial flavoprotein oxidoreductase that translocates first to the cytosol and then to the nucleus (Ferri *et al.*, 2000; Bahi *et al.*, 2006). Once in the nucleus, AIF interacts with nucleic acids, causing caspase-independent chromatin condensation and DNA fragmentation (Lorenzo and Susin, 2007).

The endoplasmic reticulum (ER) can also act in the induction and regulation of apoptosis. ER is known to play a central role in protein biosynthesis and is the major intracellular organelle involved in calcium storage (Nakagawa and Yuan, 2000). Calcium homeostasis is maintained by some members of the Bcl-2 family, like Bax and Bak, which can also be associated to the ER membrane. Upon an apoptotic stimulus, these proteins induce calcium release through ER calcium channels, such as the inositol 1,4,5-triphosphate (IP_3) receptors, whose opening is mediated by the binding of IP_3. Calcium from the ER is then taken up by mitochondria, causing a calcium overload that subsequently results in the induction of mitochondrial membrane permeabilisation by mitochondria-located Bax and Bak (Lindholm *et al.*, 2006; Schwarze *et al.*, 2008). Moreover, calcium release from the ER can induce the activation of calpains and caspase-12, which by their turn lead to the activation of other caspases, resulting in the propagation of the apoptotic signal (Nakagawa and Yuan, 2000).

It should be noted that many factors are able to regulate these pathways, by activating or inhibiting them at specific sites.

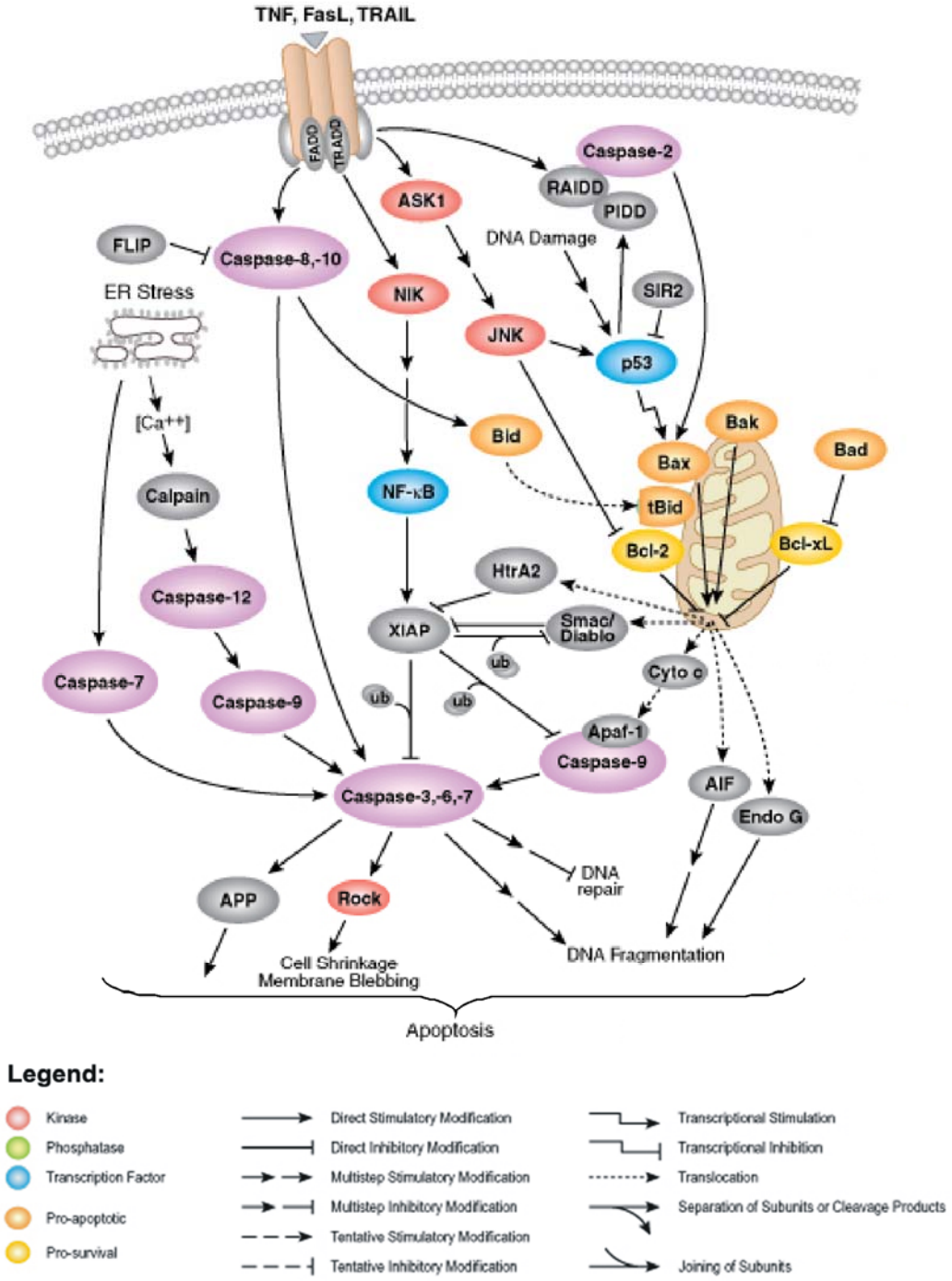


Figure 1.10 – Main intracellular pathways leading to apoptosis and some ways by which they can be regulated. The extrinsic pathway is activated by the binding of an extracellular ligand to specific death receptors, which activate caspase-8. The mitochondrial pathway is activated by Bax or Bak, which bind the mitochondrial outer membrane, inducing the release of several pro-apoptotic factors, such as cytochrome c, which then forms the apoptosome upon binding to Apaf-1 and caspase-9. These two pathways can be connected by the cleavage of Bid and both of them result in the induction of effector caspases, like caspase-3, which then activate substrate responsible for the common features of apoptosis. The caspase-independent pathway, mediated by AIF is also shown in this scheme. Adapted from Cell Signaling Technology (http://www.cellsignal.com).

Recently, a regulated lysosomal involvement in cell death has also been observed. In fact, partial lysosomal membrane permeabilisation has been described to trigger apoptosis and apoptosis-like cell death, in response to several apoptotic stimuli, such as activation of death receptors of the tumour necrosis factor (TNF) family, p53 activation, oxidative stress, among others (Kirkegaard and Jaattela, 2009). These stimuli can then lead to lysosomal membrane permeabilisation through either caspase-dependent or -independent mechanisms, which normally results in the release of a group of proteases named cathepsins (including cathepsins B, D and L) from the lysosome to the cytosol (Jaattela *et al.*, 2004). In addition, lysosomal enzymes have been found to act on mitochondria and induce the formation of mitochondrial ROS, which can then lead to further lysosomal permeabilisation (Zhao *et al.*, 2003). Moreover, it has been reported that mild stress triggers only a limited release of the lysosomal contents to the cytosol followed by apoptosis or apoptosis-like cell death, while elevated stress levels cause a generalised lysosomal rupture and rapid cellular necrosis (Kagedal *et al.*, 2001). This lysosomal apoptotic signalling pathway has recently been considered as an attracting drug target, namely in cancer therapeutics (Berndtsson *et al.*, 2008).

Occasionally, deficiencies in the cell cycle checkpoints, which by its turn lead to aberrant mitosis, may result in another type of programmed cell death, termed mitotic catastrophe (Fig. 1.9). This process is mainly characterised by enlarged and multinucleated cells, incomplete nuclear condensation, chromosome alignment defects and unequal DNA separation (Roninson *et al.*, 2001; Strauss *et al.*, 2007; de Bruin and Medema, 2008).

1.4.2. The role of ROS/RNS in the induction of cell death

The intracellular accumulation of reactive oxygen and nitrogen species also plays an important role in the initiation of cell death processes (Matés, 2000). In fact, the amount of reactive species accumulated in the cell, and the way the cell responds to that redox imbalance, can determine that same cell's fate. For example, mild oxidative stress may activate biological responses that can either lead to survival and proliferation, or can induce apoptosis, while the accumulation of high levels of ROS may promote necrosis instead (Lin *et al.*, 2004; Lopez-Sanchez *et al.*, 2007). Moreover, a sudden burst of ROS, resulting from the response to oxidative stress of cells already committed to apoptosis, can direct those cells towards a necrotic-like death (Matés, 2000; Fleury *et al.*, 2002).

The accumulation of reactive species has been described to precede changes in the mitochondrial membrane, nuclear condensation and other typical apoptotic events (Fleury *et al.*, 2002; Aquilano *et al.*, 2007). Indeed, some studies have reported that an increase in ROS induces cytochrome *c* release from mitochondria (in a voltage-dependent anion channel (VDAC)-dependent way), and caspases activation (Pias and Aw, 2002; Petrosillo *et al.*, 2003; He *et al.*, 2007).

Many studies have shown other evidences for the induction of apoptotic pathways by reactive species. Some mediators of apoptosis (*e.g.* JNK, ERK, PTEN) have been reported to lead to increased levels of ROS (Kim *et al.*, 2005; Zhu *et al.*, 2006). Similarly, it has been shown that inhibition of the mitochondrial respiratory chain at complex I (Deshpande *et al.*, 2000), or an impairment of the electron transfer chain by mutations in mitochondrial DNA, prevent the accumulation of ROS and consequently protect cells against apoptosis (Cui *et al.*, 2004; Hiona and Leeuwenburgh, 2008). In addition, lipid peroxidation has also been reported to occur following an apoptotic signal (Matés and Sanchez-Jimenez, 2000; Hiroi *et al.*, 2005). Moreover, and perhaps most important, inhibition of apoptosis by the addition of antioxidants has already been described (Gil *et al.*, 2003a; Jang *et al.*, 2004; Jung *et al.*, 2007; Choi *et al.*, 2007). For example, the antioxidant enzyme MnSOD has been reported to inhibit apoptosis during ischemia/reperfusion injury (Marczin *et al.*, 2003), and “classical” antioxidants such as α-tocopherol and GSH have shown to prevent apoptosis induced by ascorbate-iron (Hiroi *et al.*, 2005). Thus, these studies point towards a wide range of actions for ROS/RNS and add further importance to the use of antioxidants to prevent apoptosis and treat several disorders.

1.4.3. Intracellular regulation of cell death pathways

The intracellular pathways leading to apoptotic cell death can be regulated at several levels, including their blockade at the Death-Inducing Signalling Complex (DISC), which is responsible for the recruitment of procaspase-8, or the inhibition of their enzymatic activity (Figure 1.10). These regulators comprise inhibitors-of-apoptosis proteins (IAPs), the FLICE-like inhibitor protein (FLIP) and calpains (Chowdhury *et al.*, 2008). IAPs are a family of cellular proteins, including eight mammalian family members with highly conserved and differential expression patterns in various tissues, which bind to the surface of caspases, blocking the catalyzing grooves of caspases. These proteins do not inhibit caspase-8, but they inhibit its substrate, procaspase-3, instead, thus arresting the death-receptor

pathway. In the mitochondrial pathway, XIAP, c-IAP1 and c-IAP2 bind directly to procaspase-9, preventing its activation (Cain *et al.*, 2002; Chowdhury *et al.*, 2008). FLIP proteins have been described mainly as inhibitors of the death receptor pathway, since they are able, when overexpressed, to inhibit the activation of procaspase-8 at the DISC (Krueger *et al.*, 2001; Golks *et al.*, 2005).

Calpains represent a family of calcium-dependent, nonlysosomal, cysteine proteases that share many substrates with caspase-3, including fodrin, calcium-dependent protein kinase and PARP. In addition, due to the presence of a calmodulin-like calcium-binding site in their structure, these proteases are involved in various calcium-regulated processes, like signal transduction, cell proliferation, platelet activation and apoptosis (Lorenzo and Susin, 2007; Chowdhury *et al.*, 2008). Moreover, calpains can also cleave Bcl-xL, an antiapoptotic protein, converting it into a pro-apoptotic molecule (Nakagawa and Yuan, 2000).

Ceramides, which represent the structural backbone of sphingolipids/sphingomyelin, are important second messengers in several cell processes, including apoptosis. Some of their targets in apoptotic signalling pathways include mitochondria, jun kinases (JNK), lysosomal cathepsin D, p38 mitogen-activated protein kinase (MAPK), Bcl-2 family members, among others (Futerman and Hannun, 2004; Lee and Thévenod, 2008). Although the exact mechanism is not yet fully understood, ceramides seem to induce apoptosis by inducing the release of cyt *c* from mitochondria to the cytoplasm (Grether-Beck *et al.*, 2003), which might be related to their ability to form protein permeable channels in the mitochondrial membranes (Kim *et al.*, 2001; Lee and Thévenod, 2008).

The release of cathepsins (a group of proteases that stand for “lysosomal proteolytic enzymes”) from lysosomes to cytosol has also been found to be implicated in the regulation of apoptosis (Chwieralski *et al.*, 2006). This lysosomal permeabilisation seems to be an early event, occurring prior to the loss of mitochondrial transmembrane potential. Bcl-2 family members, particularly Bid, seem to be the main targets of cathepsins. Bid cleavage then leads to the activation of Bax, resulting in the consequent release of apoptogenic factors from mitochondria (Lorenzo and Susin, 2007; Conus and Simon, 2008).

The activation of the tumour suppressor protein p53 can induce DNA damage. Once activated, p53 may induce the expression of genes that prevent cell division and cause apoptosis (L'Ecuyer *et al.*, 2006). This may lead to the activation of pro-apoptotic Bcl-2 family members, like Bax, resulting in the permeabilisation of the

outer mitochondrial membrane and subsequent activation of the above mentioned mitochondrial pathway (Cui *et al.*, 2002; L'Ecuyer *et al.*, 2006; Gartel, 2008).

A small peptide derived from the carboxyl terminus of p21, a protein identified as a cyclin-dependent kinase inhibitor and that was known to play an important role in the regulation of cell growth and differentiation, has recently been found to also activate apoptosis by a process involving mitochondria, although the exact pathways are still unclear (Dong *et al.*, 2005).

1.5. Oxidative stress and disease

In normal conditions, cells can deal with mild levels of oxidative stress. They do so by upregulating the expression of genes responsible for the synthesis of antioxidant defence mechanisms. However, the intracellular accumulation of high levels of ROS/RNS can result in damage to all types of biomolecules, and ultimately lead to cell death, which has been associated with many pathological conditions (Halliwell and Gutteridge, 1999; Valko *et al.*, 2007). However, it should be noted that whereas some diseases may indeed be caused by oxidative damage to biomolecules, in others oxidative stress is a consequence (and not a cause) of the disease. However, even as a secondary event, oxidative stress is of major importance, since it can induce the aggravation of tissue damage in several disorders (Wilcox *et al.*, 2004).

A redox imbalance in the cell can lead to oxidative DNA damage, causing mutations that may result in cancer (Collins *et al.*, 2003; Bartsch and Nair, 2006). The oxidative burst induced by neutrophils and macrophages to kill pathogens may result in chronic inflammation, seen in immune disorders, such as rheumatoid arthritis and inflammatory bowel diseases (Riso *et al.*, 2006; Filippin *et al.*, 2008). Glycation of proteins leading to accumulation of advanced glycation end-products (AGEs), reduced antioxidant levels and oxidative DNA damage seem to be present in diabetic complications (Collins *et al.*, 1998; Rolo and Palmeira, 2006). The decline in $CD4^+$ lymphocytes (whose functioning is regulated by redox potential) counts has been described to contribute to the progress of HIV infection to AIDS (Gil *et al.*, 2003b; Deshmane *et al.*, 2009). Moreover, oxidative stress is involved in ophthalmologic disorders, such as advanced macular degeneration (AMD) and glaucoma (Mozaffarieh *et al.*, 2008).

Reactive species also participates in the normal process of aging. Oxidative damage accumulates with age, which leads to an increased impairment of cellular

function. This results in a markedly decreased ability for the organism to neutralise free radicals and cope with oxidative stress, as it grows older (Bertram and Hass, 2008). In addition, it has been reported that the cellular response to oxidants seems to be associated with the mechanisms that regulate longevity. Three gene products, including Forkhead transcription factors, the adaptor protein p66Shc and the hystone deacetylase Sir2, are described as being involved either in the regulation of intracellular ROS concentrations or in the increase in resistance to oxidative stress (Finkel, 2003).

Oxidative stress also seems to be important in the etiology of several cardiovascular diseases, like atherosclerosis, ischemic heart disease, cardiomyopathies and congestive heart failure, among others (Valko *et al.*, 2007; Berthiaume and Wallace, 2007). In fact, the heart is one of the most energy demanding tissues in the body and is totally dependent upon oxidative phosphorylation to supply the large amount of ATP required for beat-by-beat contraction and relaxation, which makes it quite susceptible to oxidative stress (Halestrap *et al.*, 2007). The enzymes xanthine oxidoreductase, NAD(P)H oxidase and nitric oxide synthase, as well as the mitochondrial cytochromes and haemoglobin may act as the main sources of oxidative stress in these diseases (Berry and Hare, 2004; Hare and Stamler, 2005). Furthermore, high levels of cholesterol and uptake of oxidised low-density lipoproteins (oxLDL), the main carriers of cholesterol in plasma, is a primary risk for development of atherosclerosis (Pryor, 2000; Podrez *et al.*, 2000). One of the most clinically relevant cardiac problems is ischemia-reperfusion injury. This type of injury involves an impairment of the blood flow to the heart as a result of damage to the myocardium (ischemia), in which the source of oxygen is removed, causing the cessation of oxidative phosphorylation. After a short period of ischemia, blood flow restoration occurs (reperfusion), which can, paradoxically, result in the aggravation of damage occurring during the ischemic period, and can lead to both apoptotic and necrotic cell death. An intracellular calcium overload and oxidative stress are two mechanisms that have been proposed to explain the pathogenesis of ischemia/reperfusion injury. Although the origin of ROS present during these events is yet to be well understood, it has been suggested that xanthine oxidase and NADPH oxidase, as well as mitochondria (via the electron transport chain) are mainly responsible for the massive ROS burst occurring during ischemia/reperfusion (Dhalla *et al.*, 2000; Halestrap *et al.*, 2007). In addition, increased oxidative stress causes abnormalities in the myocyte function, including inhibition of ATPases

present in the sarcolemma that, together with calcium release from the sarcoplasmic reticulum, can also contribute to the intracellular calcium overload (Valko *et al.*, 2007).

To date, many studies have demonstrated the involvement of oxidative stress in neurodegenerative diseases, such as Alzheimer's and Parkinson's (Cui *et al.*, 2004). For example, in the brains of patients with Alzheimer's disease, a significant amount of ROS/RNS has been detected, in association with a marked accumulation of amyloid-β peptide (the main constituent of senile plaques) and deposition of neurofibrillary tangles and neurophil threads (abnormal neurites) (Butterfield *et al.*, 2002). Several observations support the notion that the brain is particularly susceptible to oxidative stress: 1) this organ is known to consume about a fifth of the total oxygen used by the living organism, which increases the probability of ROS formation at the mitochondrial electron transport chain level; 2) neurons are post-mitotic (non-replicating) cells and any damage to brain tissues by ROS tends to be cumulative over time; 3) the high abundance in polyunsaturated fatty acids, which are particularly vulnerable to ROS damage; 4) the presence of high levels of transition metal ions (*e.g.* iron) that catalyse ROS formation through the Fenton reaction and the reduced levels of antioxidants able to segregate these transition metal ions, like transferrin and ceruloplasmin; 5) the release of excitatory neurotransmitters, such as glutamate, that induce a sequence of events in the post-synaptic neuron, which results in the formation of ROS; 6) the release of ROS during the oxidation of dopamine by monoamine oxidase in the nerve terminals of dopaminergic neurons may produce increased oxidative stress in brain regions, such as substantia nigra, which has been suggested to be a causative role in Parkinson's disease; 7) ascorbic acid, which is present at elevated levels in both white and grey matters, additionally to its antioxidant properties, can act as a pro-oxidant, when the free iron in brain regions increases due to intra-cerebral hemorrhage, for example; 8) in comparison with other tissues, there are low levels of antioxidant defences, such as catalase and glutathione peroxidase, in the brain (Cui *et al.*, 2004; Wilcox *et al.*, 2004; Lovell and Markesbery, 2007).

1.6. Aims of the study

The implication of oxidative stress in many pathological conditions has drawn the attention to the development of new molecules with antioxidant potential that can act beyond and/or potentiate the cells natural defence mechanisms.

In this work, we studied the pharmacological potential of four nitrogen compounds synthesised *de novo* (FMA4, FMA7, FMA762 and FMA796) on different situations involving oxidative stress and using different cell models. The compounds represented in Fig. 1.11 have an amidine group and a phenol unit. The association of these two moieties in the same molecule was expected to give them a superior antioxidant capacity, as previously referred (page 18).

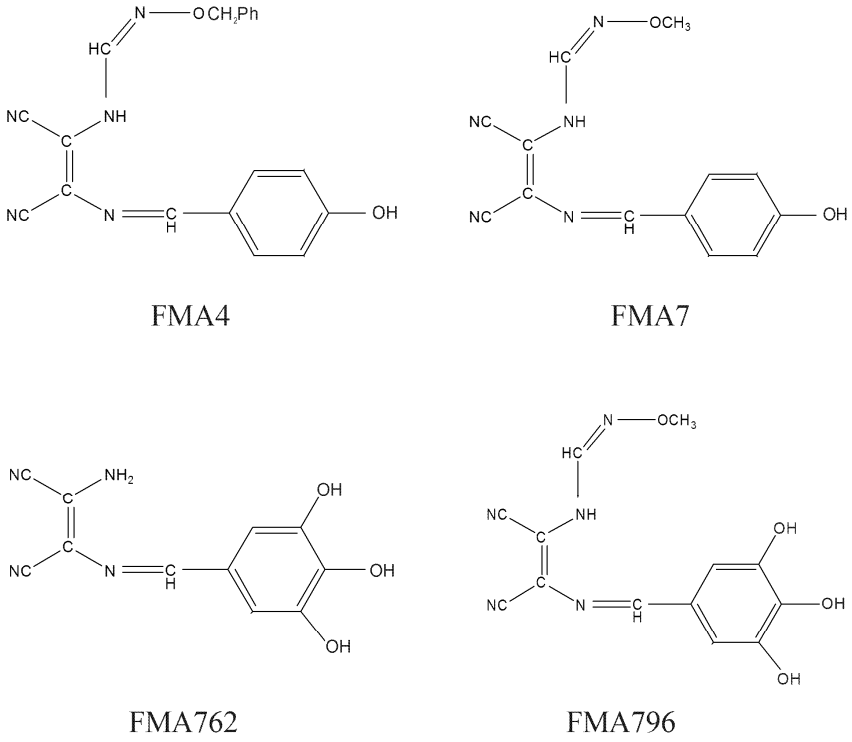


Figure 1.11 - Schematic structure of the newly synthesised nitrogen compounds in study. A phenolic unit is linked to an amidine function through a linear chain containing a nitrogen atom in three carbon atoms. FMA762 and FMA796 main difference from FMA4 and FMA7 is the presence of three hydroxyl groups within their phenol ring. The compounds were synthesised by F. Areias, Group of Organic Chemistry, University of Minho (Areias, 2006).

FMA4, FMA7 and FMA796 are (*Z*)-*N*-[1,2-dicyano-2-(arylidenamino)vinyl]-*O*-alkylformamidoximes synthesised from (*Z*)-*N*-(2-amino-1,2-dicyanovinyl)-*O*-alkylformamidoximes. While FMA4 has a benzyl substituent linked to the amidine moiety, FMA7 and FMA796 possess a methyl group instead. In addition, FMA796 contains three hydroxyl groups at the positions 3', 4' and 5' of the phenol structure, in comparison to just one hydroxyl group present in the phenol rings of FMA4 and FMA7. FMA762 is a 1-amino-2-arylidenamino-1,2-(dicyano)ethene synthesised from

diaminomaleonitrile (DAMN) and, similarly to FMA796, contains three hydroxyl groups within its phenol ring, linked to positions C-3, C-4 and C-5. The presence of more hydroxyl groups in FMA762 and FMA796 was expected to enhance the antioxidant activity of the phenol ring. In fact, in a study with modified flavones, Cotelle and co-workers concluded that the structures containing 2',3',4'-OH substituents presented higher antioxidant activities (Cotelle *et al.*, 1996). In accordance with that previous work, we have demonstrated low oxidation potential values and high antiradical activities, as determined by the DPPH discolouration test (Silva *et al.*, 2006; Areias, 2006).

This study intended to continue that previous work performed in our lab (Silva *et al.*, 2006), from which FMA4 and FMA7 emerged as promising molecules with good potential to fight off oxidative injury in mammalian cell models. In an attempt to improve the antioxidant potential of these nitrogen compounds, two other related molecules, FMA762 and FMA796, that emerged as promising in which regards their antioxidant capacities (Areias, 2006), were also studied.

Given that, our first objective was to further characterise those four compounds, at the biochemical level, in which concerns their protective effects against oxidative damage (**Chapter 2**). Two cell-free assays (DPPH discolouration and 2-deoxy-D-ribose degradation assays) were employed, in order to assess the compounds' antiradical activity. Their protective role against oxidative damage induced to cells was then evaluated by assessing the levels of intracellular ROS/RNS formation, lipid peroxidation and their effects on intracellular antioxidant defences, like GSH/GSSG, SOD and GPx. In these studies, PC12 cells, a neuronal cell line established from an adrenal pheochromocytoma that allows the correlation between oxidative stress and neurodegenerative diseases, was used.

Since oxidative DNA damage has been described as the type of damage most likely to occur in neuronal cells, we proceeded our investigation by assessing, using the Comet assay, the protective effects of these compounds on this specific type of damage, both at strand breaks and oxidised bases levels. In addition, by addressing specific repair enzymes, we intended to further characterise that protection and investigate the compounds' involvement in the mechanisms responsible for DNA repair. This was also achieved by assessing the expression of two DNA repair genes. The results obtained for the nitrogen compounds in this context are presented in **Chapter 3**.

The protective effects provided by three polyphenolic compounds from natural origin - luteolin, quercetin and rosmarinic acid - on oxidative DNA damage were also

evaluated during the course of this work (**Chapter 4**). In this way, a comparative study (at the level of oxidative DNA damage protection) between the new synthetic compounds and the polyphenols was performed.

Taking into consideration that oxidative stress is present in many other pathological conditions, namely in cardiac diseases (for example, as a result of ischemia/reperfusion), we intended to explore the spectrum of action of these compounds, in order to find them a more physiological application. So, in **Chapter 5** we present the results of the investigation of their protective role on oxidative injury and apoptosis induced to H9c2 cells. This cell line shows electrophysiological and biochemical properties of both skeletal and cardiac tissues, and has been considered a suitable model for the study of molecular responses to oxidative damage, particularly at the cardiac level. The mechanisms by which the compounds prevent *t*-BHP-induced cell death, with a particular focus on the mitochondrial-mediated pathway leading to apoptotic cell death, in addition to the compounds' effects on the associated morphological modifications, were investigated.

1.7. References

Ahkang, S., Jang, Y.J. and Park, H., 1998. *In vivo* dual effects of vitamin C on paraquat-induced lung damage: dependence on released metals from the damaged tissue. *Free Radic. Res.* **28:** 93-107.

Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y.Z., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y. and Yazaki, Y., 1997. Oxidative stress activates extracellular signal-regulated kinases through Src and ras in cultured cardiac myocytes of neonatal rats. *J. Clin. Invest.* **100:** 1813-1821.

Andreyev, A.Y., Kushnareva, Y.E. and Starkov, A.A., 2005. Mitochondrial metabolism of reactive oxygen species. *Biochemistry Mosc.* **70:** 200-214.

Antunes, F., Nunes, C., Laranjinha, J. and Cadenas, E., 2005. Redox interactions of nitric oxide with dopamine and its derivatives. *Toxicology* **208:** 207-212.

Aquilano, K., Filomeni, G., di Renzo, L., di Vito, M., di Stefano, C., Salimei, P.S., Ciriolo, M.R. and Marfe, G., 2007. Reactive oxygen and nitrogen species are involved in sorbitol-induced apoptosis of human erithroleukaemia cells K562. *Free Radic. Res.* **41:** 452-460.

Areias, F. M. 2006. Novos compostos heterocíclicos de azoto com unidades fenólicas: síntese e actividade biológica. *Ph.D. Thesis*. University of Minho, Braga, Portugal. 259p. Available online at: http://hdl.handle.net/1822/5941.

Arrigoni, O. and De Tullio, M.C., 2002. Ascorbic acid: much more than just an antioxidant. *Biochim. Biophys. Acta* **1569:** 1-9.

Azzi, A., Davies, K.J.A. and Kelly, F., 2004. Free radical biology - terminology and critical thinking. *FEBS Lett.* **558:** 3-6.

Babior, B.M., Takeuchi, C., Ruedi, J., Gutierrez, A. and Wentworth, P., 2003. Investigating antibody-catalyzed ozone generation by human neutrophils. *Proc. Natl. Acad. Sci. U. S. A.***100:** 3031-3034.

Bahi, N., Zhang, J.S., Llovera, M., Ballester, M., Comella, J.X. and Sanchis, D., 2006. Switch from caspase-dependent to caspase-independent death during heart development - Essential role of endonuclease G in ischemia-induced DNA processing of differentiated cardiomyocytes. *J. Biol. Chem.* **281:** 22943-22952.

Barañano, D.E. and Snyder, S.H., 2001. Neural roles for heme oxygenase: Contrasts to nitric oxide synthase. *Proc. Natl. Acad. Sci. U. S. A.* **98:** 10996-11002.

Barja, G., 1999. Mitochondrial oxygen radical generation and leak: Sites of production in state 4 and 3, organ specificity, and relation to aging and longevity. *J. Bioenerg. Biomembr.* **31:** 347-366.

Bartsch, H. and Nair, J., 2006. Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. *Langenbecks Arch. Surg.* **391:** 499-510.

Batinic-Haberle, I., Spasojevic, I., Stevens, R.D., Bondurant, B., Okado-Matsumoto, A., Fridovich, I., Vujaskovic, Z. and Dewhirst, M.W., 2006. New PEG-ylated Mn(III) porphyrins approaching catalytic activity of SOD enzyme. *Dalton Trans.* **4:** 617-624.

Baty, J.W., Hampton, M.B. and Winterbourn, C.C., 2005. Proteomic detection of hydrogen peroxide-sensitive thiol proteins in Jurkat cells. *Biochem. J.* **389:** 785-795.

Beal, M.F., 2002. Oxidatively modified proteins in aging and disease. *Free Radic. Biol. Med.* **32:** 797-803.

Beere, H.M., 2005. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J. Clin. Invest.* 115: 2633-2639.

Behl, C., 1999. Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches. *Prog. Neurobiol.* 57: 301-323.

Behl, C. and Moosmann, B., 2002. Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. *Free Radic. Biol. Med.* **33:** 182-191.

Berndtsson, M., Beaujoin, M., Rickardson, L., Havelka, A.M., Larsson, R., Liaudet-Coopman, E. and Linder, S., 2008. Induction of the lysosomal apoptosis pathway by inhibitors of the ubiquitin-proteasome system. *Int. J. Cancer* **124:** 1463-1469.

Berry, C.E. and Hare, J.M., 2004. Xanthine oxicloreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J. Physiol. (Lond.)* **555:** 589-606.

Berthiaume, J.M. and Wallace, K.B., 2007. Adriamycin-induced oxidative mitochondrial cardiotoxicity. *Cell Biol. Toxicol.* **23:** 15-25.

Bertram, C. and Hass, R., 2008. Cellular responses to reactive oxygen species-induced DNA damage and aging. *Biol. Chem.* **389:** 211-220.

Bitto, A., Minutoli, L., Squadrito, F., Polito, F. and Altavilla, D., 2007. Raxofelast, (+/-)5-(acetyloxy)-2,3-dihydro-4,6,7-trimethyl-2-benzofuranacetic acid: A new antioxidant to modulate the inflammatory response during ischemia-reperfusion injury and impaired wound healing. *Mini Rev. Med. Chem.* **7:** 339-343.

Bjelland, S. and Seeberg, E., 2003. Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutat. Res.* **531:** 37-80.

Boots, A.W., Li, H., Schins, R.P.F., Duffin, R., Heemskerk, J.W.M., Bast, A. and Haenen, G.R.M.M., 2007. The quercetin paradox. *Toxicol. Appl.Pharmacol.* **222:** 89-96.

Bouchier-Hayes, L., Lartigue, L. and Newmeyer, D.D., 2005. Mitochondria: pharmacological manipulation of cell death. *J. Clin. Invest.* **115:** 2640-2647.

Boujrad, H., Gubkina, O., Robert, N., Krantic, S. and Susin, S.A., 2007. AIF-mediated programmed necrosis - A highly regulated way to die. *Cell Cycle* **6:** 2612-2619.

Buettner, G.R., Ng, C.F., Wang, M., Rodgers, V.G.J. and Schafer, F.Q., 2006. A new paradigm: Manganese superoxide dismutase influences the production of H_2O_2 in cells and thereby their biological state. *Free Radic. Biol. Med.* **41:** 1338-1350.

Butterfield, D.A., Castegna, A., Lauderback, C.M. and Drake, J., 2002. Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol. Aging* **23**: 655-664.

Butterfield, D.A., Howard, B., Yatin, S., Koppal, T., Drake, J., Hensley, K., Aksenov, M., Aksenova, M., Subramaniam, R., Varadarajan, S., Harris-White, M.E., Pedigo, N.W., Jr. and Carney, J.M., 1999. Elevated oxidative stress in models of normal brain aging and Alzheimer's disease. *Life Sci.* **65:** 1883-1892.

Butts, E.D., Houde, C. and Mehmet, H., 2008. Maturation-dependent sensitivity of oligodendrocyte lineage cells to apoptosis: implications for normal development and disease. *Cell Death Diff.* **15:** 1178-1186.

Cadenas, E. and Davies, K.J.A., 2000. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic. Biol. Med.* **29:** 222-230.

Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J.P., Ravanat, J.L. and Sauvaigo, S., 1999. Hydroxyl radicals and DNA base damage. *Mutat. Res.* **424:** 9-21.

Cai, J. and Jones, D., 1999. Mitochondrial Redox Signaling during Apoptosis. *J. Bioenerg. Biomembr.* **31:** 327-334.

Cain, K., Bratton, S.B. and Cohen, G.M., 2002. The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie* **84:** 203-214.

Carr, A. and Frei, B., 1999. Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J.* **13:** 1007-1024.

Cemeli, E., Baumgartner, A. and Anderson, D., 2008. Antioxidants and the Comet assay. *Mutat. Res.* doi:10.1016/j.mrrev.2008.05.002.

Cherubini, A., Ruggiero, C., Polidori, M.C. and Mecocci, P., 2005. Potential markers of oxidative stress in stroke. *Free Radic. Biol. Med.* **39:** 841-852.

Choi, M.M., Kim, E.A., Hahn, H.G., Dal Nam, K., Yang, S.J., Choi, S.Y., Kim, T.U., Cho, S.W. and Huh, J.W., 2007. Protective effect of benzothiazole derivative KHG21834 on amyloid beta-induced neurotoxicity in PC12 cells and cortical and mesencephalic neurons. *Toxicology* **239:** 156-166.

Chowdhury, I., Tharakan, B. and Bhat, G.K., 2008. Caspases - An update. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* **151:** 10-27.

Chwieralski, C.E., Welte, T. and Buhling, F., 2006. Cathepsin-regulated apoptosis. *Apoptosis* **11:** 143-149.

Clingen, P.H., Arlett, C.F., Roza, L., Mori, T., Nikaido, O. and Green, M.H.L., 1995. Induction of cyclobutane pyrimidine dimers, pyrimidine(6-4)pyrimidone photoproducts, and Dewar valence isomers by natural sunlight in normal human mononuclear cells. *Cancer Res.* **55:** 2245-2248.

Collins, A.R. and Gaivão, I., 2007. DNA base excision repair as a biomarker in molecular epidemiology studies. *Mol. Asp. Med.* **28:** 307-322.

Collins, A.R., Harrington, V., Drew, J. and Melvin, R., 2003. Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis* **24:** 511-515.

Collins, A.R., Ralová, K., Somorovská, M., Petrovská, H., Ondruová, A., Vohnout, B., Fábry, R. and Duinská, M., 1998. DNA Damage in Diabetes: Correlation with a Clinical Marker. *Free Radic. Biol. Med.* **25:** 373-377.

Conner, E.M. and Grisham, M.B., 1996. Inflammation, free radicals, and antioxidants. *Nutr.* **12:** 274-277.

Conus, S. and Simon, H.U., 2008. Cathepsins: Key modulators of cell death and inflammatory responses. *Biochem. Pharmacol.* **76:** 1374-1382.

Cooke, M.S., Evans, M.D., Dizdaroglu, M. and Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.* **17:** 1195-1214.

Coppedè, F., Mancuso, M., Lo Gerfo, A., Manca, M.L., Petrozzi, L., Migliore, L., Siciliano, G. and Murri, L., 2007. A Ser326Cys polymorphism in the DNA repair gene hOGG1 is not associated with sporadic Alzheimer's disease. *Neurosci. Lett.* **414:** 282-285.

Cotelle, N., Bernier, J.L., Catteau, J.P., Pommery, J., Wallet, J.C. and Gaydou, E.M., 1996. Antioxidant properties of hydroxy-flavones. *Free Radic. Biol. Med.* **20:** 35-43.

Cui, H., Schroering, A. and Ding, H.F., 2002. p53 Mediates DNA damaging drug-induced apoptosis through a caspase-9-dependent pathway in SH-SY5Y Neuroblastoma Cells. *Mol. Cancer Ther.* **1:** 679-686.

Cui, J., Holmes, E.H., Greene, T.G. and Liu, P.K., 2000. Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain. *FASEB J.* 14: 955-967.

Cui, K., Luo, X., Xu, K. and Ven Murthy, M.R., 2004. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and

nutraceutical antioxidants. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **28:** 771-799.

Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R. and Milzani, A., 2006. Protein carbonylation, cellular dysfunction, and disease progression. *J. Cell. Mol. Med.* **10:** 389-406.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. and Colombo.R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **329:** 23-38.

Dalton, T.P., Shertzer, H.G. and Puga, A., 1999. Regulation of gene expression by reactive oxygen. *Annu. Rev. Pharmacol. Toxicol.* **39:** 67-101.

Day, B.J., 2009. Catalase and glutathione peroxidase mimics. *Biochem. Pharmacol.* **77:** 285-296.

de Bruin, E.C. and Medema, J.P., 2008. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treat. Rev.* **34:** 737-749.

de Waard, H., Sonneveld, E., de Wit, J., Lange, R.E., Hoeijmakers, J.H.J., Vrieling, H. and van der Horst, G.T.J., 2008. Cell-type-specific consequences of nucleotide excision repair deficiencies: Embryonic stem cells versus fibroblasts. *DNA Repair* **7:** 1659-1669.

Depeint, F., Bruce, W.R., Shangari, N., Mehta, R. and O'Brien, P.J., 2006. Mitochondrial function and toxicity: Role of the B vitamin family on mitochondrial energy metabolism. *Chem. Biol. Int.***163:** 94-112.

Deshmane, S.L., Mukerjee, R., Fan, S., Del Valle, L., Michiels, C., Sweet, T., Rom, I., Khalili, K., Rappaport, J., Amini, S. and Sawaya, B.E., 2009. Activation of the oxidative stress pathway by HIV-1 Vpr leads to induction of hypoxia inducible factor 1 alpha expression. *J. Biol. Chem.* 10.1074/jbc.M809266200: M809266200.

Deshpande, S.S., Angkeow, P., Kuang, J.P., Ozaki, M. and Irani, K., 2000. Rac1 inhibits TNF-alpha-induced endothelial cell apoptosis: dual regulation by reactive oxygen species. *FASEB J.* **14:** 1705-1714.

Dhalla, N.S., Elmoselhi, A.B., Hata, T. and Makino, N., 2000. Status of myocardial antioxidants in ischemia-reperfusion injury. *Cardiovasc. Res.* **47:** 446-456.

Di Carlo, G., Mascolo, N., Izzo, A.A. and Capasso, F., 1999. Flavonoids: Old and new aspects of a class of natural therapeutic drugs. *Life Sci.* **65:** 337-353.

Dobra, K. and Hjerpe, A., 2008. Targeted therapy - possible new therapeutic option for malignant mMesothelioma? *Connect. Tissue Res.* **49:** 270-272.

Dodson, M.L. and Lloyd, R.S., 2002. Mechanistic comparisons among base excision repair glycolsylases. *Free Radic. Biol. Med.* **32:** 678-682.

Dong, C., Li, Q., Lyu, S.C., Krensky, A.M. and Clayberger, C., 2005. A novel apoptosis pathway activated by the carboxyl terminus of p21. *Blood* **105:** 1187-1194.

Douki, T., Reynaud-Angelin, A., Cadet, J. and Sage, E., 2003. Bipyrimidine photoproducts rather than oxidative lesions are the main type of DNA damage involved in the genotoxic effect of solar UVA radiation. *Biochemistry* **42:** 9221-9226.

Drake, J., Sultana, R., Aksenova, M., Calabrese, V. and Butterfield, D.A., 2003. Elevation of mitochondrial glutathione by gamma-glutamylcysteine ethyl ester protects mitochondria against peroxynitrite-induced oxidative stress. *J. Neurosci. Res.* **74:** 917-927.

Droge, W., 2002. Free radicals in the physiological control of cell function. *Physiol. Rev.* **82:** 47-95.

Ehrlich, K., Viirlaid, S., Mahlapuu, R., Saar, K., Kullisaar, T., Zilmer, M., Langel, U. and Soomets, U., 2007. Design, synthesis and properties of novel powerful antioxidants, glutathione analogues. *Free Radic. Res.* **41:** 779-787.

Elbling, L., Weiss, R.M., Teufelhofer, O., Uhl, M., Knasmueller, S., Schulte-Hermann, R., Berger, W. and Micksche, M., 2005. Green tea extract and (-)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. *FASEB J.* **19:** 807-833.

ESCODD - European Standard Committee on Oxidative DNA Damage, 2003. Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. *Free Radic. Biol. Med.* **34:** 1089-1099.

Evans, M.D., Dizdaroglu, M. and Cooke, M.S., 2004. Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res.* **567:** 1-61.

Fagan, J., Sleczka, B.G. and Sohar, I., 1999. Quantitation of oxidative damage to tissue proteins. *Int. J. Biochem. Cell Biol.* **31:** 751-757.

Fantini, D., Vascotto, C., Deganuto, M., Bivi, N., Gustincich, S., Marcon, G., Quadrifoglio, F., Damante, G., Bhakat, K.K., Mitra, S. and Tell, G., 2008. APE1/Ref-1 regulates PTEN expression mediated by Egr-1. *Free Radic. Res.* **42:** 20-29.

Ferguson, L.R., 2001. Role of plant polyphenols in genomic stability. *Mutat. Res.* **475:** 89-111.

Ferri, K.F., Jacotot, E., Blanco, J., Este, J.A., Zamzami, N., Susin, S.A., Xie, Z.H., Brothers, G., Reed, J.C., Penninger, J.M. and Kroemer, G., 2000. Apoptosis control in syncytia induced by the HIV type 1-envelope glycoprotein complex: Role of mitochondria and caspases. *J. Exp. Med.* **192:** 1081-1092.

Filippin, L.I., Vercelino, R., Marroni, N.P. and Xavier, R.M., 2008. Redox signalling and the inflammatory response in rheumatoid arthritis. *Clin. Exp. Immunol.* **152:** 415-422.

Finkel, T., 2003. Oxidant signals and oxidative stress. *Curr. Opin. Cell Biol.* **15:** 247-254.

Fishel, M.L., Vasko, M.R. and Kelley, M.R., 2007. DNA repair in neurons: So if they don't divide what's to repair? *Mutat. Res.* **614:** 24-36.

Fleury, C., Mignotte, B. and Vayssiere, J.L., 2002. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* **84:** 131-141.

Frosina, G., 2006. Prophylaxis of oxidative DNA damage by formamidopyrimidine-DNA glycosylase. *Int. J. Cancer* **119:** 1-7.

Futerman, A.H. and Hannun, Y.A., 2004. The complex life of simple sphingolipids. *EMBO Rep.* **5:** 777-782.

Gartel, A.L., 2008. Transcriptional inhibitors, p53 and apoptosis. *Biochim. Biophys. Acta* **1786:** 83-86.

Gedik, C.M. and Collins, A., 2004. Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study. *FASEB J.* **18:** 82-104.

Gil, J., Almeida, S., Oliveira, C.R. and Rego, A.C., 2003a. Cytosolic and mitochondrial ROS in staurosporine-induced retinal cell apoptosis. *Free Radic. Biol. Med.* **35:** 1500-1514.

Gil, L., Martínez, G., González, I., Tarinas, A., Álvarez, A., Giuliani, A., Molina, R., Tápanes, R., Pérez, J. and León, O.S., 2003b. Contribution to characterization of oxidative stress in HIV/AIDS patients. *Pharmacol. Res.* **47:** 217-224.

Girotti, A.W., 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J. Lipid Res.* **39:** 1529-1542.

Golks, A., Brenner, D., Fritsch, C., Krammer, P.H. and Lavrik, I.N., 2005. c-FLIPR, a new regulator of death receptor-induced apoptosis. *J. Biol. Chem.* **280:** 14507-14513.

Gorbunova, V., Seluanov, A., Mao, Z. and Hine, C., 2007. Changes in DNA repair during aging. *Nucleic Acids Res.* **35:** 7466-7474.

Grether-Beck, S., Felsner, I., Brenden, H. and Krutmann, J., 2003. Mitochondrial cytochrome c release mediates ceramide-induced activator protein 2 activation and gene expression in keratinocytes. *J. Biol. Chem.* **278:** 47498-47507.

Griendling, K.K., Sorescu, D., Lassegue, B. and Ushio-Fukai, M., 2000. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler. Thromb. Vasc. Biol.* **20:** 2175-2183.

Gupta, M., Dobashi, K., Greene, E.L., Orak, J.K. and Singh, I., 1997. Studies on hepatic injury and antioxidant enzyme activities in rat subcellular organelles following in vivo ischemia and reperfusion. *Mol. Cell. Biochem.* **176:** 337-347.

Halestrap, A.P., Clarke, S.J. and Khaliulin, I., 2007. The role of mitochondria in protection of the heart by preconditioning. *Biochim. Biophys. Acta* **1767:** 1007-1031.

Halliwell, B., 2001. Role of free radicals in the neurodegenerative diseases - Therapeutic implications for antioxidant treatment. *Drugs Aging* **18:** 685-716.

Halliwell, B., Aeschbach, R., Loliger, J. and Aruoma, O.I., 1995. The characterization of antioxidants. *Food Chem. Toxicol.* **33:** 601-617.

Halliwell, B. and Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. 3rd edition, Oxford University Press, New York.

Hanawalt, P.C., 2002. Subpathways of nucleotide excision repair and their regulation. *Oncogene* **21:** 8949-8956.

Hare, J.M. and Stamler, J.S., 2005. NO/redox disequilibrium in the failing heart and cardiovascular system. *J. Clin. Invest.* **115:** 509-517.

He, A., Wangi, J.A., Guil, C., Jiang, Y., Sun, Y. and Chen, T., 2007. Changes of mitochondrial pathway in hypoxia/reoxygenation induced cardiomyocytes apoptosis. *Folia Histochem. Cytobiol.* **45:** 397-400.

Helleday, T., 2003. Pathways for mitotic homologous recombination in mammalian cells. *Mutat. Res.* **532:** 103-115.

Hendriks, J.J.A., Alblas, J., van der Pol, S.M., van Tol, E.A., Dijkstra, C.D. and de Vries, H.E., 2004. Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. *J. Exp. Med.* **200:** 1667-1672.

Hengartner, M.O., 2000. The biochemistry of apoptosis. *Nature* 407: 770-776.

Henriquez, M., Armisen, R., Stutzin, A. and Quest, A.F.G., 2008. Cell death by necrosis, a regulated way to go. *Curr. Mol. Med.* **8:** 187-206.

Hiona, A. and Leeuwenburgh, C., 2008. The role of mitochondrial DNA mutations in aging and sarcopenia: Implications for the mitochondrial vicious cycle theory of aging. *Exp. Gerontol.* **43:** 24-33.

Hiroi, M., Ogihara, T., Hirano, K., Hasegawa, M., Morinobu, T., Tamai, H. and Niki, E., 2005. Regulation of apoptosis by glutathione redox state in PC12 cells exposed simultaneously to iron and ascorbic acid. *Free Radic. Biol. Med.* **38:** 1057-1072.

Inoue, M., Sato, E.F., Nishikawa, M., Park, A.M., Kira, Y., Imada, I. and Utsumi, K., 2003. Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Curr. Med. Chem.* **10:** 2495-2505.

Jaattela, M., Cande, C. and Kroemer, G., 2004. Lysosomes and mitochondria in the commitment to apoptosis: a potential role for cathepsin D and AIF. *Cell Death Differ.* **11:** 135-136.

Jang, J.H., Aruoma, O.I., Jen, L.S., Chung, H.Y. and Surh, Y.J., 2004. Ergothioneine rescues PC12 cells from beta-amyloid-induced apoptotic death. *Free Radic.Biol Med.* **36:** 288-299.

Jezek, P. and Hlavata, L., 2005. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int. J. Biochem. Cell Biol.* **37:** 2478-2503.

Jung, J.Y., Han, C.R., Jeong, Y.J., Kim, H.J., Lim, H.S., Lee, K.H., Park, H.O., Oh, W.M., Kim, S.H. and Kim, W.J., 2007. Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. *Neurosci. Lett.* **411:** 222-227.

Kagedal, K., Zhao, M., Svensson, I. and Brunt, U.T., 2001. Sphingosine-induced apoptosis is dependent on lysosomal proteases. *Biochem. J.* **359:** 335-343.

Kim, D.S., Kim, H.R., Woo, E.R., Hong, S.T., Chae, H.J. and Chae, S.W., 2005. Inhibitory effects of rosmarinic acid on adriamycin-induced apoptosis in H9c2 cardiac muscle cells by inhibiting reactive oxygen species and the activations of c-Jun N-terminal

kinase and extracellular signal-regulated kinase. *Biochem. Pharmacol.* **70:** 1066-1078.

Kim, H.J., Mun, J.Y., Chun, Y.J., Choi, K.H. and Kim, M.Y., 2001. Bax-dependent apoptosis induced by ceramide in HL-60 cells. *FEBS Lett.* **505:** 264-268.

Kirkegaard, T. and Jaattela, M., 2009. Lysosomal involvement in cell death and cancer. *Biochim. Biophys. Acta*, doi: 10.1016/j.bbamcr.2008.09.008.

Kohen, R. and Nyska, A., 2002. Invited review: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol. Pathol.* **30:** 620-650.

Korkmaz, A., Reiter, R.J., Topal, T., Manchester, L.C., Oter, S. and Tan, D.-X., 2009. Melatonin: an established antioxidant worthy of use in clinical trials. *Mol. Med.* **15:** 43-50.

Kourtis, N. and Tavernarakis, N., 2008. Autophagy and cell death in model organisms. *Cell Death Differ.* **16:** 21-30.

Krantic, S., Mechawar, N., Reix, S. and Quirion, R., 2007. Apoptosis-inducing factor: A matter of neuron life and death. *Progr. Neurobiol.* **81:** 179-196.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., El-Deiry, W.S., Golstein, P., Green, D.R., Hengartner, M., Knight, R.A., Kumar, S., Lipton, S.A., Malorni, W., Nunez, G., Peter, M.E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B. and Melino, G., 2008. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ.* **16:** 3-11.

Krueger, A., Schmitz, I., Baumann, S., Krammer, P.H. and Kirchhoff, S., 2001. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J. Biol. Chem.* **276:** 20633-20640.

Kruman, I.I., 2004. Why do neurons enter the cell cycle? *Cell Cycle* **3:** 769-773.

L'Ecuyer, T., Sanjeev, S., Thomas, R., Novak, R., Das, L., Campbell, W. and Vander Heide, R., 2006. DNA damage is an early event in doxorubicin-induced cardiac myocyte death. *Am. J. Physiol. Heart Circ. Physiol.* 291: H1273-H1280.

Lambert, J.D., Hong, J., Yang, G.Y., Liao, J. and Yang, C.S., 2005. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *Am. J. Clin. Nutr.* **81:** 284S-291.

Lanza, V. and Vecchio, G., 2008. New conjugates of superoxide dismutase/catalase mimetics with cyclodestrins. *J. Inorg. Biochem.* doi:10.1016/j.jinorgbio.2008.11.017.

Lebeau, J., Furman, C., Bernier, J.L., Duriez, P., Teissier, E. and Cotelle, N., 2000. Antioxidant properties of di-*tert*-butylhydroxylated flavonoids. *Free Radic. Biol. Med.* **29:** 900-912.

Lee, D.H., Folsom, A.R., Harnack, L., Halliwell, B. and Jacobs, D.R., Jr., 2004. Does supplemental vitamin C increase cardiovascular disease risk in women with diabetes? *Am. J. Clin. Nutr.* **80:** 1194-1200.

Lee, W.K. and Thévenod, F., 2008. Novel roles for ceramides, calpains and caspases in kidney proximal tubule cell apoptosis: Lessons from in vitro cadmium toxicity studies. *Biochem. Pharmacol.* **76:** 1323-1332.

Lehuédé, J., Fauconneau, B., Barrier, L., Ourakow, M., Piriou, A. and Vierfond, J.-M., 1999. Synthesis and antioxidant activity of new tetraarylpyrroles. *Eur. J. Med. Chem.* **34:** 991-996.

Leist, M. and Jaattela, M., 2001. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat. Rev. Mol. Cell Biol.* **2:** 589-598.

Leung, H.W.C., Wu, C.H., Lin, C.H. and Lee, H.Z., 2005. Luteolin induced DNA damage leading to human lung squamous carcinoma CH27 cell apoptosis. *Eur. J. Pharmacol.* **508:** 77-83.

Levi, S., Santambrogio, P., Corsi, B., Cozzi, A. and Arosio, P., 1996. Evidence that residues exposed on the three-fold channels have active roles in the mechanism of ferritin iron incorporation. *Biochem. J.* **317**: 467-473.

Lien, E.J., Ren, S., Bui, H.H. and Wang, R., 1999. Quantitative structure-activity relationship analysis of phenolic antioxidants. *Free Radic. Biol. Med.* **26:** 285-294.

Lillig, C.H., Lonn, M.E., Enoksson, M., Fernandes, A.P. and Holmgren, A., 2004. Short interfering RNA-mediated silencing of glutaredoxin 2 increases the sensitivity of HeLa cells toward doxorubicin and phenylarsine oxide. *Proc. Natl. Acad. Sci U.S.A.* **101:** 13227-13232.

Lima, C.F., Fernandes-Ferreira, M. and Pereira-Wilson, C., 2006. Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. *Life Sci.* **79:** 2056-2068.

Limón-Pacheco, J. and Gonsebatt, M.E., 2008. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutat. Res.* doi:10.1016/j.mrgentox.2008.09.015.

Lin, Y., Choksi, S., Shen, H.M., Yang, Q.F., Hur, G.M., Kim, Y.S., Tran, J.H., Nedospasov, S.A. and Liu, Z.G., 2004. Tumor Necrosis Factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J. Biol. Chem.* **279:** 10822-10828.

Lindholm, D., Wootz, H. and Korhonen, L., 2006. ER stress and neurodegenerative diseases. *Cell Death Differ.* **13:** 385-392.

Loft, S. and Moller, P., 2006. Oxidative DNA damage and human cancer: Need for cohort studies. *Antioxid. Redox Signal.* **8:** 1021-1031.

Lopez-Sanchez, N., Rodriguez, J.R. and Frade, J.M., 2007. Mitochondrial c-Jun NH2-terminal kinase prevents the accumulation of reactive oxygen species and reduces necrotic damage in neural tumor cells that lack trophic support. *Mol. Cancer Res.* **5:** 47-60.

Lorenzo, H.K. and Susin, S.A., 2007. Therapeutic potential of AIF-mediated caspase-independent programmed cell death. *Drug Resist. Updat.* **10:** 235-255.

Lovell, M.A. and Markesbery, W.R., 2007. Oxidative damage in mild cognitive impairment and early Alzhelimer's disease. *J. Neurosci. Res.* **85:** 3036-3040.

Macdonald, J., Galley, H.F. and Webster, N.R., 2003. Oxidative stress and gene expression in sepsis. *Br. J. Anaesth.* **90:** 221-232.

Manach, C., Scalbert, A., Morand, C., Remesy, C. and Jimenez, L., 2004. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **79:** 727-747.

Marczin, N., El-Habashi, N., Hoare, G.S., Bundy, R.E. and Yacoub, M., 2003. Antioxidants in myocardial ischemia-reperfusion injury: therapeutic potential and basic mechanisms. *Arch. Biochem. Biophys.* **420:** 222-236.

Martin, L.J., 2008. DNA damage and repair: Relevance to mechanisms of neurodegeneration. *J. Neuropathol. Exp. Neurol.* **67:** 377-387.

Martinez-Ruiz, A. and Lamas, S., 2004. S-nitrosylation: a potential new paradigm in signal transduction. *Cardiovasc. Res.* 62: 43-52.

Masella, R., Di Benedetto, R., Vari, R., Filesi, C. and Giovannini, C., 2005. Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes. *J. Nutr. Biochem.* **16:** 577-586.

Matés, J.M., 2000. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* 153: 83-104.

Matés, J.M., Pérez-Gómez, C. and De Castro, I.N., 1999. Antioxidant enzymes and human diseases. *Clin. Biochem.* **32:** 595-603.

Matés, J.M. and Sanchez-Jimenez, F.M., 2000. Role of reactive oxygen species in apoptosis: implications for cancer therapy. *Int. J. Biochem. Cell Biol.* **32:** 157-170.

McCall, M.R. and Frei, B., 1999. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic. Biol. Med.* **26:** 1034-1053.

Mccord, J.M. and Fridovich, I., 1969. Superoxide Dismutase an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244:** 6049-6055.

Meagher, E.A. and FitzGerald, G.A., 2000. Indices of lipid peroxidation in vivo: strengths and limitations. *Free Radic. Biol. Med.* **28:** 1745-1750.

Mehta, R., Shangari, N. and O'Brien, P.J., 2008. Preventing cell death induced by carbonyl stress, oxidative stress or mitochondrial toxins with vitamin B anti-AGE agents. *Mol. Nutr. Food Res.* **52:** 379-385.

Montiel-Duarte, C., Ansorena, E., Lopez-Zabalza, M.J., Cenarruzabeitia, E. and Iraburu, M.J., 2004. Role of reactive oxygen species, glutathione and NF-kappaB in apoptosis induced by 3,4-methylenedioxymethamphetamine ("Ecstasy") on hepatic stellate cells. *Biochem. Pharmacol.* **67:** 1025-1033.

Moon, Y.J., Wang, X. and Morris, M.E., 2006. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. *Toxicol. in Vitro* **20:** 187-210.

Mozaffarieh, M., Grieshaber, M.C., Orgul, S. and Flammer, J., 2008. The Potential Value of Natural Antioxidative Treatment in Glaucoma. *Surv. Ophthalmol.* **53:** 479-505.

Munteanu, A., Zingg, J.M., Ogru, E., Libinaki, R., Gianello, R., West, S. and Azzi, A., 2004. Modulation of cell proliferation and gene expression by alpha-tocopheryl phosphates - Relevance to atherosclerosis and inflammation. *Biochem. Biophys. Res. Comm.* **1031:** 405-411.

Mwanjewe, J., Hui, B.K., Coughlin, M.D. and Grover, A.K., 2001. Treatment of PC12 cells with nerve growth factor increases iron uptake. *Biochem. J.* **357:** 881-886.

Nagayama, T., Lan, J., Henshall, D.C., Chen, D.X., O'Horo, C., Simon, R.P. and Chen, J., 2000. Induction of oxidative DNA damage in the peri-infarct region after permanent focal cerebral ischemia. *J. Neurochem.* **75:** 1716-1728.

Nair, U., Bartsch, H. and Nair, J., 2007. Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans. *Free Radic. Biol. Med.* **43:** 1109-1120.

Nakagawa, T. and Yuan, J., 2000. Cross-talk between two cysteine protease families: activation of xaspase-12 by xalpain in apoptosis. *J. Cell Biol.* **150:** 887-894.

Neri, S., Gardini, A., Facchini, A., Olivieri, F., Franceschi, C., Ravaglia, G. and Mariani, E., 2005. Mismatch repair system and aging: Microsatellite instability in peripheral blood cells from differently aged participants. *J. Gerontol. A Biol. Sci. Med. Sci.* **60:** 285-292.

Niki, E., 2000. Oxidative stress and aging. *Intern. Med.* **39:** 324-326.

Niki, E., Yoshida, Y., Saito, Y. and Noguchi, N., 2005. Lipid peroxidation: Mechanisms, inhibition, and biological effects. *Biochem. Biophys. Res. Comm.* **338:** 668-676.

Nordberg, J. and Arner, E.S., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* **31:** 1287-1312.

Nunes, C., Almeida, L. and Laranjinha, J., 2008. 3,4-Dihydroxyphenylacetic acid (DOPAC) modulates the toxicity induced by nitric oxide in PC-12 cells via mitochondrial dysfunctioning. *Neurotoxicology* **29:** 998-1007.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Jr., Valanis, B., Williams, J., Jr., Barnhart, S., Cherniack, M.G., Brodkin, C.A. and Hammar, S., 1996. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. *J. Natl. Cancer Inst.* **88:** 1550-1559.

Petrosillo, G., Ruggiero, F.M. and Paradies, G., 2003. Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. *FASEB J.* **17:** 2202-2208.

Pias, E.K. and Aw, T.Y., 2002. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC12 cells. *Cell Death Differ.* **9:** 1007-1016.

Podrez, E.A., Abu-Soud, H.M. and Hazen, S.L., 2000. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic. Biol. Med.* **28:** 1717-1725.

Powell, C.L., Swenberg, J.A. and Rusyn, I., 2005. Expression of base excision DNA repair genes as a biomarker of oxidative DNA damage. *Cancer Lett.* **229:** 1-11.

Pryor, W.A., 2000. Vitamin E and heart disease:: Basic science to clinical intervention trials. *Free Radic. Biol. Med.* **28:** 141-164.

Radi, R., Peluffo, G., Alvarez, M.N., Naviliat, M. and Cayota, A., 2001. Unraveling peroxynitrite formation in biological systems. *Free Radic. Biol. Med.* **30:** 463-488.

Rao, A.V. and Rao, L.G., 2007. Carotenoids and human health. *Pharmacol. Res.* **55:** 207-216.

Rao, K.S., 2007. DNA repair in aging rat neurons. *Neurosci.* **145:** 1330-1340.

Reeve, J.L.V., Szegezdi, E., Logue, S.E., Chonghaile, T.N., O'Brien, T., Ritter, T. and Samali, A., 2007. Distinct mechanisms of cardiomyocyte apoptosis induced by doxorubicin and hypoxia converge on mitochondria and are inhibited by Bcl-XL. *J. Cell. Mol. Med.* **11:** 509-520.

Rice-Evans, C., Miller, N.J. and Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids . *Free Radic. Biol. Med.* **20:** 933-956.

Riso, P., Visioli, F., Grande, S., Guarnieri, S., Gardana, C., Simonetti, P. and Porrini, M., 2006. Effect of a tomato-based drink on markers of inflammation, immunomodulation, and oxidative stress. *J. Agric. Food Chem.* **54:** 2563-2566.

Robichova, S., Slamenova, D., Chalupa, I. and Sebova, L., 2004. DNA lesions and cytogenetic changes induced by N-nitrosomorpholine in HepG2, V79 and VH10 cells: the protective effects of Vitamins A, C and E. *Mutat. Res.* **560:** 91-99.

Rolo, A. and Palmeira, C.M., 2006. Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress. *Toxicol. Appl. Pharmacol.* **212:** 167-178.

Romieu, I., 2005. Nutrition and lung health. *Int. J. Tuberc. Lung Dis.* **9:** 362-374.

Roninson, I.B., Broude, E.V. and Chang, B.D., 2001. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist. Updat.* **4:** 303-313.

Rubbo, H., Radi, R., Anselmi, D., Kirk, M., Barnes, S., Butler, J., Eiserich, J.P. and Freeman, B.A., 2000. Nitric oxide reaction with lipid peroxyl radicals spares alpha-tocopherol during lipid peroxidation. Greater oxidant protection from the pair nitric oxide/alpha-tocopherol than alpha-tocopherol/ascorbate. *J. Biol. Chem.* **275:** 10812-10818.

Ruiz, P.A. and Haller, D., 2006. Functional diversity of flavonoids in the inhibition of the proinflammatory NF-kappa B, IRF, and Akt signaling pathways in murine intestinal epithelial cells. *J. Nutr.* **136:** 664-671.

Santocono, M., Zurria, M., Berrettini, M., Fedeli, D. and Falcioni, G., 2007. Lutein, zeaxanthin and astaxanthin protect against DNA damage in SK-N-SH human neuroblastoma cells induced by reactive nitrogen species. *J. Photochem. Photobiol. B* **88:** 1-10.

Schafer, F.Q. and Buettner, G., 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30**: 1191-1212.

Schafer, Z.T. and Kornbluth, S., 2006. The Apoptosome: physiological, developmental, and pathological modes of regulation. *Develop. Cell* **10:** 549-561.

Schneider, C., 2005. Chemistry and biology of vitamin E. *Mol. Nutr. Food Res.* **49:** 7-30.

Schwarze, S.R., Lin, E.W., Christian, P.A., Gayheart, D.T. and Kentucky, L., 2008. Intracellular death platform steps-in: Targeting prostate tumors via endoplasmic reticulum (ER) apoptosis. *The Prostate* **68:** 1615-1623.

Sellak, H., Franzini, E., Hakim, J. and Pasquier, C., 1994. Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood* **83:** 2669-2677.

Seluanov, A., Mittelman, D., Pereira-Smith, O.M., Wilson, J.H. and Gorbunova, V., 2004. DNA end joining becomes less efficient and more error-prone during cellular senescence. *Proc. Natl. Acad. Sci. U.S.A.* **101:** 7624-7629.

Serafini, M., Laranjinha, J., Almeida, L.M. and Maiani, G., 2000. Inhibition of human LDL lipid peroxidation by phenol-rich beverages and their impact on plasma total antioxidant capacity in humans. *J. Nutr. Biochem.* **11:** 585-590.

Semenza, G.L., 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J. Appl. Physiol.* **88:** 1474-1480.

Shapiro, S.S. and Saliou, C., 2001. Role of vitamins in skin care. *Nutr.* **17:** 839-844.

Siddaiah, V., Maheswara, M., Rao, C.V., Venkateswarlu, S. and Subbaraju, G.V., 2007. Synthesis, structural revision, and antioxidant activities of antimutagenic homoisoflavonoids from *Hoffmanosseggia intricata*. *Bioorg. Med. Chem. Lett.* **17:** 1288-1290.

Sies, H., 1997. Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* **82:** 291-295.

Silva, J.P., Areias, F.M., Proença, M.F. and Coutinho, O.P., 2006. Oxidative stress protection by newly synthesized nitrogen compounds with pharmacological potential. *Life Sci.* **78:** 1256-1267.

Somayajulu, M., McCarthy, S., Hung, M., Sikorska, M., Borowy-Borowski, H. and Pandey, S., 2005. Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. *Neurobiol. Dis.* **18:** 618-627.

Spiteller, G., 2001. Lipid peroxidation in aging and age-dependent diseases. *Exp. Gerontol.* **36:** 1425-1457.

Stadtman, E.R., 2004. Role of oxidant species in aging. *Curr. Med. Chem.* **11:** 1105-1112.

Strauss, S.J., Higginbottom, K., Juliger, S., Maharaj, L., Allen, P., Schenkein, D., Lister, T. and Joel, S.P., 2007. The proteasome inhibitor Bortezomib acts independently of p53 and induces cell death via apoptosis and mitotic catastrophe in B-cell lymphoma cell lines. *Cancer Res.* **67:** 2783-2790.

Suh, H.-J., Chung, M.-S., Cho, Y.-H., Kim, J.-W., Kim, D.-H., Han, K.-W. and Kim, C.-J., 2005. Estimated daily intakes of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyl hydroquinone (TBHQ) antioxidants in Korea. *Food Addit. Contam.* **22:** 1176-1188.

Tamimi, R.M., Hankinson, S.E., Campos, H., Spiegelman, D., Zhang, S., Colditz, G.A., Willett, W.C. and Hunter, D.J., 2005. Plasma carotenoids, retinol, and tocopherols and risk of breast cancer. *Am. J. Epidemiol.* **161:** 153-160.

Tang, D., Lahti, J.M. and Kidd, V.J., 2000. Caspase-8 activation and bid cleavage contribute to MCF7 cellular execution in a caspase-3-dependent manner during staurosporine-mediated apoptosis. *J. Biol. Chem.* **275:** 9303-9307.

The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group, 1994. The effect of vitamin E and Beta carotene on the incidence of lung cancer and other cancers in male smokers. *N. Engl. J. Med.* **330:** 1029-1035.

Tomasetti, M., Alleva, R. and Collins, A.R., 2001. In vivo supplementation with coenzyme Q(10) enhances the recovery of human lymphocytes from oxidative DNA damage. *FASEB J.* **15:** 1425-1451.

Trova, M.P., Gauuan, P.J., Pechulis, A.D., Bubb, S.M., Bocckino, S.B., Crapo, J.D. and Day, B.J., 2003. Superoxide dismutase mimetics. Part 2: synthesis and structure-Activity relationship of glyoxylate- and glyoxamide-Derived metalloporphyrins. *Bioorg. Med. Chem.* **11:** 2695-2707.

Tudek, B., Swoboda, M., Kowalczyk, P. and Olinski, R., 2006. Modulation of oxidative DNA damage repair by the diet, inflammation and neoplastic transformation. *J. Physiol. Pharm.* **57:** 33-49.

Turrens, J.F., 2003. Mitochondrial formation of reactive oxygen species. *J. Physiol. (Lond.)* **552:** 335-344.

Uemura, M., Manabe, H., Yoshida, N., Fujita, N., Ochiai, J., Matsumoto, N., Takagi, T., Naito, Y. and Yoshikawa, T., 2002. Alpha-tocopherol prevents apoptosis of vascular endothelial cells via a mechanism exceeding that of mere antioxidation. *Eur. J. Pharmacol.* **456:** 29-37.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J. and Telser, J., 2004. Role of oxygen radicals in DNA damage and cancer incidence. *Mol. Cell. Biochem.* **266:** 37-56.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39:** 44-84.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem. Biol. Int.* **160:** 1-40.

Vanasco, V., Cimolai, M.C., Evelson, P. and Alvarez, S., 2008. The oxidative stress and the mitochondrial dysfunction caused by endotoxemia are prevented by alpha-lipoic acid. *Free Radic. Res.* **42:** 815-823.

Vogel, S., Ohmayer, S., Brunner, G. and Heilmann, J., 2008. Natural and non-natural prenylated chalcones: Synthesis, cytotoxicity and anti-oxidative activity. *Bioorg. Med. Chem.* **16:** 4286-4293.

Wentrup, C., 1984. Reactive molecules: the neutral reactive intermediates in organic chemistry. 23-161. New York: Wiley; 1984. p 23-161.

Wilcox, J.K., Ash, S.L. and Catignani, G.L., 2004. Antioxidants and prevention of chronic disease. *Crit. Rev. Food Sci. Nutr.* **44:** 275-295.

Williamson, G. and Manach, C., 2005. Bioavailability and bioefficacy of polyphenols in humans II: Review of 93 intervention studies. *Am. J. Clin. Nutr.* **81:** 243-255.

Young, I.S. and McEneny, J., 2001. Lipoprotein oxidation and atherosclerosis. *Biochem. Soc. Trans.* **29:** 358-362.

Zhao, M., Antunes, F., Eaton, J.W. and Brunt, U.T., 2003. Lysosomal enzymes promote mitochondrial oxidant production, cytochrome *c* release and apoptosis. *Eur. J. Biochem.* **270:** 3778-3786.

Zhu, H. and Bunn, H.F., 1999. Oxygen sensing and signaling: impact on the regulation of physiologically important genes. *Respir. Physiol.* **115:** 239-247.

Zhu, Y., Hoell, P., Ahlemeyer, B. and Krieglstein, J., 2006. PTEN: A crucial mediator of mitochondria-dependent apoptosis. *Apoptosis* **11:** 197-207.

Zimmermann, K.C., Bonzon, C. and Green, D.R., 2001. The machinery of programmed cell death. *Pharmacol. Ther.* **92:** 57-70.

CHAPTER: 2

PROTECTIVE ROLE OF NEW NITROGEN COMPOUNDS ON ROS/RNS-MEDIATED DAMAGE TO PC12 CELLS

Silva, J.P., Proença, M.F. and Coutinho, O.P., 2008. Protective role of new nitrogen compounds on ROS/RNS-mediated damage to PC12 cells. *Free Radic. Res.* **42:** 57-69.

Abstract

Reactive oxygen (ROS) and nitrogen (RNS) species are known to be involved in many degenerative diseases. Here, we report the study of four new nitrogen compounds from organic synthesis, identified as FMA4, FMA7, FMA762 and FMA796, which differ mainly by the number of hydroxyl groups within their phenolic unit. Their potential role as antioxidants was evaluated in PC12 cells by assessing their protection against oxidative and nitrosative insults. The four compounds, and particularly FMA762 and FMA796, were able to protect cells against lipid peroxidation and intracellular ROS/RNS formation to a great extent. Their protective effects were likely mediated by their free radicals scavenge ability, as they appeared to be involved neither in the induction of natural antioxidant enzymes like GSH-PX and SOD, nor in the inhibition of NOS. Nevertheless, these results suggest a promising potential for these compounds as ROS/RNS scavengers in pathologies where oxidative/nitrosative stress are involved.

2.1. Introduction

Reactive oxygen (ROS) and nitrogen (RNS) species are produced during normal cellular function and in response to various stimuli (Mates, 2000; Somayajulu *et al.*, 2005). Vital beneficial physiological cellular use of ROS, which include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) has been demonstrated in different functions including intracellular signalling, host defence, and redox regulation (Lopez-Lluch *et al.*, 1998; Nordberg and Arner, 2001; Behl and Moosmann, 2002; Valko *et al.*, 2006). Moreover, nitric oxide ($^{\bullet}NO$), a RNS, can act as an important neurotransmitter or neuromodulator in the central and peripheral nervous systems (Calabrese *et al.*, 2000; Jung *et al.*, 2007).

Endogenous and exogenous factors may trigger the overproduction of ROS/RNS (Wilcox *et al.*, 2004). To withstand these sudden stressful changes, organisms have developed a variety of enzymatic (*e.g.* superoxide dismutase, catalase and glutathione-related enzymes) and non enzymatic (*e.g.* glutathione and vitamin E) antioxidant systems that can either prevent the formation of ROS/RNS or convert them to inactive derivatives (Fleury *et al.*, 2002; Stadtman, 2004; Doulias *et al.*, 2007). Nevertheless, when an imbalance occurs between oxidants and antioxidants in favour of the oxidants, we come to a situation defined as oxidative stress, which leads to cellular damage responsible for degenerative conditions like those that occur in Alzheimer, Parkinson and atherosclerosis, among others (Tamagno *et al.*, 1998; Halliwell, 2001; Jang *et al.*, 2004; Piga *et al.*, 2005; Tang *et al.*, 2005).

In an effort to prevent or diminish ROS/RNS-induced damage, many investigators have been focused on the evaluation of either natural or synthetic compounds that can act beyond and/or potentiate the cells natural defence mechanisms (Sasaki *et al.*, 2002; Nie *et al.*, 2002; Lin *et al.*, 2006; Siddaiah *et al.*, 2007).

In this study, we used new nitrogen compounds from organic synthesis, which are nitrogenated structures composed of an amidine unit and a phenol ring (Figure 2.1). The choice of these molecules was based both on the knowledge that the hydroxyl groups of the phenol ring are usually responsible for the antioxidant properties and on the knowledge that nitrogen compounds (in particular, nitrogen heterocycles incorporating an imidazole or an amidine unit) can easily interact with active centres responsible for different functions in living organisms (Areias, 2006).

The association of these two moieties in the same molecule was expected to result in new structures capable of acting as antioxidants in living systems. Molecules incorporating conjugated systems with nitrogen atoms are also known to stabilize free radicals (Wentrup, 1984) and this combination was also expected to enhance the antioxidant activity of the phenolic unit (Areias *et al.*, 2001).

We previously reported (Silva *et al.*, 2006) a good antioxidant potential for two of these new nitrogen compounds, named FMA4 and FMA7. Furthermore, in a parallel study involving the synthesis of nitrogen structures (Areias, 2006), two other compounds, FMA762 and FMA796, emerged as promising in which concerns their antioxidant capacities. That study suggested that the presence of three hydroxyl groups in C-3, -4 and -5 positions of the aromatic substituent, as is the case of these two structures, improved the antiradical activity of the compounds.

In the present study, we intended to further characterize these four new nitrogen structures (FMA4, FMA7, FMA762 and FMA796) in which respects to ROS/RNS-mediated damage induced in a neuronal cell model. PC12 cells were used since they have some advantages over primary cultured neuronal cells, including the homogeneity of the cell population (Hong and Liu, 2004; Colognato *et al.*, 2006).

2.2. Materials and methods

2.2.1. Compounds

The new compounds used in this study (Figure 2.1) were prepared in the group of Organic Synthesis, Chemistry Department, University of Minho, from the reaction of an appropriate phenolic aldehyde with a substituted amidine, as described in (Areias, 2006). The experimental procedure was adapted from previous work (Booth *et al.*, 1999) carried out on a selection of mono-substituted aldehydes. All of these structures present a linear chain, differing mainly by the presence of one (FMA4 and FMA7) or three (FMA762 and FMA796) hydroxyl groups present within the phenol ring.

The compounds were provided as a yellowish powder, which was reconstituted in DMSO, aliquoted and maintained frozen at -80ºC until utilization. Each aliquot was thawed only once.

Foetal bovine serum (FBS) was from BioChrom KG (Berlin, Germany); horse serum donor herd was purchased from Gibco (Paisley, UK). 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was purchased from Molecular Probes (Eugene, OR, USA). NitroBlue Tetrazolium was purchased from Diagnostica Merck (Darmstadt, Germany). 6-hydroxi-2,5,7,8-tetrametilcromano-2-carboxylic acid 97% (trolox) were purchased from Sigma-Aldrich Chemie (Berlin, Germany). All other reagents, including those for cell culture, were purchased from Sigma Chemical Company (St Louis, USA).

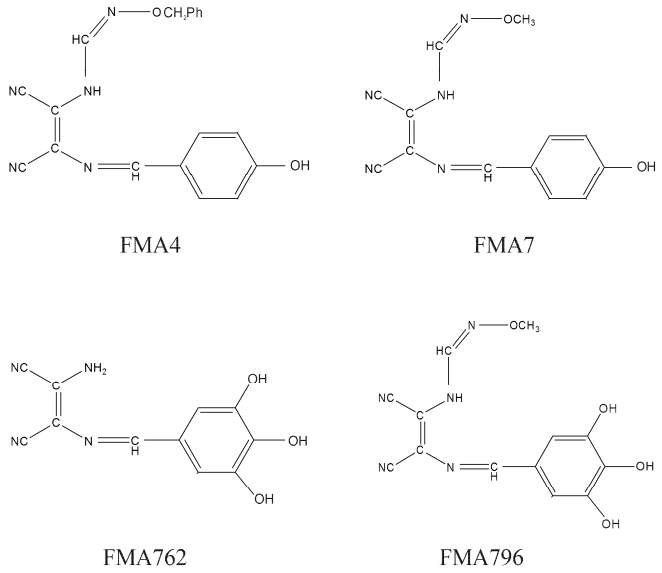


Figure 2.1 – Schematic structure of the newly synthesized nitrogen compounds. Two phenolic units are linked to an amidine function through a linear chain containing a nitrogen atom in three carbon atoms. FMA762 and FMA796 differ mainly from FMA4 and FMA7 by the presence of three hydroxyl groups. The compounds were prepared by F. Areias, Group of Organic Chemistry, University of Minho (Areias, 2006).

2.2.2. PC12 cell culture and treatment conditions

PC12, a neuronal cell line established from a rat adrenal pheochromocytoma (Greene and Tischler, 1976) was used in this study. There are many reports of the use of this cell line as a model for the study of neurodegenerative disorders in which oxidative stress is involved, such as Parkinson's or Alzheimer disease (Oliveira *et al.*, 2002; Wang *et al.*, 2003; Hiroi *et al.*, 2005; Piga *et al.*, 2005; Jung *et al.*, 2007).

Cells were cultured in suspension in 75 cm^2 flasks, in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) of an antibiotic/antimycotic solution. Cultures were maintained in a humidified incubator containing 95% air and 5% CO_2, and passed twice a week. Before each assay, the cell aggregates were carefully disrupted by gently pipetting and the separated cells plated in poly-D-lysine-coated multiwells, at a density of 2.5×10^5 cells/cm^2, for the MTT assay, and at a density of 1.6×10^5 cells/cm^2 for other studies. After plating, cells were left for adhesion overnight. The compounds in study were usually added to the cells three hours prior to the addition of the deleterious stimuli.

2.2.3. Determination of the radical scavenging effect — DPPH assay

Free radical scavenging capacity of the compounds was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) discoloration method, according to the procedure previously described (Silva *et al.*, 2006). The reduction of this radical by an antioxidant compound results in a decrease in absorbance and is proportional to the number of electrons absorbed (Parejo *et al.*, 2000), indicating the antiradical capacity of the substances in study.

Briefly, the discoloration rate of a 0.002% ethanolic solution of DPPH was followed at 517nm, along the time, in a SpectraMax 340PC microplate reader, versus a control containing ethanol instead of the compound in study. The inhibition of discoloration was expressed as a percentage, towards the control, and the IC_{50} were then obtained from the inhibition curve. The absorbance stabilization time was also determined. Antiradical Efficiency (AE) was determined according to the formula

$$AE = 1 / (IC_{50} \times T_{IC50}) \quad (2.1)$$

where IC_{50} is the concentration needed to reduce the DPPH discoloration by 50% and T_{IC50} is the time needed to reach the discoloration steady state at IC_{50} concentration (Sanchez-Moreno *et al.*, 1998).

2.2.4. Measurement of 2-deoxy-D-ribose degradation

The compounds scavenging activity against the hydroxyl radical was evaluated by the 2-deoxy-D-ribose degradation assay. Hydroxyl radicals formed in this method through a mixture of ascorbic acid, H_2O_2 and EDTA-Fe^{3+} (Haber-Weiss reaction) degrade deoxyribose in a series of fragments, which react on heating with thiobarbituric acid to give a pink chromogen. If a compound is added to the reaction mixture, that can scavenge the hydroxyl radical more efficiently than deoxyribose, then its degradation will be slower, as well as the chromogen formation (Halliwell *et al.*, 1995).

A reaction mixture was prepared in 10 mM KH_2PO_4-KOH, pH 7.4, containing 2.8 mM deoxyribose, 20 μM $FeCl_3$ (dissolved in 2 mM Na_2EDTA), 1.42 mM H_2O_2, 50 μM ascorbic acid, in the presence or absence of the compounds in study at their IC_{50} concentrations (obtained by the DPPH discoloration method). After 1 h incubation at 37ºC, the reaction was stopped by adding 1% thiobarbituric acid in 50 mM NaOH and 2.8% trichloroacetic acid. The reaction mixture was then heated at 100ºC for 15 minutes. After cooling, absorbance values were determined at 535 nm in a microplate reader (SpectraMax 340PC).

2.2.5. Evaluation of the degree of hydrophobicity

The drugs hydrophobicity was determined by measuring the partition coefficients (PC), in an *n*-octanol/HEPES system. The nitrogen compounds were dissolved in *n*-octanol at a concentration of 20 μM, and 1 ml of each solution was shaken with 20 ml HEPES (20 mM, pH 7.4) for about 10 min, at room temperature. The two different phases formed were then separated by centrifugation. The absorbance peaks of each drug, needed to assess the concentrations in each solution, were determined as 380 nm for FMA762 and 415 nm for FMA796. PC values were then calculated using the formula:

$$PC = \log (C_O / C_H) \qquad (2.2)$$

where C_O and C_H are the concentrations of the drugs in *n*-octanol and in HEPES, respectively. The C_H values were indirectly determined by calculating the difference between the initial and the final concentrations of the drug in octanol.

2.2.6. Analysis of cell survival

Cell viability in the presence of the compounds was evaluated by the MTT reduction test, as previously described (Silva *et al.*, 2006). Briefly, 0.5 ml MTT (final concentration 0.5 mg/ml, in Krebs medium, pH = 7.4), prepared just before usage and maintained in the dark, was added to the PC12 cells. The plate, wrapped in aluminium foil, was left incubating for 2 hours. Hydrogen chloride 0.04 M in isopropanol was then added, followed by 2 hours more of orbital shaking, in the dark, to dissolve the formazan crystals. The survival of PC12 cells was expressed as the percentage of OD towards control cells, containing the same amount of the drug solvent, DMSO.

2.2.7. Measurement of the extent of lipid peroxidation – TBARS assay

Lipid peroxidation was evaluated by measuring the levels of Thiobarbituric Acid-Reactive Substances (TBARS), which are expressed in terms of malondialdehyde (MDA) equivalents that react with thiobarbituric acid (TBA) (Hodges *et al.*, 1999). TBARS formation was induced by the oxidant pair ascorbate/iron for 1h at 37ºC, as previously described (Silva *et al.*, 2006). Absorbance was read at 530 nm in a multiplate reader (Spectramax 340PC). The amount of TBARS produced was calculated using the molar absorption coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$, corrected for the total protein content (Sedmak and Grossberg, 1977) and expressed as nmol TBARS/mg protein.

The antioxidant capacity of the drugs was evaluated determining the percentage of protection offered by each drug against the lipid peroxidation induced by the oxidant pair, by using the normalization proposed by Singh (1998) (Singh *et al.*, 1998):

$$\% \text{ Protection} = 1 - [(D-C) / OP] \times 100 \qquad (2.3)$$

where D is the amount of TBARS in the presence of the drug, C is the basal lipid peroxidation (negative control) and OP is the amount of TBARS in the presence of the oxidant pair.

2.2.8. Measurement of intracellular reactive oxygen and nitrogen species

Levels of intracellular ROS were measured by flow cytometry as the fluorescence of DCF, which is the oxidation product of $DCFH_2$-DA that is an ester that freely permeates the cells membranes. After entering the cells, $DCFH_2$-DA loses its diacetate group (becoming $DCFH_2$), by esterase action, and can then be oxidized to highly fluorescent DCF. ROS and RNS like H_2O_2, NO and its reaction product with $O_2^{-}\cdot$, which is the highly reactive peroxynitrite ($ONOO^-$), can oxidize $DCFH_2$ (Possel *et al.*, 1997; Sharikabad *et al.*, 2001).

PC12 cells were incubated with 10 μM $DCFH_2$-DA for 45 min, in the dark and at 37ºC, after being subjected to the oxidative insult (2 mM ascorbate/100 μM Fe^{2+}) for 1h or with the NO donor Sodium Nitroprusside (SNP) for 20h. After two washes with PBS, cells were scrapped and collected for flow cytometry analysis in an Epics® XL-MCL™ (Beckman Coulter) flow cytometer equipped with an argon-ion laser emitting a 488-nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass. At least 20 000 cells were analysed per sample at a low flow rate. Data were analysed by WinMDI 2.8 software.

2.2.9. Nitrite assay

Accumulation of extracellular nitrites (NO_2^-), an indicator of NO synthase activity, was measured in the culture medium by the Griess reaction. This assay relies on a simple colorimetric reaction between nitrite and Griess reagent [0.1% N-(naphthyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid] to produce a pink azo product (Jang and Suhr, 2005; Tsikas, 2007). Concentrations of nitrite, the end-product of NO production, were quantified 20h after addition of the compounds and/or SNP to the cells. The Griess reagent (100 μl) was then added to 100 μl of sample of the cell culture medium, mixed and incubated for 20 min at room temperature. The optical density (OD) was measured at 540 nm in a microplate reader (SpectraMax 340PC). Nitrites concentrations were determined from a calibration curve of the absorbances obtained for a set of standards of sodium nitrite prepared in culture medium.

2.2.10. Determination of GSH/GSSG content

The glutathione (GSH) content of PC12 cells, incubated for 2 h 30 min in the presence of 1 mM *tert*-butylhydroperoxide (*t*-BHP), was assessed by the DTNB-GSSG reductase recycling assay, based in the methodology of Anderson (Anderson, 1985), with some slight modifications. Briefly, 360 µl of culture medium was added to 40 µl of 50% (w/v) 5-sulfosalicylic acid (SSA) for protein precipitation and centrifuged for 1 min at 10 000 *g*. These samples allowed the quantification of GSH and oxidized glutathione (GSSG) in the extracellular medium. The cells that remained attached to the wells were scrapped in PBS and 360 µl of this cell suspension were added to 40 µl of 50% (w/v) SSA and then centrifuged. This procedure allowed for quantification of intracellular GSH and GSSG. Total glutathione levels were measured in the supernatants, by following the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) oxidation, at 415 nm, and comparing it to a GSH standard curve. For GSSG measurement, 100 µl of supernatant was derivatized with 2 µl of 2-vinylpyridine and 10 µl of 50% (v/v) triethanolamine and continuously mixed for 1 h. GSSG levels were then quantified in the same way as previously described for total glutathione, but using a GSSG standard curve instead. GSH content was calculated by subtracting GSSG content from the total glutathione level.

2.2.11. Determination of antioxidant enzymes activity

The activity of antioxidant enzymes was measured after a pre-incubation period, with the compounds, at 3 h. PC12 cells were then subjected to 1 mM *t*-BHP for 2.5h more. After that, cells were washed and suspended in ice-cold PBS (pH 7.4) and, after a short-pulse sonication to promote cell lysis, centrifuged at 13 000 *g* for 1 min. All enzyme activities were measured in the supernatants. Determination of glutathione peroxidase (GSH-Px) activity is based on the oxidation of reduced glutathione by GSH-Px, using *t*-BHP as a substrate, coupled to the disappearance of NADPH by glutathione reductase (Flohé and Gunzler W.A., 1984; Alia *et al.*, 2006). This reaction was monitored by following the decrease in absorbance at 340nm, on a microplate reader (SpectraMax 340PC). One mili-unit (mU) of GSH-Px was defined as the amount of enzyme that catalyses the oxidation of 1nmol of NADPH per minute. Superoxide dismutase (SOD) activity was determined

spectrophotometrically at 546 nm by following the reduction of NitroBlue Tetrazolium (NBT) by the xanthine/xanthine oxidase system (McCord and Fridovich, 1969; Pias *et al.*, 2003). One unit (U) of SOD was defined as the amount of enzyme needed to inhibit the reduction rate of NBT in 50%. This assay measures the activities of the three most discussed types of SOD, namely Cu/Zn-SOD, Mn-SOD, and Fe-SOD. All the enzymes activities were expressed relatively to the amount of protein in the cell extracts.

2.2.12. Total protein quantification

Protein content was measured with the Bradford Reagent purchased from Sigma (St. Louis, Missouri) using a bovine serum albumin standard.

2.2.13. Statistical analysis

Data are expressed as the mean ± S.E.M., of the indicated number of experiments. The significance of the differences between the means observed was evaluated using the unpaired two-tailed Student's *t*-test. A difference of $p \leq 0.05$ was considered significant.

2.3. Results

2.3.1. Determination of the radical scavenging effect

It is well established that the antioxidant activity of a drug depends on its ability to scavenge free radicals (Beckman and Ames, 1998). The DPPH discoloration assay, a method frequently used to evaluate the radical scavenging activity of the compounds by themselves (Okawa *et al.*, 2001; Molyneux, 2004), was used as a first approach. The first step was to determine the stabilization time for the discoloration of a DPPH ethanolic solution, as previously reported (Silva *et al.*, 2006). We considered 40 min as a relative steady state discoloration time for all the compounds. With the absorbance results obtained at this timepoint we calculated the percentages of inhibition of discoloration for each drug concentration. For the antiradical efficiency calculations we considered two parameters: the IC_{50} obtained

for each drug (directly taken from the dose-response curves) and the time required by each compound to reach the steady state of DPPH discoloration, at this IC_{50} concentration. This time of stabilization at the IC_{50} concentration was found to be 15 and 35 min for FMA762 and FMA796, respectively. Results in Table 2.1 show that FMA762 and FMA796 have higher antiradical efficiency values than the antioxidant trolox, used as a reference, and than FMA4 and FMA7 (Silva *et al.*, 2006). This is the first indication of their improved ability to scavenge free radicals.

Table 2.1 - Free radical scavenging parameters of the different compounds tested.

Compounds	DPPH discoloration assay			2-Deoxy-D-ribose degradation (%)
	IC_{50} (μM)	T_{IC50} (min)	Antiradical Efficiency ($\times 10^{-3}$)	
Control	---	---	---	**100.0 ± 4.5**
FMA762	**3.7 ± 0.7**	**15**	**18.02**	**37.9 ± 2.3** ***
FMA796	**3.4 ± 0.3**	**35**	**8.40**	**38.7 ± 2.1** ***
FMA4[(a)]	**19.8 ± 0.1**	**40**	**1.26**	---
FMA7[(a)]	**20.4 ± 0.2**	**40**	**1.23**	---
Trolox	**9.0 ± 0.2**	**20**	**5.56**	**76.6 ± 2.6** **

Different concentrations of each drug were added to the ethanolic solution of DPPH and the discoloration measured spectrophotometrically, at 517 nm, after 40 min. Results were expressed as the percentage of DPPH discoloration towards a positive control containing only the DPPH solution. The antiradical efficiency was calculated as described in Materials and Methods. Degradation of 2-deoxy-D-ribose degradation was assessed by adding the compounds, at the IC_{50} concentrations, to a reaction mixture as described in Materials and Methods. Results were expressed as the percentage, relatively to the control (containing DMSO instead of the compound) of 2-deoxy-D-ribose degradation occurring in the presence of Fe^{3+} and ascorbic acid. Data represent mean ± S.E.M for at least three different experiments. ** $p \leq 0.01$, *** $p \leq 0.001$, compared to control. [(a)] FMA4 and FMA7 results in Silva *et al.* (2006).

The scavenging effect of the compounds on hydroxyl radicals was assayed by measuring the effect on the 2-deoxy-D-ribose degradation produced by the reaction of Fe^{3+} with ascorbic acid, in the presence of EDTA. Table 2.1 shows that FMA762 and FMA796, at their IC_{50} concentrations, attenuated the iron plus ascorbic acid

mediated deoxyribose degradation by 62.1 ± 2.3 and 61.3 ± 2.1%, respectively. This attenuation was higher than the one observed for trolox (23.4 ± 2.6%), indicating the compounds greater ability to scavenge hydroxyl radicals when compared with the traditional antioxidant, trolox. These results indicate that FMA762 and FMA796 are good scavengers of hydroxyl radicals and validate the ones obtained for the DPPH discoloration assay.

2.3.2. Determination of intracellular reactive oxygen species

An effective antioxidant should be able to scavenge intracellular reactive oxygen/nitrogen species (ROS/RNS) in order to stop radical chain reactions, or to inhibit the reactive oxidants from being formed in the first place (Behl and Moosmann, 2002; Huang *et al.*, 2005).

The effects of the new compounds on intracellular ROS content were assessed with the fluorescent probe DCF, by flow cytometry analysis. The pair ascorbate/ Fe^{2+}, which has been described as adequate to induce oxidative stress in neuronal models such as retinal cells (Rego *et al.*, 1998; Areias *et al.*, 2001) and was also used in our previous studies (Silva *et al.*, 2006), was used as a free radical generator. In this case, the ferrous iron (Fe^{2+}) outside the cells is easily oxidized to ferric iron (Fe^{3+}). Ascorbate acts as a pro-oxidant agent, reducing Fe^{3+} and originating Fe^{2+}. The interaction of this Fe^{2+} with the hydrogen peroxide inside the cells originates hydroxyl radicals through the Fenton reaction, which are susceptible of inducing the oxidative stress cascade of events (Shapiro and Saliou, 2001; Hiroi *et al.*, 2005).

Our data in Figure 2.2 shows the increase in fluorescence originated by the reaction of DCF with ROS. In the presence of the oxidant pair 2 mM ascorbate/100 μM Fe^{2+} the cells display an increase in fluorescence (2.73 ± 0.21, $p \leq 0.001$) relatively to the control (cells alone). Pre-treatment with FMA762 and FMA796 for 3 hours resulted in a statistically significant reduction in fluorescence intensity, when compared to the cells treated with the oxidant pair. This decrease in intracellular ROS formation was similar to the one observed for α-tocopherol, the liposoluble form of vitamin E, used as a positive control due to its known ROS scavenging properties (Hong and Liu, 2004). FMA7 and FMA4 were not able to reduce intracellular ROS levels induced by the oxidant pair.

Thus, we can say that among the new compounds tested, FMA762 and FMA796 are the most efficient ones, showing a high protective effect on ascorbate/iron-induced intracellular ROS formation.

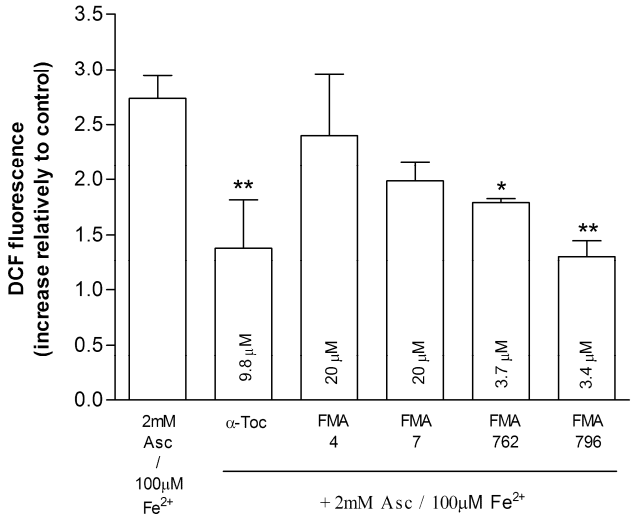


Figure 2.2 - Inhibitory effect of the selected compounds on intracellular ROS formation. Compounds were added to the cells 3 hours prior to the incubation with the oxidant pair 2 mM ascorbate / 100 μM Fe^{2+} for 1 h. ROS formation was assayed by flow cytometry after 45 min incubation with 10 μM DCFH-DA. α-Tocopherol (α-Toc) was used as a positive control. Results were expressed as the increase in fluorescence relatively to control. For each bar is represented the mean ± S.E.M for at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, compared to oxidant pair.

2.3.3. Effect of the compounds on ascorbate/iron-induced lipid peroxidation

It is known that metal-induced generation of oxygen radicals results in the attack of different cellular components, including polyunsaturated fatty acid residues of phospholipids which are, because of their multiple double bonds, excellent targets for free radical attacks (Nordberg and Arner, 2001; Valko *et al.*, 2006).

The extent of protection on lipid peroxidation presented by the compounds in study is shown in Figure 2.3. The new compounds were tested at their IC_{50} concentrations and at a concentration 5-fold their IC_{50}. For the lower concentrations, the protective effect was of about 40-50%. Furthermore, for the higher ones (17 μM and 18.5 μM), FMA762 and FMA796 protection (64.9 ± 5.7% and 50.5 ± 2.0%,

respectively) is higher than the one of trolox (37.5 ± 3.1%) and, in the case of FMA762 is even similar to the one of α-tocopherol (68.3 ± 4.7%).

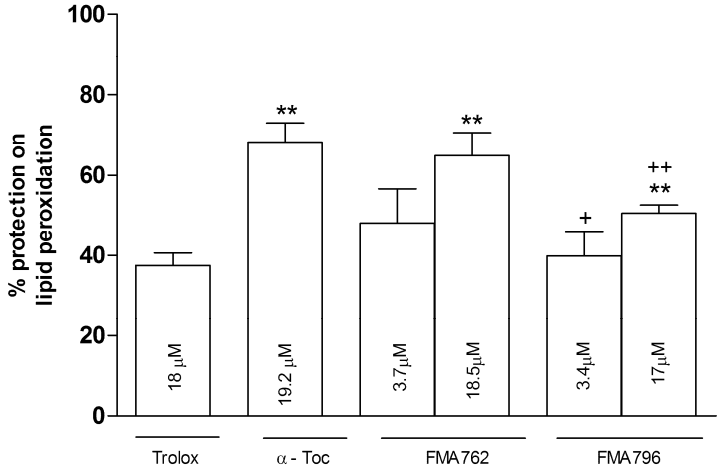


Figure 2.3 - Relative drug protection on lipid peroxidation, in PC12 cell model. The oxidant pair concentration used was 2 mM ascorbate/100 μM Fe^{2+}. Cells, at a density of 1.6 × 10^5 cells/cm^2, were pre-incubated with the drugs for 3 h, prior to the addition of the oxidant pair, which was left for 1 h more. Each column represents the mean ± S.E.M., considering the results obtained for at least 3 different experiments. ** $p \leq 0.01$, compared to trolox. $^{+}$ $p \leq 0.05$, $^{++}$ $p \leq 0.01$, compared to α-tocopherol.

In comparison to our results previously obtained with FMA4 and FMA7 (Silva *et al.*, 2006), FMA762 and FMA796, which differ mainly by the presence of three hydroxyl groups within their phenol ring, show an improved ability to protect cells from the ascorbate/iron-induced lipid peroxidation, which is in agreement with their greater capacity to prevent intracellular ROS formation (Figure 2.2).

2.3.4. Measurement of the compounds liposolubility

The partition coefficient is a physico-chemical property of a compound that can be linked to its biological behaviour (Bhat *et al.*, 2002; Rogachev *et al.*, 2003). Therefore, the degree of hydrophobicity of the new compounds was measured by determining this parameter in an *n*-octanol/HEPES system. Partition coefficients for FMA762 and FMA796 were 1.30 ± 0.02 and 1.91 ± 0.12, respectively. These results are indicative of the relative liposolubility of the new compounds in study, which is intermediate between the hydrosoluble form of vitamin E, trolox, and the liposoluble

one, α-tocopherol (Silva *et al.*, 2006). This fact could explain the intermediate protection (between these two forms of vitamin E) previously observed on lipid peroxidation (Figure 2.3).

Among the tested compounds, FMA796 presents a higher affinity for the octanol phase (highest PC value). This is even higher than the PCs previously obtained for FMA4 and FMA7 (Silva *et al.*, 2006), which again confirms its increased ability to act intracellularly.

2.3.5. Effect of the compounds on SNP-induced toxicity

Nitric oxide production is commonly associated with degenerative conditions such as Alzheimer and Parkinson's diseases (Araujo *et al.*, 2003; Jang and Suhr, 2005). Therefore, an antioxidant compound would be of greater value if it could also decrease the levels of nitrosative stress.

In this context, Sodium Nitroprusside (SNP) was used as a NO generator inside the cells. However, it has been previously reported that toxicity induced by SNP is associated with a decrease in cell viability, as a consequence of the stimulation of reactive oxygen and nitrogen species related to the release of NO, and subsequent generation of even more reactive molecules, like peroxynitrite (Bastianetto and Quirion, 2002; Jung *et al.*, 2007).

Indeed, treatment of PC12 cells with 400 μM SNP resulted in a decrease in cell viability of 51.5 % (MTT assay), as it is shown in Figure 2.4A. Both FMA762 and FMA796 led to a significant increase in cell survival, in the presence of SNP, revealing a good ability to attenuate the NO-induced cytotoxicity. Their beneficial effect was even better than the one observed for the vitamin E hydrosoluble analogue, trolox. As expected, the DCF assay indicated that 400 μM SNP caused a significant increase in ROS/RNS accumulation (2.4 ± 0.2 above control values, $p \leq 0.001$) 20 h after its addition to the culture medium (Figure 2.4B). SNP-induced ROS/RNS production was significantly attenuated by FMA762 (1.7 ± 0.2, $p \leq 0.05$) and FMA796 (1.6 ± 0.3, $p \leq 0.05$). On the other hand, both FMA4 and FMA7 were unable to attenuate the effect of SNP on ROS/RNS intracellular accumulation. The lack of inhibitory effects of the former against toxic events initiated by SNP were shared by 45μM trolox (used as control), which is in accordance with other published papers (Bastianetto *et al.*, 2000). So, FMA762 and FMA796 seem to be effective also as RNS scavengers.

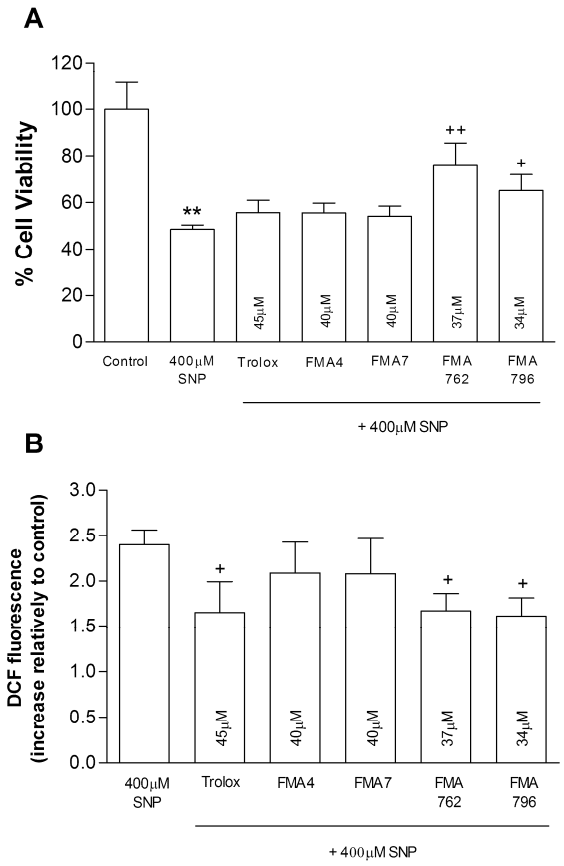


Figure 2.4 - Effects of the tested compounds on SNP-induced toxicity and intracellular ROS/RNS formation. Cells, at a density of 1.6×10^5 cells/cm^2, were incubated in the presence of 400 μM SNP together with the compounds for 20 h. A) Cell viability assessed by the MTT assay. B) Intracellular ROS/RNS formation, assessed by flow cytometry, using the fluorescent probe DCF. For each bar is represented the mean ± S.E.M for at least three independent experiments. ** $p \leq 0.01$, compared to control cells; $^{+}$ $p \leq 0.05$, $^{++}$ $p \leq 0.01$, compared to 400 μM SNP.

The fact that these new compounds are nitrogenated has raised a question regarding whether these structures *per se* could, directly or indirectly, lead to nitric oxide production inside the cells, which could somehow influence the results.

As previously reported (Bastianetto and Quirion, 2002), an exposure to SNP leads to an increase in nitrites in the culture medium. For this reason, the increase in nitrites concentration induced in the presence or absence of SNP was evaluated for each experimental condition. So, first we determined if the compounds led to an increase in the accumulation of nitrites, which are the end-products of NO

production. SNP was used as a positive control, leading to an increase from 0.7 to 21.6 μM in nitrites concentration, as shown in Figure 2.5A. Results in this figure clearly show that the new compounds in study do not act as substrates for nitric oxide synthase and do not contribute, *per se*, to the increase of intracellular nitrite levels. Moreover, there are no differences in nitrites production between them.

Additionally, none of the compounds tested decreased the SNP-induced accumulation of nitrites, indicating that they were unable to modulate the activity of nitric oxide synthase elicited by SNP (Figure 2.5B).

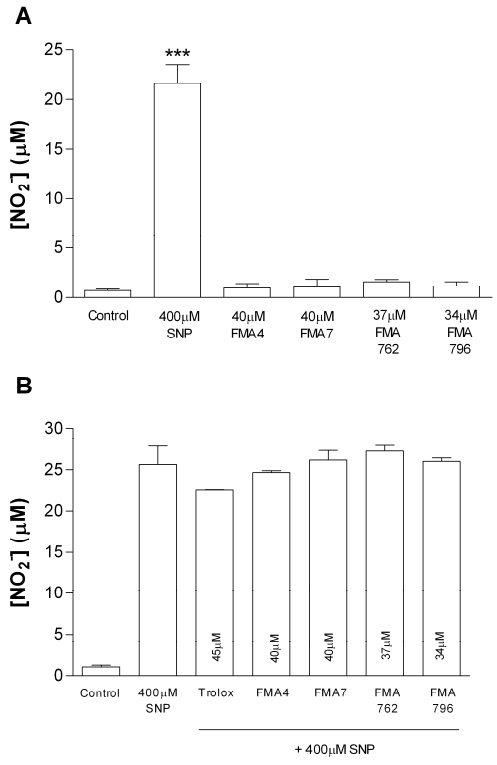


Figure 2.5 – Effects of the compounds on nitrite accumulation in the culture medium. Cells were incubated in the presence of 400 μM SNP and/or the compounds for 20 h. A) Effect of the compounds alone on nitrite levels. In this case, 400 μM SNP was used as a positive control. B) Effects of the compounds on nitric oxide synthase activity induced by SNP. For each bar is represented the mean ± S.E.M for at least three independent experiments. *** $p \leq 0.001$, compared to control cells.

2.3.6. Effects of the nitrogen compounds on cellular total glutathione levels and on the activities of GSH-Px and SOD

Besides the effect of the compounds as ROS/RNS scavenging agents we wanted to investigate their role on enzymatic and non-enzymatic intracellular defences against oxidative stress.

So, as an indicator of the intracellular non enzymatic antioxidant defences, the GSH content was measured in cells treated with 1 mM *t*-BHP for 2.5 h. The thiol-oxidizing agent, *t*-BHP, has been widely used as an inducer of oxidative stress (Ahmed-Choudhury *et al.*, 1998), being its use reported to study changes in the levels of antioxidant defences (Pias and Aw, 2002; Alia *et al.*, 2005).

Our results show that in the presence of *t*-BHP there was a decrease in cytoplasmic GSH content (Table 2.2). A 3 h pre-incubation with the compounds prior to the addition of *t*-BHP was not able to revert this *t*-BHP-induced decrease in GSH content, since no significant changes were observed between the compounds and *t*-BHP. There were neither significant changes in oxidized glutathione (GSSG) content (Table 2.2) produced by pre-incubation of the cells with the nitrogen compounds.

In order to study the effects of the nitrogen compounds on the antioxidant defence enzymes in our model, the activities of GSH-Px and SOD were determined after incubation with 1 mM *t*-BHP for 2.5 h.

Results in Table 2.2 show that 1 mM *t*-BHP induced a statistically significant decrease in enzymes activities (about 35% and 42% for GSH-Px and SOD, respectively) as expected. However, this decrease was not attenuated by pre-incubating cells with the nitrogen compounds. This indicates that the compounds antioxidant effects are not mainly due to a modulation of the studied natural antioxidant enzymes.

Table 2.2 - Effect of the compounds on glutathione levels and antioxidant enzymes activities.

Compound	1mM *t*-BHP, 2.5 h	GSH (nmol / mg protein)	GSSG (nmol GSH equiv/ mg protein)	GSH-Px (mU /mg protein)	SOD (U/mg protein)
---	-	24.3 ± 4.4	0.7 ± 0.4	14.3 ± 0.5	2.4 ± 0.5
---	+	5.8 ± 1.8 **	9.0 ± 1.9 **	9.3 ± 0.2 **	1.4 ± 0.2 *
40µM FMA4	+	6.1 ± 0.7	6.3 ± 0.9	10.2 ± 0.1	1.2 ± 0.2
40µM FMA7	+	5.9 ± 1.4	9.3 ± 0.6	10.5 ± 1.9	1.4 ± 0.3
37µM FMA762	+	9.3 ± 2.5	10.5 ± 2.6	10.1 ± 0.7	1.3 ± 0.1
34µM FMA796	+	7.7 ± 2.2	12.1 ± 2.3	10.5 ± 0.7	1.2 ± 0.1

Cells were pre-incubated in the presence of the compounds for 3 h prior to the addition of 1 mM *t*-BHP for 2h30. Glutathione content was determined by the DTNB-GSSG reductase recycling assay. Enzymatic activities were performed as described in Materials and Methods. For each condition is represented the mean ± S.E.M for at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, compared to control cells.

2.4. Discussion

In a previous study, a broad range of nitrogen compounds were synthesized and chemically characterized regarding their antioxidant potential (Areias, 2006). From that study, which also tried to determine the ideal structures an antioxidant should have in order to be effective, two compounds emerged as promising in which concerns to their antioxidant potential. The present work intended to continue that study, by focusing on four structurally related new nitrogen compounds with slight structural differences, FMA4, FMA7, FMA762 and FMA796. Our first results with FMA4 and FMA7 had shown an ability to scavenge free radicals (Silva *et al.*, 2006). However, modifications made to their structures resulted in more promising compounds in terms of antioxidant activity (FMA762 and FMA796), as the results obtained with chemical approaches like the DPPH discoloration and 2-deoxy-D-ribose degradation assays, demonstrate (Table 2.1). Essentially, FMA762 and FMA796 differ from FMA4 and FMA7 by the presence of three hydroxyl groups within the phenol ring, instead of just one, which seems very important since the substituents present in the molecule, and particularly their positions, contribute to the antiradical activity differences seen between compounds (Yokozawa *et al.*,

1998; Arora *et al.*, 1998; Lebeau *et al.*, 2000). In addition, the compounds in study differ by the presence or absence of functional groups in the amidine unit which have the intent to make them more liposoluble and thus permeate biological membranes more easily. Previous studies with flavonoids indicated that the more hydroxyl substitutions in a molecule, the stronger the antioxidant activities observed (Cao *et al.*, 1997). Therefore, the presence of three hydroxyl groups in FMA762 and FMA796 is expected to improve their free radical scavenging ability, when compared to FMA4 and FMA7 (see Figure 2.1).

The extent of oxidative stress induced by ascorbate/iron (II) was determined by following the changes in DCF fluorescence, which is a measure of intracellular ROS formation. As the oxidant stimulus, we used the pair ascorbate/Fe^{2+}, which has been shown to significantly increase the formation of intracellular free radicals in PC12 (Gassen *et al.*, 1998) and retinal (Areias *et al.*, 2001) cells. It is described that iron is present in very high concentrations in different regions of the brain and that an increase in free iron concentration occurs in many neuropathological situations, like Parkinson and Alzheimer's diseases, as a result of a disruption of iron homeostasis (Halliwell *et al.*, 1995; Berg *et al.*, 2001; Mwanjewe *et al.*, 2001). Furthermore, free iron can be reduced by ascorbate that exists at a high (millimolar) concentration in the nervous tissue (Sen *et al.*, 2006; Qiu *et al.*, 2007), generating hydroxyl radicals by the Fenton reaction, making the pair adequate to our cell model. As expected from the results regarding their higher ability to scavenge the DPPH radical and to inhibit 2-deoxy-D-ribose degradation, FMA762 and FMA796, as opposite to FMA4 and FMA7, were effective in decreasing ascorbate/iron-induced intracellular ROS formation, at their IC_{50} concentrations (previously determined by the DPPH discoloration method). This decrease was similar to the one observed for α-tocopherol, commonly used as an antioxidant. These results are of great relevance since the pair ascorbate/iron, used in this study as the free radical generator, as previously stated, is known to induce an excessive production of free radicals in several pathological conditions where it is involved an alteration of the intracellular iron homeostasis.

There seems to be a relationship between the compounds structure, their antiradical activity and their ability to protect cells from intracellular ROS formation. When compared with FMA4 and FMA7, which have only one hydroxyl group in their structures, the presence of three hydroxyl groups in the phenol ring of FMA762 and FMA796 provides them a higher ability to scavenge free radicals, as determined by the chemical approaches (DPPH discoloration and 2-deoxy-D-ribose degradation

assays). This was also observed in our biological model, by assessing their greater ability to prevent the formation of ROS inside PC12 cells. The protection profile of the compounds in study against increased ROS formation induced by the oxidant pair ascorbate/iron seems to correlate with the protective effect observed against lipid peroxidation.

FMA762 and FMA796 also presented an ameliorated ability, in comparison to FMA4 and FMA7, to prevent lipid peroxidation induced by ascorbate/iron. The amount of protection observed for FMA762 and FMA796 was higher when compared to trolox, the hydrosoluble form of vitamin E, and FMA762 was similar to the one of α-tocopherol. It should be noted that α-tocopherol is the most active form of vitamin E in humans, has been considered the major membrane bound antioxidant employed by the cell, whose main function is to protect cells against lipid peroxidation (Valko *et al.*, 2006). Therefore, the ability of the compounds to match α-tocopherol's protection on lipid peroxidation, as well as to prevent the intracellular ROS formation, could be indicative of a superior antioxidant potential of the compounds in study.

Since lipid peroxidation occurs mainly due to the free radicals formed intracellularly it is important that the antioxidant compounds have the ability to cross the lipid bilayer and prevent those radicals from inducing the peroxidation of membrane lipids (Spiteller, 2001). The partition coefficients obtained for the new compounds are indicative of their relative liposolubility, which enables them to permeate cells, further contributing to their intracellular action. Their lipophilic profiles were similar to some common natural antioxidant compounds (Areias *et al.*, 2001; Kitagawa *et al.*, 2005). It seems difficult to establish a correlation between the nitrogen compounds structures and their liposolubility profiles, since the presence of more polar groups (*e.g.* hydroxyl) does not, by itself, contribute to a lower liposolubility. For example, FMA796 despite presenting an added number of hydroxyl groups, shows an increased liposolubility when compared to FMA7 (see figure 1). However, the hydroxyl groups in the phenolic unit of FMA796 are engaged in a highly favourable intramolecular H-bonding. Consequently, the interaction with water molecules (HEPES solution) will be reduced and the solubility in the non-polar solvent *n*-octanol is favoured, compared to what is observed for compound FMA7, where the hydroxyl group interacts exclusively with the water molecules. Nevertheless, the liposolubility results can at least partially explain the good performance of the compounds in scavenging ascorbate/iron-induced intracellular ROS and in preventing lipid peroxidation. In fact, all the compounds, which

presented higher PCs than trolox (0.49 ± 0.02) and lower than α-tocopherol (6.76 ± 0.39) [25], were more effective on the prevention of lipid peroxidation than the water-soluble analogue of vitamin E, but were not able to surpass the protection offered by its lipophilic form (α-tocopherol). The liposoluble form of vitamin E, α-tocopherol, in contrast to the more hydrosoluble one, trolox, is known to bind the cells plasma membrane, where it exerts its main function of protecting cells against lipid peroxidation (Valko *et al.*, 2006). Therefore, the ability of the compounds to match α-tocopherol's protection on lipid peroxidation, associated to their action on ROS scavenging at the intracellular level (Figure 2.2), makes them promising antioxidants with a broader action than α-tocopherol and suggests their ability to act intracellularly.

Besides ROS, reactive nitrogen species (RNS), like nitric oxide (NO) and peroxinitrite can also exert harmful effects to cells (Antunes *et al.*, 2005; Valko *et al.*, 2006). NO is a short-lived radical that is generated during the conversion of L-arginine to citrulline by nitric oxide synthase. In the first step of the reaction, arginine is hydroxylated to *N*-hydroxy-arginine, which is then oxidized to citrulline and NO (Cadenas and Cadenas, 2002; Li *et al.*, 2007). Although at physiological concentrations NO acts as an important second messenger, when present at concentrations higher than physiological ones, NO can initiate a toxic cascade that leads to cell death (Dawson and Dawson, 1996).

Among the nitrogen compounds herein studied, FMA762 and FMA796 showed the highest protective effect against SNP-induced toxicity as a result of NO generation (Figure 2.4), as they significantly reversed the decrease in cell viability induced by SNP (assessed by the MTT assay) and also significantly diminished the increase in intracellular RNS formation.

Since the new compounds are nitrogenated, a question raised regarding whether these structures *per se* could, directly or indirectly, lead to nitric oxide production inside the cells. In fact, with the exception of FMA762, all of them possess an amidine group in their structure, similar to the one of L-arginine, which could be oxidized inside the cells, originating nitric oxide. Depending on the concentration produced, this could be either harmful or beneficial to cells (Valko *et al.*, 2006).

However, it was observed that in the presence of the NO donor none of the compounds was able to reduce the extracellular nitrite accumulation (see Figure 2.5). This is in accordance with results obtained for common natural compounds with antioxidant activity, which were also reported to exert no effect on NOS

(Bastianetto *et al.*, 2000; Bastianetto and Quirion, 2002). As it can be observed in Figure 2.5, the concentration of nitrites did not increase in the presence of the compounds alone, indicating that they do not act as substrates for NO synthase and thus do not lead, by themselves, to an increased production of NO inside the cells. Altogether, these findings indicate that the protective effects of these compounds on nitrosative stress are not likely associated to the inhibition of nitric oxide synthase, but with their ability to scavenge reactive nitrogen species.

In addition to directly scavenge free radicals (ROS and RNS), another way by which an antioxidant can act is through the induction of expression of cells natural antioxidant defences (Behl and Moosmann, 2002). It is well known that cells are well equipped with defence mechanisms against oxidative stress-induced cell damage (Halliwell and Gutteridge, 1999). These cellular antioxidant systems can be divided into two major groups: enzymatic (comprising SOD, catalase and GSH-related enzymes) and nonenzymatic (which include the low molecular-weight molecule GSH) (Nordberg and Arner, 2001; Wilcox *et al.*, 2004). Glutathione (GSH) is the major intracellular low-molecular-weight thiol that plays a critical role in the non-enzymatic cellular defence against oxidative stress in mammalian cells (Schafer and Buettner, 2001; Guan *et al.*, 2006) and plays an important role in protecting brain tissue from oxidative stress (Drake *et al.*, 2003; Yu *et al.*, 2005). Despite the cells ability to deal with mild oxidative stress through an upregulation of the antioxidant defence mechanisms, cell injury may occur when adaptation is not adequate for the build up of oxidation products, leading to oxidative damage to all types of biomolecules, including proteins (Willcox *et al.*, 2004). In our case, the cells incubation with 1mM *t*-BHP for 2.5 h caused a decrease in cell integrity of about 50% (data not shown), which may have induced the inactivation of the natural antioxidant enzymes and thus leading to a decrease in their activity. Such results have also been observed in other published papers using the same cell model (Yu *et al.*, 2005; Sun *et al.*, 2005; Guan *et al.*, 2006; Wu *et al.*, 2006).

We tested the effects of the new compounds on these antioxidant defence systems. No statistically significant protective effect could be observed against *t*-BHP-induced decrease in GSH-Px and SOD activities. In the same way, no beneficial effect was observed on the decrease in intracellular GSH. Overall, these results indicate that the compounds protective effect against oxidative stress is not due to an involvement on the natural antioxidant defence systems but, again, rather to a direct scavenging of free radicals.

In conclusion, we have shown that the new nitrogenated compounds herein studied are capable of rescuing PC12 cells against oxidative and nitrosative stress. These effects are likely mediated by their ability to scavenge free radicals, as they proved to be quite effective in reducing lipid peroxidation, intracellular ROS and RNS formation in association with their ability to scavenge the DPPH radical and inhibit 2-deoxy-D-ribose degradation and do not appear to be involved neither in the induction of intracellular antioxidant enzymes such as GSH-Px and SOD, nor in the inhibition of NOS. Nevertheless, our results suggest a real interest of these compounds as potent free radical scavengers and so with a possible pharmacological application against pathological situations in which oxidative/nitrosative stress is involved.

Further studies on basic structure modification regarding other biological targets are under current investigation.

2.5. References

Ahmed-Choudhury, J., Orsler, D.J. and Coleman, R., 1998. Hepatobiliary effects of *tertiary*-butylhydroperoxide (tBOOH) in isolated rat hepatocyte couplets. *Toxicol. Appl.Pharmacol.* **152**: 270-275.

Alia, M., Ramos, S., Mateos, R., Bravo, L. and Goya, L., 2005. Response of the antioxidant Defense system to *tert*-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *J. Biochem. Mol. Toxicol.* **19**: 119-128.

Alia, M., Ramos, S., Mateos, R., Granado-Serrano, A.B., Bravo, L. and Goya, L., 2006. Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicology and Applied Pharmacology* **212**: 110-118.

Anderson, M.E., 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.* **113**: 548-555.

Antunes, F., Nunes, C., Laranjinha, J. and Cadenas, E., 2005. Redox interactions of nitric oxide with dopamine and its derivatives. *Toxicology* **208**: 207-212.

Araujo, I.M., Ambrosio, A.F., Leal, E.C., Santos, P.F., Carvalho, A.P. and Carvalho, C.M., 2003. Neuronal nitric oxide synthase proteolysis limits the involvement of nitric oxide in kainate-induced neurotoxicity in hippocampal neurons. *J. Neurochem.* **85**: 791-800.

Areias, F.M. 2006. Novos compostos heterocíclicos de azoto com unidades fenólicas: síntese e actividade biológica. *Ph.D. Thesis*. University of Minho, Braga, Portugal. 259p. Available online at: http://hdl.handle.net/1822/5941.

Areias, F.M., Rego, C., Oliveira, C. and Seabra, R.M., 2001. Antioxidant effect of flavonoids after ascorbate/Fe^{2+}-induced oxidative stress in cultured retinal cells. *Biochem. Pharmacol.* **62**: 111-118.

Arora, A., Nair, M.G. and Strasburg, S., 1998. Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. *Arch. Biochem. Biophys.* **356**: 133-141.

Bastianetto, S. and Quirion, R., 2002. Natural extracts as possible protective agents of brain aging. *Neurobiol. Aging* **5687**: 1-7.

Bastianetto, S., Zheng, W.-H. and Quirion, R., 2000. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br. J. Pharm.* **131**: 711-720.

Beckman, K.B. and Ames, B.N., 1998. The free radical theory of aging matures. *Physiol. Rev.* **78**: 547-581.

Behl, C. and Moosmann, B., 2002. Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. *Free Radic. Biol. Med.* **33**: 182-191.

Berg, D., Gerlach, M., Youdim, M.B.H., Double, K.L., Zecca, L., Riederer, P. and Becker, G., 2001. Brain iron pathways and their relevance to Parkinson's disease. *J. Neurochem.* **79**: 225-236.

Bhat, K., Garg, A. and Bock, C., 2002. Calculated values of the octanol–water partition coe.cient and aqueous solubility for aminoazobenzene dyes and related structures. *Dyes Pigments* **52**: 145-159.

Booth, B.L., Costa, F.A.T., Mahmood, Z., Pritchard, R.G. and Proenca, M.F., 1999. Synthesis of (Z)-N-(2-amino-1,2-dicyanovinyl)formamide O-alkyloximes and a study of their cyclization in the presence of base. *J. Chem. Soc. [Perkin 1]* 1853-1858.

Cadenas, S. and Cadenas, A.M., 2002. Fighting the stranger-antioxidant protection against endotoxin toxicity. *Toxicology* **180**: 45-63.

Calabrese, V., Copani, A., Testa, D., Ravagna, A., Spadaro, F., Tendi, E., Nicoletti, V.G. and Giuffrida Stella, A.M., 2000. Nitric oxide synthase induction in astroglial cell cultures: effect on heat shock protein 70 synthesis and oxidant/antioxidant balance. *J. Neurosci. Res.* **60**: 613-622.

Cao, G., Sofic, E. and Prior, R.L., 1997. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic. Biol. Med.* **22**: 749-760.

Colognato, R., Laurenza, I., Fontana, I., Coppede, F., Siciliano, G., Coecke, S., Aruoma, O.I., Benzi, L. and Migliore, L., 2006. Modulation of hydrogen peroxide-induced DNA damage, MAPKs activation and cell death in PC12 by ergothioneine. *Clin. Nutr.* **25**: 135-145.

Dawson, V.L. and Dawson, T.M., 1996. Nitric oxide neurotoxicity. *J. Chem. Neuroanat.* **10**: 179-190.

Doulias, P.T., Kotoglou, P., Tenopoulou, M., Keramisanou, D., Tzavaras, T., Brunk, U., Galaris, D. and Angelidis, C., 2007. Involvement of heat shock protein-70 in the mechanism of hydrogen peroxide-induced DNA damage: The role of lysosomes and iron. *Free Radic. Biol. Med..* **42**: 567-577.

Drake, J., Sultana, R., Aksenova, M., Calabrese, V. and Butterfield, D.A., 2003. Elevation of mitochondrial glutathione by gamma-glutamylcysteine ethyl ester protects mitochondria against peroxynitrite-induced oxidative stress. *J. Neurosci. Res.* **74**: 917-927.

Fleury, C., Mignotte, B. and Vayssiere, J.L., 2002. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* **84**: 131-141.

Flohé, L. and Gunzler W.A., 1984. Assays of Glutathione Peroxidase. *Methods Enzymol.* **105**: 114-115.

Gassen, M., Pergande, G. and Youdim, M.B.H., 1998. Antioxidant properties of the triaminopyridine, flupirtine. *Biochem. Pharmacol.* **56**: 1323-1329.

Greene, L.A. and Tischler, A.S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **73**: 2424-2428.

Guan, S., Bao, Y.-M., Jiang, B. and An, L.-J., 2006. Protective effect of protocatechuic acid from Alpinia oxyphylla on hydrogen peroxide-induced oxidative PC12 cell death. *Eur. J. Pharmacol.* **538**: 73-79.

Halliwell, B., 2001. Role of free radicals in the neurodegenerative diseases - Therapeutic implications for antioxidant treatment. *Drugs & Aging* **18**: 685-716.

Halliwell, B., Aeschbach, R., Loliger, J. and Aruoma, O.I., 1995. The Characterization of antioxidants. *Food Chem. Toxicol.* **33**: 601-617.

Halliwell, B. and Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. 3rd edition, Oxford University Press, New York.

Hiroi, M., Ogihara, T., Hirano, K., Hasegawa, M., Morinobu, T., Tamai, H. and Niki, E., 2005. Regulation of apoptosis by glutathione redox state in PC12 cells exposed simultaneously to iron and ascorbic acid. *Free Radic. Biol. Med.* **38**: 1057-1072.

Hodges, D.M., DeLong, J.M., Forney, C.F. and Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *PLANTA* **207**: 604-611.

Hong, H. and Liu, G.Q., 2004. Protection against hydrogen peroxide-induced cytotoxicity in PC12 cells by scutellarin. *Life Sci.* **74**: 2959-2973.

Huang, D., Ou, B. and Prior, R.L., 2005. The Chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **53**: 1841-1856.

Jang, J.H., Aruoma, O.I., Jen, L.S., Chung, H.Y. and Surh, Y.J., 2004. Ergothioneine rescues PC12 cells from *beta*-amyloid-induced apoptotic death. *Free Radic. Biol. Med.* **36**: 288-299.

Jang, J.H. and Suhr, Y.J., 2005. AP-1 mediates *b*-amyloid-induced iNOS expression in PC12 cells via the ERK2 and p38 MAPK signaling pathways. *Biochem. Biophys. Res. Comm.* **331**: 1421-1428.

Jung, J.Y., Han, C.R., Jeong, Y.J., Kim, H.J., Lim, H.S., Lee, K.H., Park, H.O., Oh, W.M., Kim, S.H. and Kim, W.J., 2007. Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. *Neurosci. Lett.* **411**: 222-227.

Kitagawa, S., Nabekura, T., Takahashi, T., Nakamura, Y., Sakamoto, H., Tano, H., Hirai, M. and Tsukahara, G., 2005. Structure-activity relationships of the inhibitory effects of flavonoids on P-glycoprotein-mediated transport in KB-C2 cells. *Biol. Pharm. Bull.* **28**: 2274-2278.

Lebeau, J., Furman, C., Bernier, J.L., Duriez, P., Teissier, E. and Cotelle, N., 2000. Antioxidant properties of di-*tert*-butylhydroxylated flavonoids. *Free Radic. Biol. Med.* **29**: 900-912.

Li, D., Kabir, M., Stuehr, D.J., Rousseau, D.L. and Yeh, S.-R., 2007. Substrate- and isoform-specific dioxygen complexes of nitric oxide synthase. *J. Am. Chem. Soc.* **129**: 6943-6951.

Lin, C.H., Kuo, S.C., Huang, L.J. and Gean, P.W., 2006. Neuroprotective effect of N-acetylcysteine on neuronal apoptosis induced by a synthetic gingerdione compound: Involvement of ERK and p38 phosphorylation. *J. Neurosci. Res.* **84**: 1485-1494.

Lopez-Lluch, G., Buron, M.I., Alcain, F.J., Quesada, J.M. and Navas, P., 1998. Redox regulation of cAMP levels by ascorbate in 1,25-dihydroxy- vitamin D3-induced differentiation of HL-60 cells. *Biochem. J.* **331 (Pt 1)**: 21-27.

Mates, J.M., 2000. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* **153**: 83-104.

McCord, J.M. and Fridovich, I., 1969. Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**: 6049-6055.

Molyneux, P., 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J. Sci. Technol.* **26**: 211-219.

Mwanjewe, J., Hui, B.K., Coughlin, M.D. and Grover, A.K., 2001. Treatment of PC12 cells with nerve growth factor increases iron uptake. *Biochem. J.* **357**: 881-886.

Nie, G., Jin, C., Cao, Y., Shen, S. and Zhao, B., 2002. Distinct effects of tea catechins on 6-hydroxydopamine-induced apoptosis in PC12 cells (vol 397, pg 84, 2002). *Arch. Biochem. Biophys.* **399**: 116-116.

Nordberg, J. and Arner, E.S., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* **31**: 1287-1312.

Okawa, M., Kinjo, J., Nohara, T. and Ono, M., 2001. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of flavonoids obtained from some medicinal plants. *Biol. Pharm. Bull.* **24**: 1202-1205.

Oliveira, M.T., Rego, A.C., Morgadinho, M.T., Macedo, T.R. and Oliveira, C.R., 2002. Toxic effects of opioid and stimulant drugs on undifferentiated PC12 cells. *Ann. N. Y. Acad. Sci.* **965**: 487-496.

Parejo, I., Codina, C., Petrakis, C. and Kefalas, P., 2000. Evaluation of scavenging activity assessed by Co(II)/EDTA-induced luminol chemiluminescence and DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) free radical assay. *J. Pharmacol. Toxicol. Methods* **44**: 507-512.

Pias, E.K. and Aw, T.Y., 2002. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC-12 cells. *Cell Death Differ.* **9**: 1007-1016.

Pias, E.K., Ekshyyan, O.Y., Rhoads, C.A., Fuseler, J., Harrison, L. and Aw, T.Y., 2003. Differential effects of superoxide dismutase isoform expression on hydroperoxide-induced apoptosis in PC-12 cells. *J. Biol. Chem.* **278**: 13294-13301.

Piga, R., Saito, Y., Chen, Z., Yoshida, Y. and Niki, E., 2005. Characterization of monochloramine toxicity on PC12 cells and protective effect of tocopherol via antioxidative function. *Arch. Biochem. Biophys.* **436**: 101-109.

Possel, H., Noack, H., Augustin, W., Keilhoff, G. and Wolf, G., 1997. 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. *FEBS Lett.* **416**: 175-178.

Qiu, S., Li, L., Weeber, E.J. and May, J.M., 2007. Ascorbate transport by primary cultured neurons and its role in neuronal function and protection against excitotoxicity. *J. Neurosci. Res.* **85**: 1046-1056.

Rego, A.C., Santos, M.S., Proença, M.T. and Oliveira, C.R., 1998. Influence of vitamin E succinate on retinal cell survival. *Toxicology* **128**: 113-124.

Rogachev, I., Serra, C., Farran, A., Cortina, J.L., Gressel, J. and Warshawsky, A., 2003. Prediction of the octanol–water distribution of dithiocarbamate derivatives. *React. Funct. Polymers* **54**: 17-24.

Sanchez-Moreno, C., Larrauri, J.A. and Saura-Calixto, F., 1998. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **76**: 270-276.

Sasaki, N., Toda, T., Kaneko, T., Baba, N. and Matsuo, M., 2002. Flavonoids suppress the cytotoxicity of linoleic acid hydroperoxide toward PC12 cells. *Biol. Pharm. Bull.* **25**: 1093-1096.

Schafer, F.Q. and Buettner, G., 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30**: 1191-1212.

Sedmak, J.J. and Grossberg, S.E., 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* **79**: 544-552.

Sen, T., Sen, N., Tripathi, G., Chatterjee, U. and Chakrabarti, S., 2006. Lipid peroxidation associated cardiolipin loss and membrane depolarization in rat brain mitochondria. *Neurochem. Int.* **49**: 20-27.

Shapiro, S.S. and Saliou, C., 2001. Role of vitamins in skin care. *Nutrition* **17**: 839-844.

Sharikabad, M.N., Ostbye, K.M., Lyberg, T. and Brors, O., 2001. Effect of extracellular Mg^{2+} on ROS and Ca^{2+} accumulation during reoxygenation of rat cardiomyocytes. *Am. J. Physiol.-Heart Circ. Physiol.* **280**: H344-H353.

Siddaiah, V., Maheswara, M., Rao, C.V., Venkateswarlu, S. and Subbaraju, G.V., 2007. Synthesis, structural revision, and antioxidant activities of antimutagenic homoisoflavonoids from Hoffmanosseggia intricata. *Bioorg. Med. Chem. Lett.* **17**: 1288-1290.

Silva, J.P., Areias, F.M., Proença, M.F. and Coutinho, O.P., 2006. Oxidative stress protection by newly synthesized nitrogen compounds with pharmacological potential. *Life Sci.* **78**: 1256-1267.

Singh, B., Saxena, A.K., Chandan, B.K., Anand, K.K., Suri, O.P., Suri, K.A. and Sattti, N.K., 1998. Hepatoprotective activity of verbenalin on experimental liver damage in rodents. *Fitoterapia* **LXIX**: 135-140.

Somayajulu, M., McCarthy, S., Hung, M., Sikorska, M., Borowy-Borowski, H. and Pandey, S., 2005. Role of mitochondria in neuronal cell death induced by oxidative stress: neuroprotection by Coenzyme Q10. *Neurobiol. Dis.* **18**: 618-627.

Spiteller, G., 2001. Lipid peroxidation in aging and age-dependent diseases. *Exp. Gerontol.* **36**: 1425-1457.

Stadtman, E.R., 2004. Role of oxidant species in aging. *Curr. Med. Chem.* **11**: 1105-1112.

Sun, C., Shan, C.Y., Gao, X.D. and Tan, R.X., 2005. Protection of PC12 cells from hydrogen peroxide-induced injury by EPS2, an exopolysaccharide from a marine filamentous fungus *Keissleriella* sp. YS4108. *J. Biotechnol.* **115**: 137-144.

Tamagno, E., Aragno, M., Boccuzzi, G., Gallo, M., Parola, S., Fubini, B., Poli, G. and Danni, O., 1998. Oxygen free radical scavenger properties of dehydroepiandrosterone. *Cell Biochem. Funct.* **16**: 57-63.

Tang, X.Q., Zhi, J.L., Cui, Y., Feng, J.Q. and Chen, P.X., 2005. Hydrogen peroxide preconditioning protects PC12 cells against apoptosis induced by dopamine. *Life Sci.* **78**: 61-66.

Tsikas, D., 2007. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *J. Chromatogr. B* **851**: 51-70.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem.-Biol. Int.* **160**: 1-40.

Wang, L., Nishida, H., Ogawa, Y. and Konishi, T., 2003. Prevention of oxidative injury in PC12 cells by a traditional Chinese medicine, Shengmai San, as a model of an antioxidant-based composite formula. *Biol. Pharm. Bull.* **26**: 1000-1004.

Wentrup, C., 1984. Reactive molecules: the neutral reactive intermediates in organic chemistry. 23-161. New York: Wiley; 1984. p 23-161.

Wilcox, J.K., Ash, S.L. and Catignani, G.L., 2004. Antioxidants and prevention of chronic disease. *Crit. Rev. Food Sci. Nutr.* **44**: 275-295.

Wu, J.H., Xu, C., Shan, C.Y. and Tan, R.X., 2006. Antioxidant properties and PC12 cell protective effects of APS-1, a polysaccharide from *Aloe vera* var. *chinensis*. *Life Sci.* **78**: 622-630.

Yokozawa, T., Chen, C.P., Dong, E., Tanaka, T., Nonaka, G.I. and Nishioka, I., 1998. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2 picrylhydrazyl radical. *Biochem. Pharmacol.* **56**: 213-222.

Yu, D., Duan, Y., Bao.Y., Wei, C. and An, L., 2005. Isoflavonoids from *Astragalus mongholicus* protect PC12 cells from toxicity induced by l-glutamate. *J. Ethnopharmacol.* **98**: 89-94.

CHAPTER: 3

NOVEL NITROGEN COMPOUNDS ENHANCE PROTECTION AND REPAIR OF OXIDATIVE DNA DAMAGE IN A NEURONAL CELL MODEL

Silva, J.P., Gomes, A.C., Proença, M.F. and Coutinho, O.P. Novel nitrogen compounds enhance protection and repair of oxidative DNA damage in a neuronal cell model. *Submitted*.

Abstract

Oxidative DNA damage has been described as the type of damage most likely to occur in neuronal cells and is involved in many neurodegenerative diseases and in aging. This study evaluates the protection of four new synthetic nitrogen compounds that we have been characterizing (FMA4, FMA7, FMA762 and FMA796). In this case, we studied their effects on oxidative DNA damage induced in PC12 cells, using the Comet assay. FMA762 and FMA796 were the most effective in preventing *t*-BHP-induced formation of DNA strand breaks and in improving the cells' capacity to repair this kind of damage. Moreover, they increased the repair capacity of oxidised bases induced with the photosensitiser Ro 19-8022, which seems to be mediated by an increase in DNA repair enzymes activity, assessed by the *in vitro* BER assay. However, no regulation at the expression of *OGG1* and *APE1* genes was detected. In addition to other properties previously found for these compounds, they now prove their effectiveness against oxidative stress-induced DNA damage in a neuronal cell model.

3.1. Introduction

DNA lesions are constantly being produced in living cells by the deleterious action of both endogenous and environmental DNA-damaging agents (Kruman, 2004). A great amount of this damage is caused by the oxidation of DNA by reactive oxygen species (ROS), which are generated during normal cell metabolism and in response to exogenous factors (Gedik *et al.*, 2002; Wilcox *et al.*, 2004). Oxidative DNA lesions include the formation of single and double-strand breaks, abasic (apurinic/apyrimidinic) sites and DNA-protein cross-links, all of which are cytotoxic and/or mutagenic (Powell *et al.*, 2005; Youn *et al.*, 2005; Hazra *et al.*, 2007).

Neuronal cells, because of their high rate of oxidative metabolism and low levels of antioxidant enzymes, are quite susceptible to damage by ROS (Englander and Ma, 2006; Rolseth *et al.*, 2008). In fact, oxidative DNA damage has been described by some authors as the type of damage most likely to occur in neuronal cells (Fishel *et al.*, 2007; Bohr *et al.*, 2007), with severe implications in neurodegenerative diseases (Gabbita *et al.*, 1998; Brooks, 2002; Cui *et al.*, 2004), as well as in aging (Rao, 2007).

To cope with the deleterious consequences of DNA lesions, cells are equipped with efficient defence mechanisms to remove this kind of damage by DNA repair pathways (Kruman, 2004). In this way, DNA damage is predominantly corrected by the base excision repair (BER) pathway (Coppede *et al.*, 2007), although certain types of oxidative lesions also appear to be repaired by nucleotide excision repair (NER) (Dusinska *et al.*, 2006) and mismatch repair (MMR) (Neri *et al.*, 2005).

BER is a major pathway used to repair base lesions and abasic sites. The first steps are the recognition of damaged bases by specific DNA glycosylases, hydrolysis of the glycosidic bond between base and deoxyribose and incision of the affected DNA strand by an apurinic/apyrimidinic (AP) endonuclease at the resulting abasic site, thus creating a DNA single-strand break. The appropriate nucleotides are then inserted by polymerases before the nick is sealed (Burkle, 2006; Collins and Gaivão, 2007; Dobbs *et al.*, 2008). Two enzymes that play an important role in DNA repair are 8-oxoguanine DNA glycosylase 1 (OGG1) and AP endonuclease 1 (APE1, also known as APEX, HAP1 and Ref-1 (Saitoh *et al.*, 2001)). In mammals, OGG1 is responsible for the removal of 8-oxoguanine, a lesion that arises from the oxidation of guanine base by ROS. This enzyme also has an AP lyase activity, which is slow and limits the overall rate of repair (Alamo *et al.*, 1998; Dodson and Lloyd, 2002). So, following the glycosylase reaction, APE1, which is an AP

endonuclease, bypasses the AP lyase activity of OGG1 and enhances OGG1 turnover, thus having an important role in the regulation of base excision repair of oxidative DNA damage (Hill *et al.*, 2001; Vidal *et al.*, 2001).

As part of a broader research project regarding the development process of drugs that can be useful in the improvement of human health, we have been investigating the potential of new nitrogen compounds, from organic synthesis, in different conditions where oxidative stress is involved (Silva *et al.*, 2006; Silva *et al.*, 2008b). In fact, we have previously reported an elevated ROS scavenging activity for four of them, named FMA4, FMA7, FMA762 and FMA796. These molecules are composed of an amidine unit and a phenol ring (Figure 3.1) and were synthesised based on the knowledge that the hydroxyl groups of the phenol ring are usually responsible for the antioxidant properties of a compound and that nitrogen compounds can easily interact with active centres responsible for different functions in living organisms (Wentrup, 1984; Areias *et al.*, 2001). Therefore, the association of these two moieties in the same molecule was expected to result in new structures capable of acting even better as antioxidants in living systems (Areias, 2006).

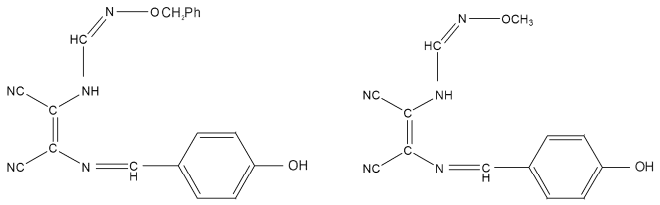
In this work, we evaluated the protective potential of those four new nitrogen compounds (FMA4, FMA7, FMA762 and FMA796) on *tert*-butylhydroperoxide (*t*-BHP)-induced oxidative DNA damage in a neuronal cell model, by the Comet assay, a technique which has been commonly used to assess the genotoxicity of antioxidants (Cemeli *et al.*, 2008). In addition, we intended to further characterise that protection and investigate the involvement of these compounds in DNA repair mechanisms, by addressing specific repair enzymes both at activity and gene expression levels. The rat pheochromocytoma PC12 cell line, used in this study, is derived from chromaffin cells of the adrenal medulla and have been previously used as a simple model system in which a correlation between oxidative stress and neurodegeneration, characteristic of Parkinson's or Alzheimer's diseases, has been established (Piga *et al.*, 2005; Guan *et al.*, 2006; Jung *et al.*, 2007).

3.2. Materials and methods

3.2.1. Compounds

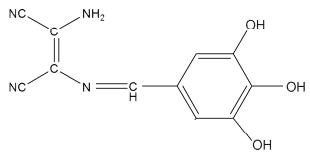
The compounds used in this study (Figure 3.1) were prepared in the group of Organic Synthesis, Chemistry Department, University of Minho, from the reaction of an appropriate phenolic aldehyde with a substituted amidine, as previously

described (Areias, 2006). The experimental procedure was adapted from previous work (Booth *et al.*, 1999) carried out on a selection of mono-substituted aldehydes. The compounds were provided as a yellowish powder, which was reconstituted in DMSO, aliquoted and maintained frozen at -80ºC until use. Each aliquot was thawed only once. When incubated with cells, the final solution contains no more than 1% DMSO. In this case, controls were performed with the same volume of DMSO. Cells were incubated in the presence of these nitrogen compounds for the indicated time periods (1h or 24h) in a final solution containing no more than 1% DMSO. The same volume of DMSO was also added to control cells for each experiment in which the nitrogen compounds were used.

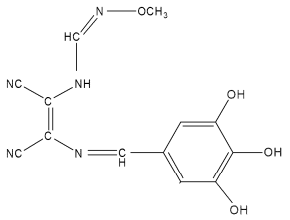


FMA4

FMA7



FMA762



FMA796

Figure 3.1. – Schematic structure of the newly synthesized nitrogen compounds. A phenolic unit is linked to an amidine function through a linear chain containing a nitrogen atom in three carbon atoms. FMA762 and FMA796 differ mainly from FMA4 and FMA7 by the presence of three hydroxyl groups within the phenol ring, instead of one. The compounds were prepared by F. M. Areias, Group of Organic Chemistry, University of Minho.

Foetal bovine serum (FBS) was obtained from BioChrom KG (Berlin, Germany); horse serum donor herd was purchased from Gibco (Paisley, UK). RPMI-1640 and DMEM cell culture media, dimethyl sulfoxide (DMSO), EDTA, trypsin, *tert*-butyl hydroperoxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ro 19-8022 (used to induce specific DNA damage) was kindly provided by Hoffman La-Roche (Basel, Switzerland). Primers specific for 18S, *OGG1* and *APE1* genes were

synthesised by STAB-VIDA (Oeiras, Portugal). Qiagen RNeasy total RNA isolation kit was purchased to Qiagen (Hilden, Germany). Superscript Reverse Transcriptase III kit was obtained from Invitrogen (USA). Power SYBR Green master mix was acquired from Applied Biosciences (Cheshire, UK).

3.2.2. PC12 cell culture

PC12, a cell line established from a rat adrenal pheochromocytoma (Greene and Tischler, 1976) was used in this study. When grown in serum-containing medium, these cells divide and resemble precursors of adrenal chromaffin cells and sympathetic neurons, being able to release dopamine (Sasaki *et al.*, 2003). In addition, they are a user-friendly cell model with some advantages over primary cultured neuronal cells, including the homogeneity of the cell population (Colognato *et al.*, 2006; Silva *et al.*, 2008b). Cells were cultured in suspension in 75 cm^2 flasks, in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated foetal bovine serum and 1% (v/v) of an antibiotic/antimycotic solution. Cultures were maintained in a humidified incubator containing 5% CO_2 and passed twice a week. Before each assay, the cell aggregates were carefully disrupted by gently pipetting and the separated cells plated in poly-D-lysine-coated multiwells at a density of 5×10^5 cells/well, for all studies. After plating, cells were left for adhesion overnight.

L929 cells, used as substrates for the *in vitro* base excision repair assay, were routinely grown in 75 cm^2 tissue culture flasks in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) of an antibiotic/antimycotic solution, until they reached confluence.

3.2.3. Single cell gel electrophoresis (Comet assay)

The Comet assay is a simple, sensitive, fast and cheap method that allows the assessment of DNA damage in individual cells. It identifies strand breaks, after separation of the DNA fragments by electrophoresis in alkali conditions. The protection against oxidative DNA damage conferred by the compounds herein studied was assessed using this assay, slightly modified as previously described (Silva *et al.*, 2008a). Cells were plated at a density of 7.5×10^5 cells per well and left to adhere overnight. Oxidative DNA damage was induced in the presence of either

200 μM *t*-BHP (for strand breaks assessment) or 0.6 μM Ro 19-8022, a photosensitiser compound, in order to measure oxidised purines. Briefly, after treatment with the compounds for the indicated time periods, cells were trypsinised and about 50 000 of them were centrifuged, suspended in low melting point agarose and spread on slides previously coated with normal melting point agarose. Cells were lysed, leaving just the nucleoid (which accounts for the supercoiled DNA) embedded in the agarose. This DNA was then subjected to an electrophoresis, in alkaline conditions, for 20 min at 1 V/cm, which makes the broken DNA loops, if they exist, to extend towards the anode, yielding an image that looks like a comet. Finally, slides were neutralised by washing three times with 0.4 M Tris, pH 7.5, and fixed with absolute ethanol. Comets were stained with ethidium bromide (10 μg/ml in PBS) and analysed under a fluorescence microscope. Comet quantification was performed through either of two ways: visual scoring, a method in which comets are classified into one of five classes of damage (from 0 to 4) in 100 nucleoids (range of score: 0 – 400); and/or through a computer-assisted image analysis (TriTek CometScore™ Freeware v1.5), by measuring the percentage of DNA in the tail. These two methods had previously shown a good correlation (Silva *et al.*, 2008a).

After subtracting the background levels of damage observed in the untreated cells, their repair capacity (RC) was determined by using the formula:

$$RC = 100 \times [(D_0X - D_tX) / D_0X] \quad (3.1)$$

where D_0X represents DNA damage before the recovery period in the condition X and D_tX represent DNA damage after a recovery period, for the same condition. By using this formula it is assumed that the initial rate of repair is influenced by the amount of damage present. In fact the rate of biochemical reactions depends on the substrate concentration (if not in saturating concentrations). So, if a compound decreases the initial damage level, as is the case with the ones we are studying, it will also reduce the repair rate. Therefore, the repair was calculated considering the initial amount of damage present for each condition (and not to the higher amount present in control cells) as also done by others (Ramos *et al.*, 2008). The increase in cells' repair capacity induced by the compounds was obtained by subtracting the percentage of repair observed in the presence of the deleterious stimulus alone, to the percentage of repair in the presence of the compounds and the deleterious stimulus.

3.2.4. Measurement of oxidised purines

To measure the amount of oxidised bases, cells were treated with the photosensitiser compound Ro 19-8022, which induces an excess of this kind of damage relatively to strand breaks. A stock of a 1 mM concentration was prepared in 70 % ethanol and stored in small aliquots at -20ºC. The working solutions were prepared immediately before use by diluting this stock solution with PBS (132 mM NaCl, 4 mM KCl, 1.2 mM NaH_2PO_4, 1.4 mM $MgCl_2$, 10 mM HEPES, 6 mM glucose, 1 mM $CaCl_2$ pH 7.4). The experiment was carried out in the dark and all solutions were kept on ice. Cells, plated as previously described, were incubated in the presence of the nitrogen compounds for the indicated time periods (1h or 24h). After that treatment, culture medium was removed, cells were rinsed with PBS and 3 ml of a Ro 19-8022 solution were added to each well at the final concentrations indicated for each condition. The plates were irradiated on ice at a distance of 33 cm from a 500 W halogen lamp, for 5 min. Control cells were incubated in PBS alone. After washing the cells with PBS to remove traces of Ro 19-8022, the Comet assay was performed as described above, with a small change: following lysis, the slides were washed 3 times (5 min each) in cold enzyme reaction buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8, 4 ºC), blotted dry and incubated with the bacterial repair enzyme formamidopyrimidine DNA glycosylase (FPG), or buffer (controls), for 30 min, at 37 ºC. FPG recognises oxidised purines, creating breaks at those sites (Duthie and Dobson, 1999; ESCODD, 2003; Gedik and Collins, 2004), and facilitating their identification. The amount of oxidised purines (FPG-sensitive sites) was then determined by subtracting the amount of strand breaks (samples incubated with buffer alone) to the total amount of breaks obtained after incubation with FPG.

3.2.5. In vitro *base excision repair assay*

The base excision repair (BER) assay measures the ability of a cell-free extract, containing the DNA repair enzymes, to recognise the damage in the DNA of substrate nucleoids and incise the DNA containing specific damage, in this case 8-oxoguanine (8-oxoGua). The increase in the amount of strand breaks produced reflects the DNA repair activity of the cell extract (Collins, 2004). This assay was performed as we previously described (Silva *et al.*, 2008a).

L929 fibroblasts were used as substrates, since these cells allow an easier measurement of comets' damage due to their greater size and lack of a tendency to form aggregates, in comparison with PC12 cells. Oxidation of purines was induced by treating L929 cells in a 75 cm^2 flask, near confluence, with the photosensitiser Ro 19-8022 plus visible light (5 min irradiation on ice at 33 cm from a 500 W halogen lamp). Cells were then collected and stored at -80ºC, in aliquots with a density of 3 × 10^6 cells/ml.

For the extract preparation, PC12 cells, plated at a density of 5 × 10^6 cells/ml, were incubated with the nitrogen compounds for either 1 or 24 h. Cells were then trypsinised, divided into aliquots and centrifuged at 14 000 *g* for 5 min, at 4ºC. The dry pellets were then flash frozen in liquid nitrogen and stored at -80ºC. On the day of the experiment, one of these aliquots was thawed and the pellet resuspended in 65 µl of lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10 % glycerol, pH 7.8 with KOH, supplemented with 0.25 % Triton X-100). The suspension, mixed by vortexing, was centrifuged at 14 000 *g* for 5 min, at 4ºC, and 55 µl of the supernatant were removed and combined with 220 µl of cold enzyme reaction buffer.

An aliquot of Ro- and light-treated substrate L929 cells was thawed, washed twice in cold PBS and spinned at 800 *g*, for 5 min, at 4ºC. The pellets were suspended in 100 µl of PBS and 35 µl of this suspension was mixed with 1.5 ml of 1 % low-melting point agarose at 37ºC. These cells were then embedded in agarose and the procedure followed according to the standard Comet assay, with a slight modification: after lysis, the slides were washed three times (for 5 min each) in cold enzyme reaction buffer and incubated with 35 µl of treated or non-treated extract for 20 min. Percentage of DNA in the comets' tails was then measured by a computer-assisted image analysis. Control slides incubated with either FPG (positive control) or buffer alone (negative control) were performed. It should be noted that, in this assay, the higher amount of strand breaks indicates a higher activity of DNA repair enzymes.

3.2.6. Quantification of rOGG1 and rAPE1 expression

Total RNA was isolated from PC12 cells treated in the same conditions as for the base excision repair assay, using a Qiagen RNeasy total RNA isolation kit (Qiagen, USA), following the standard protocol. RNA purity was confirmed by

determining the OD260/OD280 nm absorption ratio. One microgram of total RNA was reversed transcribed with the Superscript Reverse Transcriptase III kit (Invitrogen, USA), by using 50 ng/µl of random hexamers and 10 mM of a dNTP mix according to the manufacturer's instructions. cDNA integrity was verified by gel electrophoresis after PCR amplification of Gapdh, using sequence-specific primers.

PCR was performed using 18S specific primers, manufactured by STAB-VIDA, Portugal (forward: 5'- AAG TCC CTG CCC TTT GTA CAC A -3'; reverse: 5'- GCC TCA CTA AAC CAT CCA ATC G -3') as an internal reference. Specific primer pairs for r*OGG1* (forward: 5'- ACT TAT CAT GGC TTC CCA AAC C -3'; reverse: 5'- CAA CTT CCT CAG GTG GGT CTC T -3') and r*APE1* (forward: 5'- GCG GCA GCG GAA GAC -3'; reverse: 5'- GCC TCC TTC TCA GTT TTC TTT GCT -3') were used (Silva *et al.*, 2008a).

Real-time RT-PCR was performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, USA) using 1 µl of the cDNA preparation, which was added to a reaction mixture containing 12.5 µl Power SYBR Green master mix (Applied Biosciences, Cheshire, UK) plus 1 µl of each primer (25 pmol/µl) and sterile water to a final volume of 25 µl per well. The plates were covered, centrifuged and placed in the thermal cycler. The PCR conditions were: 50ºC for 2 min, 95ºC for 10 min and 40 cycles (95ºC, 15 s; 60ºC, 60 s).

The expression of *OGG1* and *APE1* mRNA in samples was determined from a standard curve constructed from serial dilutions of cDNA obtained from unstimulated PC12 cells. Target genes' transcript levels were all normalised to 18S mRNA levels. The average of at least two replicates, for each of three independent experiments, was used.

3.2.7. Statistical analysis

Data are expressed as the mean ± S.E.M., of the indicated number of experiments. The significance of the differences between the means observed was evaluated using the unpaired two-tailed Student's *t*-test. A difference of $p \leq 0.05$ was considered significant.

3.3. Results

3.3.1. Evaluation of the compounds' protection against DNA strand breaks formation

Reactive oxygen species formed intracellularly can react with DNA, leading to its oxidation, which results in the formation of several types of DNA lesions, such as strand breaks and oxidised bases (Saitoh *et al.*, 2001; Moller *et al.*, 2003).

As a first approach, we studied the protective effects of the compounds against the formation of strand breaks, induced by *t*-BHP, a widely accepted inducer of oxidative stress (Ahmed-Choudhury *et al.*, 1998; Palomba *et al.*, 2001; Pias and Aw, 2002). In fact, this is one of the major types of DNA damage, in which might occur a loss of a purine or pyrimidine base and the deoxyribose, by a cut in the phosphodiester backbone in one or two strands (single-strand or double-strand breaks, respectively) of the double helix (Martin, 2008).

PC12 cells were subjected to either one of two types of incubation with the nitrogen compounds: a co-incubation of 1 h with the compounds and *t*-BHP; or a 3 h pre-incubation with the compounds prior to the addition of the pro-oxidant agent. As it is shown in Figure 3.2G, all the compounds significantly decreased the *t*-BHP-induced formation of DNA strand breaks. FMA762 and FMA796 presented the highest protective effect, leading to a decrease between 25 and 30% in the amount of DNA damage, for either incubation conditions. So, the period of incubation seems not to be important for the protective action observed. The representative images of comets presented in Fig. 3.2 (A-F) clearly demonstrate the protection offered by the compounds, which, in this case, were added to cells at the same time as *t*-BHP. Indeed, the amount of DNA breaks observed in the tail of the comets is decreased in their presence, especially for FMA762 and FMA796 (Fig. 3.2 E and F). In addition, it should also be noted that none of the compounds, by itself, induced an increase in DNA strand breaks, in comparison with control cells, as observed in cultures treated with the nitrogen compounds alone (data not shown).

Based on these results and on our previous work describing the antioxidant properties of these compounds (Silva *et al.*, 2008b), in which FMA762 and FMA796 demonstrated ameliorated ROS scavenging effects, we selected them to proceed with further experiments regarding their action on oxidative DNA damage.

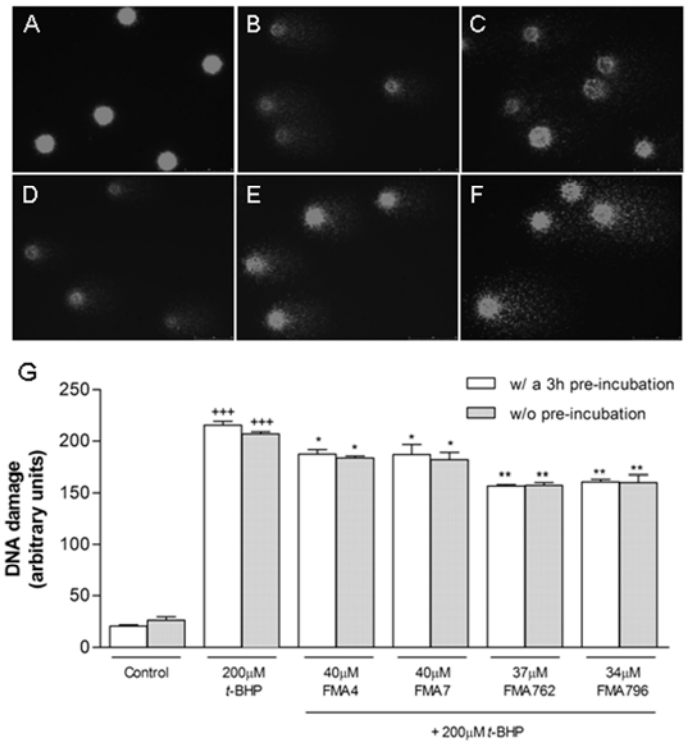


Figure 3.2 – Compounds protection against *t*-BHP-induced formation of DNA strand breaks, evaluated by the Comet assay. A-F) representative images of comets from control, 200μM *t*-BHP, 40 μM FMA4, 40 μM FMA7, 37 μM FMA762 and 34 μM FMA796 (without pre-incubation), respectively. G) Quantification of DNA damage by visual scoring. PC12 cells were incubated for 1 h in the presence of 200μM *t*-BHP. Nitrogen compounds were added to the cells either simultaneously or 3 h prior to the addition of the oxidative stimulus. Each bar represents the mean ± S.E.M. for at least three independent experiments. $^{+++}$ $p \leq 0.001$, compared to respective control cells; * $p \leq 0.05$, ** $p \leq 0.01$, compared to 200μM *t*-BHP (for each respective incubation condition).

So, we then investigated their ability to enhance the repair of strand breaks. To observe a possible effect at this level, after cells' incubation with the nitrogen compounds and *t*-BHP for 1 h, culture media was replaced and cells given a 1 h recovery period (in the absence of any compound or stimulus) to allow the repair of DNA damage, namely strand breaks. The increase in cells' repair capacity induced by the compounds was obtained by subtracting the percentage of repair observed in the presence of the deleterious stimulus alone, to the percentage of repair in the presence of the compounds and the deleterious stimulus, as described before (Materials and Methods section). In Figure 3.3, we observe that cells treated with the deleterious stimulus alone were able to repair 48.2 % of strand breaks during that recovery period (Fig. 3.3B). When incubated in the presence of the nitrogen

compounds, cells' capacity to repair strand breaks was significantly increased (in 19.9 % and 18.3 % for FMA762 and FMA796, respectively) relatively to cells treated with *t*-BHP (see figure legend). Therefore, these results demonstrate the ability of these compounds to protect PC12 cells against DNA strand breaks, not only by preventing their formation but, and perhaps most important than this, by increasing the cells' capacity to repair this kind of damage.

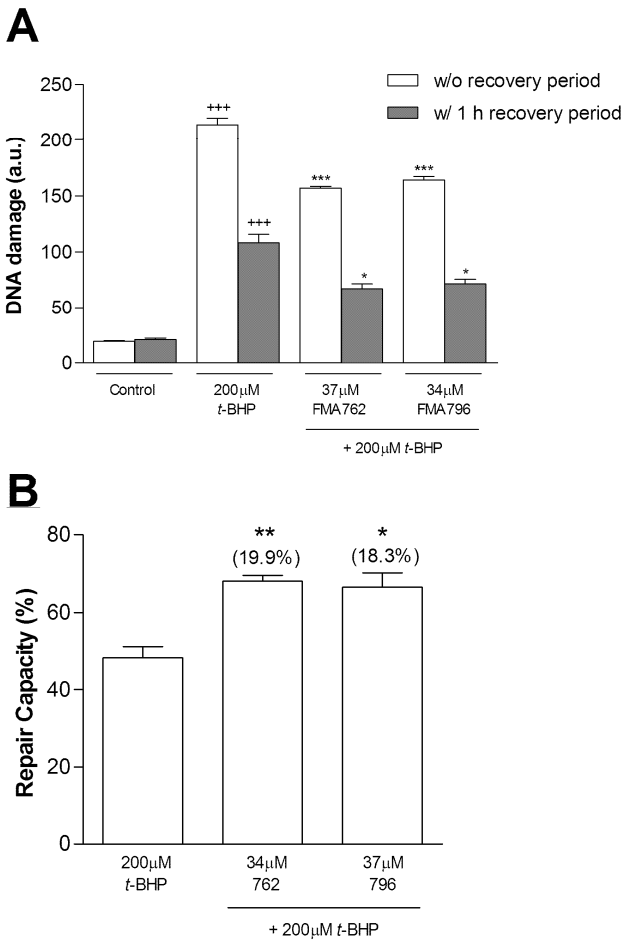


Figure 3.3 – Effects of the compounds on DNA strand breaks repair. A) After 1 h of cells' simultaneous incubation with the compounds and 200μM *t*-BHP, cell culture media was replaced and DNA damage evaluated 1 h later. Comets were visually scored and results expressed in percentage of damage relatively to the maximum obtained before the recovery period. B) Repair capacity was calculated using the formula presented in the Material and Methods section. Values in brackets represent the increase in repair capacity. For each bar is represented the mean ± S.E.M for at least three independent experiments. $^{+++}$ $p \leq 0.001$, compared to respective control cells; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, compared to 200μM *t*-BHP (for each respective incubation condition).

3.3.2. Effects of the nitrogen compounds on base oxidation

Nucleotidic bases in the DNA can also undergo structure modifications upon exposure to ROS. Oxidative base lesions are of likely importance as DNA strand breaks to overall cellular function and survival (Bjelland and Seeberg, 2003). In fact, they mispair with the other bases during DNA replication and transcription, thus becoming mutagenic (Martin, 2008). So, we continued our studies by investigating if the nitrogen compounds, were also able to prevent the oxidation of nucleotidic bases.

Bases oxidation was induced in the presence of Ro 19-8022, a photosensitiser compound that produces singlet oxygen, which is 1000-fold less reactive with DNA than hydroxyl radicals. In this way, guanine modifications sensitive to FPG, in addition to AP sites and pyrimidine modifications sensitive to endonuclease III are generated in excess over single-strand breaks, as previously described (Angelis *et al.*, 1999; Tomasetti *et al.*, 2001). In contrast with the effects previously observed using Ro 19-8022, *t*-BHP induced a great amount of strand breaks along with the oxidation of nucleotidic bases, not allowing the distinction between those two types of damage. From this point on, the % of DNA in the tail of comets was quantified by computerised image analysis, since we previously observed a good correlation between this and the semi-quantitative method of visual scoring (Silva *et al.*, 2008a)**(1)**, which is in accordance with other authors (Collins *et al.*, 1997), and also decreases the subjectivity.

As it can be observed in Figure 3.4, which presents the amount of oxidised purines only (FPG-sensitive sites), treatment of PC12 cells with 0.6 μM Ro 19-8022 led to a significant increase of the percentage of DNA in the comets' tails. This Ro 19-8022 concentration was chosen based on previous results (not shown) indicating that with 0.6 μM Ro 19-8022 a significant increase in DNA damage was observed, though not reaching the assay's saturation point, which could lead to underestimation of enzyme-sensitive sites (Gedik *et al.*, 2002). Figure 3.4 also shows that pre-incubation of the cells with the nitrogen compounds, for either 1 h or 24 h, did not lead to a significant decrease in the amount of oxidised purines.

In addition we assessed the effects of the nitrogen compounds after a recovery period, following the incubation with Ro 19-8022, since the observation of a possible protective effect could be related with the time needed for the repair of bases to occur, after the incubation period. In fact, it is described that base lesions require a longer period of time to be repaired, in comparison to strand breaks (Collins and

(1) Results presented in Fig. 4.6 (Chapter 4, page 146)

Horvathova, 2001). So, after treatment of PC12 cells with Ro 19-8022, culture media was replaced and cells allowed to recover for a period of 6 h. Previous results had shown that at this time point cells had repaired between 40 - 50 % of oxidised bases and the assay had not reached saturation (Silva *et al.*, 2008a) [(1)].

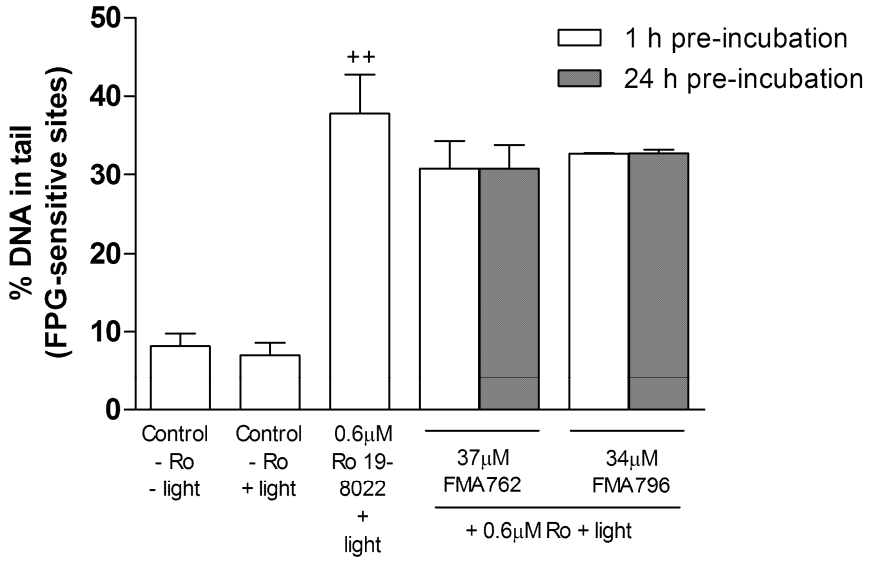


Figure 3.4 – Effect of the nitrogen compounds on Ro 19-8022-induced purines oxidation. PC12 cells were either incubated for 1 h or 24 h, prior to the treatment with 0.6 μM Ro 19-8022 plus light. The level of oxidized purines was determined through sample incubation with FPG, for 30 min. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. Each bar represents the mean ± S.E.M for at least three independent experiments. $^{++}$ $p \leq 0.01$, compared to cells in the absence of Ro 19-8022.

Results in Figure 3.5 show that after the 6 h recovery period, cells had repaired 43.8 % of the amount of oxidised purines induced in the presence of Ro 19-8022. In addition, after that same recovery period, but following a 24 h incubation of cells in the presence of the nitrogen compounds and treatment with Ro 19-8022, the levels of DNA in the comets' tails were not significantly altered by the presence of the compounds (Fig. 3.5A). However, by calculating the repair capacity induced by each compound, we observed that treatment with FMA796 leads to a statistically significant increase (6.2 %) in the cells capacity to repair oxidised purines, relatively to the Ro-treated cells (Fig. 3.5B). On the other hand, cell treatment with FMA762 did not significantly enhance the cells' repair capacity, although there seems to be a trend to do so.

[(1)] Results presented in Fig. 4.8 (Chapter 4, page 148)

Therefore, these results suggest that the nitrogen compounds, particularly FMA796, may be involved in the repair of oxidised bases.

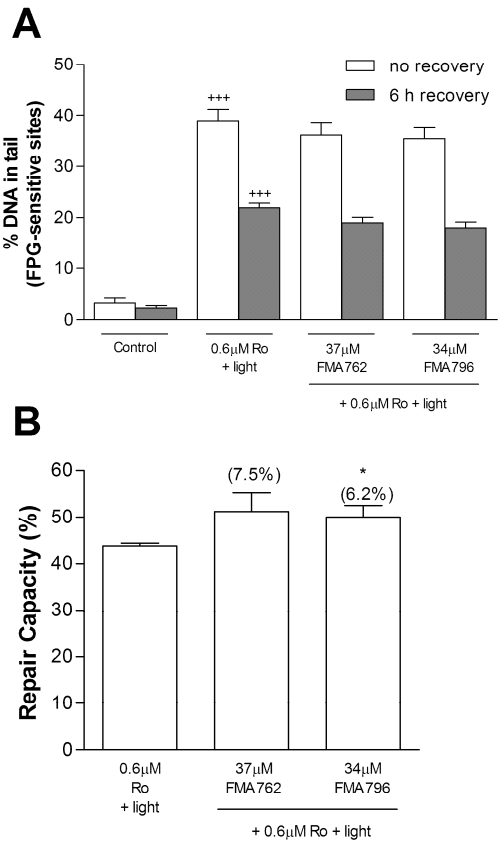


Figure 3.5 – Effects of the compounds on purine oxidation repair. A) After a 24 h incubation in the presence of the compounds, cells were treated with Ro 19-8022 plus light for 5 min, on ice. Culture media was then replaced and DNA damage evaluated by the Comet assay 6 h later. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. B) Repair capacity calculated according to the formula presented in Material and Methods. Values in brackets represent the increase in repair capacity. For each bar is represented the mean ± S.E.M. for at least three independent experiments. $^{+++}$ $p \leq 0.001$, compared to respective control cells. * $p \leq 0.05$, compared to Ro 19-8022.

3.3.3. In vitro *base excision repair assay*

Repair of DNA containing oxidised bases in vivo proceeds predominantly through the base excision repair (BER) pathway initiated by DNA glycosylases (Bjelland and Seeberg, 2003). Therefore, we investigated the ability of the compounds to enhance the activity of DNA repair enzymes, by using a modified version of the Comet assay that measures the incision activity of cell-free extracts, which were obtained from PC12 cells previously treated with the nitrogen compounds for 24 h. Damage-containing substrates were obtained by treating L929 cells with 1 μM Ro 19-8022, for 5 min on ice, under a 500 W halogen lamp. Since these substrate cells were treated with Ro 19-8022 plus light, the breaks observed reflect only the amount of oxidised nucleotidic bases.

As presented in Figure 3.6E, after a 20 min incubation of the substrate with a cell-free extract, containing the DNA repair enzymes, there is an increase in the number of DNA breaks (FPG-sensitive sites) relatively to the control (24.84 ± 0.47 % *vs* 6.12 ± 0.97 %, $p \leq 0.001$), which is indicative of the greater incision activity of the extract and is in accordance with the literature (Collins *et al.*, 2003; Dusinska *et al.*, 2006). It is also evident in the same figure that extracts from compound-treated cells showed a significant increase in their incision activity (2.44 % and 5.56% for FMA762 and FMA796, respectively) compared to the extract of non-treated cells. However, if we subtract the amount of damage in the control (containing strand-breaks alone) to all the other conditions, and consider the amount of damage in the extract of untreated cells as being 100%, then those differences effectively correspond to increases in the extracts' DNA repair capacity of 13.03 % and 29.7 %, for FMA762 and FMA796. The ameliorated incision activity of the extracts from FMA762 and FMA796-treated cells is also clearly demonstrated in the representative images (Fig. 3.6A-D), in which is possible to observe an increased amount of DNA damage in cells incubated with extracts treated with the nitrogen compounds, relatively to the non-treated extract.

These positive effects on the DNA repair enzymes' activity suggested us a possible involvement of the nitrogen compounds in the regulation of gene expression of those same enzymes.

3.3.4. Expression of DNA repair genes

We then tried to elucidate the molecular mechanisms underlying the action of the compounds in study on the DNA repair enzymes' activity. In this way, we investigated a possible direct effect of these compounds on the expression of the DNA repair genes *OGG1* and *APE1*, using *real time* RT-PCR. These genes are known to play an important role in DNA repair, namely in the base excision repair pathway, as stated in the introduction.

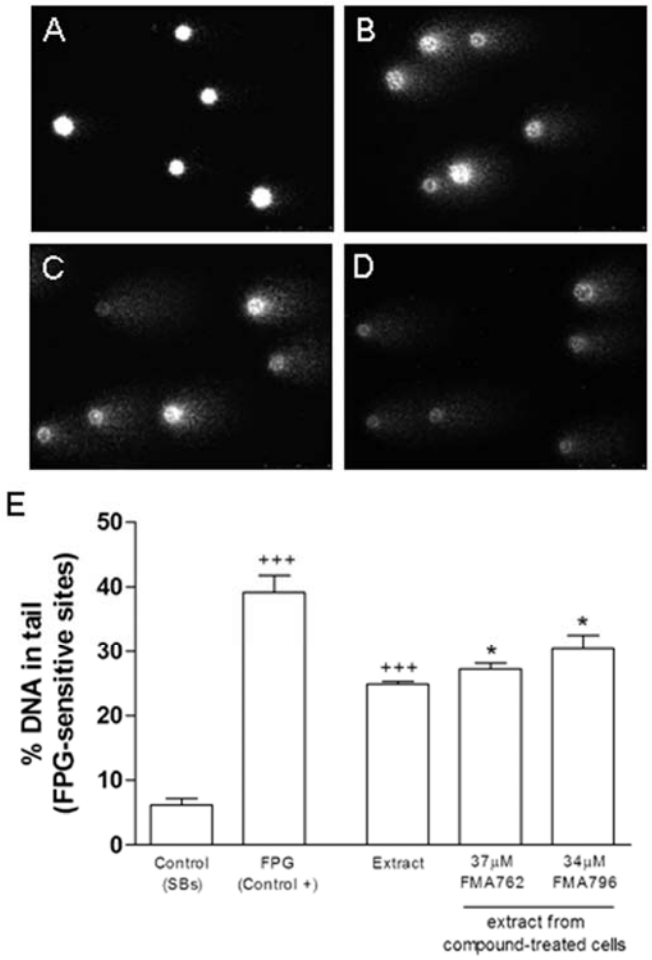


Figure 3.6 – Effect of the compounds on the *in vitro* BER assay. A-D) representative images of Comets from control (buffer alone), and extracts from non-treated, FMA762-treated and FMA796-treated cells, respectively. E) Quantification of the percentage of DNA in the comets tails after treatment of damage-containing substrate cells with a cell-free extract obtained from PC12 cells incubated with the nitrogen compounds for 24 h. The repair enzyme FPG was used as a positive control. Levels of oxidised purines were determined and quantified as previously described. For each condition is represented the mean ± S.E.M for at least three independent experiments. $^{+++}$ $p \leq 0.001$compared to control cells; * $p \leq 0.05$, relatively to non treated extract. SBs – Strand Breaks.

Results in Figure 3.7 show that no statistically significant differences could be observed between the expression of the DNA repair genes in Ro-treated and non-treated cells (control bars). In addition, treatment of PC12 cells with the nitrogen compounds did not also induce any significant changes in the expression of the genes tested, for both conditions.

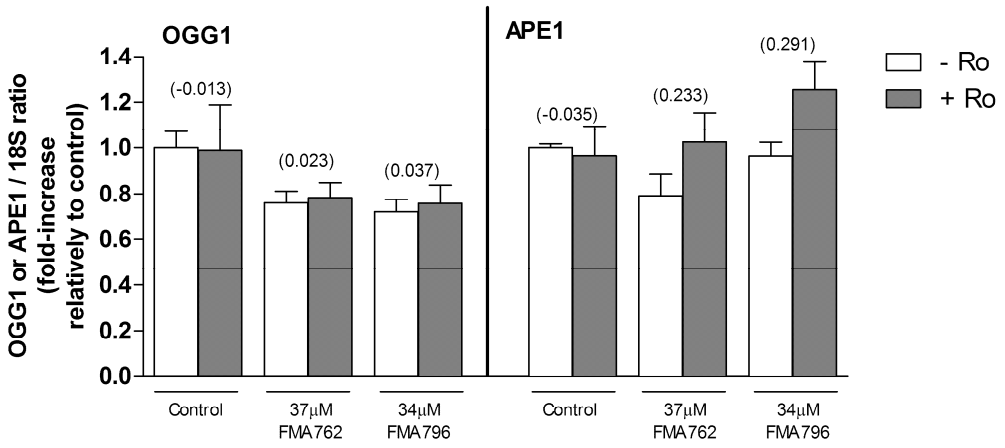


Figure 3.7 – Effects of FMA762 and FMA796 on the expression of repair genes *OGG1* and *APE1*. PC12 cells were incubated for 24 h in the presence of the nitrogen compounds, prior to the exposure to Ro 19-8022 plus visible light. Levels of genes expression was determined by *real time* RT-PCR and results expressed as the increase in the expression of each gene relatively to the control. Values in brackets represent the difference in the fold-increase expression between Ro-treated and non-treated conditions. Real control values were of 0.376 and 0.288 arbitrary units for *OGG1* and *APE1*, respectively. Each bar represents the mean ± S.E.M for at least two independent experiments.

Such results indicate that the action of the compounds on DNA repair is not mediated by a direct regulation at the level of the expression of the DNA repair genes tested, which are the most important in BER pathways, as already mentioned. These results are discussed below.

3.4. Discussion

DNA oxidation resulting from the action of reactive oxygen species has been described to play an important role in neurodegenerative diseases, aging and cancer. Thus, prevention of DNA damage has obvious and important implications for the prevention and treatment of the mentioned situations (Duthie and Dobson, 1999;

Perron *et al.*, 2008). Despite the presence of antioxidant systems that scavenge endogenous and/or exogenous free radicals, DNA damage still occurs in normal physiological conditions. However, most of this damage is removed by repair enzymes before it can interfere with the process of DNA replication, by introducing mutations and eventually stopping it. In this way, an equilibrium state between damage and repair is maintained. The implication is that DNA repair, in normal physiological conditions, maintains oxidative damage at a level that is tolerable in terms of genetic stability (Tomasetti *et al.*, 2001; Gaivão *et al.*, 2008).

To date, many compounds presenting antioxidant properties, of both natural and synthetic origin, have been tested for their ability to reduce oxidative DNA damage. However, some of them have different effects, depending on the concentrations, cell lines and oxidant stimuli used (Cemeli *et al.*, 2008). In addition, most of those studies do not reveal if the protective effects observed are attained through an improvement of DNA repair or if the compounds simply prevent the oxidation of DNA due to their antioxidant properties. Recently, we have also studied the effects of three polyphenolic compounds, namely luteolin, quercetin and rosmarinic acid on oxidative DNA damage (Silva *et al.*, 2008a). Although all of them decreased the amount of strand breaks formed, only rosmarinic acid was able to increase the cells' capacity to repair base lesions, most likely due to its involvement in the regulation of expression of some genes responsible for DNA repair.

We have been investigating the potential of new nitrogen compounds as protectors of oxidative stress conditions (Silva *et al.*, 2006; Silva *et al.*, 2008b). In this particular work, we evaluated the protective effects of four of those compounds (FMA4, FMA7, FMA762 and FMA796) on oxidative DNA damage induced in a neuronal cell model, with a special focus on the mechanisms by which they could be acting.

We began by investigating their involvement in protection and repair of *t*-BHP-induced strand breaks formation. This type of damage, and in particular double-strand breaks, is widely accepted to be the most severe form of damage, which if left un/mis-repaired may either induce gross chromosomal rearrangements or become cytotoxic (Dobbs *et al.*, 2008). The compounds were tested at concentrations previously shown in our lab to have high antioxidant activity and no cytotoxicity (Silva *et al.*, 2008b). As it might be expected from their antioxidant properties, all the four compounds were able to decrease the *t*-BHP-induced DNA strand breaks formation. In accordance with our previous results (Silva *et al.*, 2008b), FMA762 and FMA796, which differ from FMA4 and FMA7 mainly by the

presence of three hydroxyl groups within the phenol ring (instead of just one), proved to be the most efficient ones. So, we proceeded by testing only those two compounds that presented better results. No differences in protection were observed when the compounds were added simultaneously or 3 h prior to the addition of *t*-BHP, which should be taken in consideration when comparing these compounds with some natural ones. Quercetin, for example, despite having important protective effects against oxidative DNA damage, may be metabolised into a less protective compound, and even become a cause of genotoxicity when added to cells for long periods of time, as we previously reported in PC12 cells (Silva *et al.*, 2008a) and as it is also reported in lymphocytes (Duthie and Dobson, 1999). FMA762 and FMA796 also led to an increase in the cells' capacity to repair strand breaks, which was similar for both of them.

The action of these compounds against *t*-BHP-induced strand breaks formation may be attributed, at least in part, to their ROS scavenging properties. Indeed, our previous work (Silva *et al.*, 2008b) demonstrated their effects on several biomarkers of oxidative stress, such as their ability to decrease intracellular ROS formation and lipid peroxidation. In that same report, they were also shown to scavenge hydroxyl ($^{\bullet}$OH) radicals, as indicated by the 2-deoxy-D-ribose degradation assay. These results assume major importance if we take into account that, as it has been described, $^{\bullet}$OH radicals may be considered the main contributing reactive oxygen species to endogenous oxidation of cellular DNA (Cadet *et al.*, 1999; Dizdaroglu *et al.*, 2002), although this radical has a generalised action on all biomolecules. Moreover, another feature that may be contributing to these compounds' protection against DNA damage is their good lipophilicity, as indicated by their relatively high partition coefficients (Silva *et al.*, 2008b). In fact it is described that the degree of lipophilicity of a compound is of great importance for protecting cells from DNA damage, since it increases its cellular uptake (Sestili *et al.*, 2002; Spencer *et al.*, 2004; Lima *et al.*, 2006) and consequent interaction with the hydrophilic DNA.

Alternatively, the protective effects of these nitrogen compounds might be associated to an involvement in the intracellular pathways related to DNA repair. However, measuring DNA strand breaks only gives limited information, since breaks may represent the direct effect of some damaging agent, but they are generally quickly rejoined (Collins, 2004). So, it becomes important to investigate the effects of the nitrogen compounds on the protection against nucleotidic bases oxidation, a kind of damage which is more slowly repaired by the cells. In the conditions tested, none of the compounds was able to reduce the amount of oxidised purines induced

by the photosensitiser compound Ro 19-8022, regardlessly of the incubation time used in the pre-treatment. Nevertheless, at least FMA796 was able to increase the cells' capacity to repair oxidised purines. For FMA762, a trend to increase the repair of these lesions was observed, though this increase was not statistically significant.

The effect of an antioxidant on recovery from oxidative DNA damage can be explained by at least two ways: 1) it can stimulate the activity of repair enzymes or 2) it can directly protect against oxidation (Tomasetti *et al.*, 2001). The lack of a protective effect against Ro-induced DNA damage, for either of the pre-treatments tested, suggest that the nitrogen compounds do not act through the second mechanism, since they do not seem able to scavenge the singlet oxygen produced by Ro 19-8022, though they seem to scavenge the radicals produced by *t*-BHP. However, at least for FMA796 there seems to be an effect on the stimulation of the oxidised bases repair, which suggests that these compounds may be acting on the intracellular pathways responsible for the repair of DNA base lesions.

In order to better understand the mechanisms underlying the compounds action on oxidative DNA damage, we used an approach to assess the repair capacity of a sample extract provided with a DNA substrate carrying a specific type of damage (in this case, oxidised nucleotidic bases), the BER assay.

We observed that both nitrogen compounds (FMA762 and FMA796) led to an increase in the activity of DNA repair enzymes, as indicated by the greater amount of breaks formed in the presence of the extracts pre-treated with these compounds, relatively to the non-treated extract. However, this effect does not seem to be regulated at the gene expression level, since no significant changes could be observed in the levels of *OGG1* and *APE1* mRNA. Similar results were obtained in a human intervention study, in which healthy individuals were given a diet supplementation with kiwifruit. In that study, higher incision rates were observed in extracts of lymphocytes obtained after kiwifruit supplementation. However, no changes in *OGG1* and *APE1* genes expression were observed, even after diet supplementation (Collins *et al.*, 2003). It should be considered that, like in that study, the effects observed with our nitrogen compounds on DNA repair may result from an increased stability of the OGG1 protein or availability of an unknown factor, which could, for example, increase the lyase activity of OGG1. In addition, it is also possible that repair enzymes are susceptible to inhibition by ROS and that a decrease in these reactive species, resulting from exposure to the antioxidant compounds, gives rise to the higher rates of repair, as also observed by Collins and co-workers (Collins *et al.*, 2003). Moreover, it should be taken into account that by

measuring the expression of *OGG1* and *APE1* genes by *real time* RT-PCR, we are just assessing the effects of the compounds on mRNA steady state levels. However, additional factors have been demonstrated to enhance the activity of *OGG1* (Le Page *et al.*, 2000). So, it is possible that the compounds may also be involved in the regulation of DNA repair at the post-transcriptional level. Independently of the explanation, increased repair together with the increased antioxidant capacity demonstrated by these nitrogen compounds in this and in our previous studies, can account for the significant decreases in endogenous oxidative DNA damage.

It should be noted that, although the protective effects observed for the nitrogen compounds on increased repair of base oxidation appear to be small, they may in fact assume a great biological relevance. In this regard, some points should be taken into consideration: 1) DNA repair involves a complex and coordinated network of events that coordinate cell cycle arrest, DNA repair and apoptosis following DNA damage. Since those defects in the DNA damage response may result in several disorders, including cancer, a fine equilibrium is required for proper activation and inactivation of DNA damage signalling and repair mechanisms (Buscemi *et al.*, 2006; Déry and Masson, 2007); 2) Some of the genes involved in the DNA repair process, like *OGG1*, are considered housekeeping genes (Loft and Moller, 2006). Thus, a large increase in their activity could result in the deregulation of fine-tuned mechanisms vital to cells; 3) Several polymorphisms of DNA glycosylases (like OGG1) responsible for the excision of 8-oxoGua are known, and their presence in human genomes has been linked to the risk of developing specific types of cancers. However, some of them have been shown to be responsible for reducing the affinity of OGG1 to bind DNA, in comparison to the wild-type enzyme (Tudek, 2007). Thus, these polymorphic variants decrease the repair enzyme's activity, which may also be responsible for the small differences obtained for the incision activity levels; 4) Some DNA repair enzymes also participate in other cellular functions. For example, APE1 is also able to enhance DNA binding of a number of transcription factors, including p53, Egr-1 and NF-kB, which are involved in cell proliferation, by acting as transcriptional co-activators (Fantini *et al*. 2008). In this way, a deregulation of that gene could interfere with other processes vital to cells.

So, given the complex regulation of DNA damage, above mentioned, and its association with other cellular processes, it should be considered that great increases in the expression and activity of the several proteins involved in the tight regulation of those mechanisms could result in abnormalities in vital processes.

Therefore, small nitrogen compounds-induced increases in DNA repair, which assume great relevance at the biological level, should not be underestimated.

In this study, we have shown the protective effects of new nitrogen compounds against oxidative DNA damage induced to PC12 cells and investigated some of the underlying mechanisms regulating their action. The nitrogen compounds in study not only were able to prevent *t*-BHP-induced DNA strand breaks formation, but also induced an increase in the repair of different types of DNA damage, such as strand breaks and oxidised bases. However, it should be taken into account that this study intended to be the first step in the evaluation of the protective effects of these new compounds on oxidative DNA damage induced to neuronal cells. Before any correlations can be made between the protection observed *in vitro*, in this cell model, and what happens *in vivo*, more studies should be performed, since it remains unknown, for example, if they are capable of crossing the blood-brain barrier. Nevertheless, even if they are unable to do so, a role for these compounds in neuroprotection should not be ruled out, since there are ways of increasing a compound's ability to cross the blood brain barrier, like the combination of some phospholipids with flavonoids described in Dajas and colleagues (Dajas *et al.*, 2003).

In conclusion, the results herein presented confirm the great value of the nitrogen compounds in study, while opening new perspectives for their development and application in several pathological conditions, like neurodegenerative diseases, in which oxidative DNA damage is involved. Nevertheless, further investigation, which is currently being held, is required to understand the exact mechanisms of action of these compounds on DNA repair, namely which proteins may be activated in the presence of the compounds and how exactly their ROS scavenging properties may be important for that process.

3.5. References

Ahmed-Choudhury, J., Orsler, D.J. and Coleman, R., 1998. Hepatobiliary effects of tertiary-butylhydroperoxide (tBOOH) in isolated rat hepatocyte couplets. *Toxicol. Appl. Pharmacol.* **152**: 270-275.

Alamo, M.J.P., Jurado, J., Francastel, E. and Laval, F., 1998. Rat 7,8-dihydro-8-oxoguanine DNA glycosylase: substrate specificity, kinetics and cleavage mechanism at an apurinic site. *Nucleic Acids Res.* **26:** 5199-5202.

Angelis, K.J., Dusinská, M. and Collins, A.R., 1999. Single cell gel electrophoresis: Detection of DNA damage at different levels of sensitivity. *Electrophoresis* **20:** 2133-2138.

Areias, F.M., Rego, C., Oliveira, C. and Seabra, R.M., 2001. Antioxidant effect of flavonoids after ascorbate/Fe^{2+}-induced oxidative stress in cultured retinal cells. *Biochem. Pharmacol.* **62:** 111-118.

Areias, F.M. 2006. Novos compostos heterocíclicos de azoto com unidades fenólicas: síntese e actividade biológica. *Ph.D. Thesis*. University of Minho, Braga, Portugal. 259p. Available online at: http://hdl.handle.net/1822/5941.

Bjelland, S. and Seeberg, E., 2003. Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutat. Res.* **531:** 37-80.

Bohr, V.A., Ottersen, O.P. and Tonjum, T., 2007. Genome instability and DNA repair in brain, ageing and neurological disease. *Neurosci.* **145:** 1183-1186.

Booth, B.L., Costa, F.A.T., Mahmood, Z., Pritchard, R.G. and Proenca, M.F., 1999. Synthesis of (Z)-N-(2-amino-1,2-dicyanovinyl)formamide O-alkyloximes and a study of their cyclization in the presence of base. *J. Chem. Soc. [Perkin 1]* **13:** 1853-1858.

Brooks, P.J., 2002. DNA repair in neural cells: basic science and clinical implications. *Mutat. Res.* **509:** 93-108.

Burkle, A., 2006. DNA repair and PARP in aging. *Free Radic. Res.* **40:** 1295-1302.

Buscemi, G., Carlessi, L., Zannini, L., Lisanti, S., Fontanella, E., Canevari, S. and Delia, D., 2006. DNA damage-induced cell cycle regulation and function of novel Chk2 phosphoresidues. *Mol. Cell Biol.* **26:** 7832-7845.

Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J.P., Ravanat, J.L. and Sauvaigo, S., 1999. Hydroxyl radicals and DNA base damage. *Mutat. Res.* **424:** 9-21.

Cemeli, E., Baumgartner, A. and Anderson, D., 2008. Antioxidants and the Comet assay. *Mutat. Res.*, *doi:10.1016/j.mrrev.2008.05.002*.

Collins, A.R., Dusinska, M., Franklin, M., Somorovska, M., Petrovska, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslova, K. and Vaughan, N., 1997. Comet assay in human biomonitoring studies: Reliability, validation, and applications. *Environ. Mol. Mutagen.* **30:** 139-146.

Collins, A.R., 2004. The comet assay for DNA damage and repair - Principles, applications, and limitations. *Mol. Biotechnol.* **26:** 249-261.

Collins, A.R. and Gaivão, I., 2007. DNA base excision repair as a biomarker in molecular epidemiology studies. *Mol. Aspects Med.* **28:** 307-322.

Collins, A.R., Harrington, V., Drew, J. and Melvin, R., 2003. Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis* **24:** 511-515.

Collins, A.R. and Horvathova, E., 2001. Oxidative DNA damage, antioxidants and DNA repair: applications of the comet assay. *Biochem. Soc. Trans.* **29:** 337-341.

Colognato, R., Laurenza, I., Fontana, I., Coppede, F., Siciliano, G., Coecke, S., Aruoma, O. I., Benzi, L. and Migliore, L., 2006. Modulation of hydrogen peroxide-induced DNA damage, MAPKs activation and cell death in PC12 by ergothioneine. *Clin. Nutr.* **25:** 135-145.

Coppede, F., Mancuso, M., Lo Gerfo, A., Manca, M. L., Petrozzi, L., Migliore, L., Siciliano, G. and Murri, L., 2007. A Ser326Cys polymorphism in the DNA repair gene hOGG1 is not associated with sporadic Alzheimer's disease. *Neurosci. Lett.* **414:** 282-285.

Cui, K., Luo, X., Xu, K. and Ven Murthy, M.R., 2004. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **28:** 771-799.

Dajas, F., Rivera-Megret, F., Blasina, F., Arredondo, F., bin-Carriquiry, J.A., Costa, G., Echeverry, C., Lafon, L., Heizen, H., Ferreira, M. and Morquio, A., 2003. Neuroprotection by flavonoids. *Braz. J. Med. Biol. Res.* **36:** 1613-1620.

Déry, U. and Masson, J.Y., 2007. Twists and turns in the function of DNA damage signaling and repair proteins by post-translational modifications. *DNA Repair* **6:** 561-577.

Dizdaroglu, M., Jaruga, P., Birincioglu, M. and Rodriguez, H., 2002. Free radical-induced damage to DNA: Mechanisms and measurement. *Free Radic. Biol. Med.* **32:** 1102-1115.

Dobbs, T.A., Palmer, P., Maniou, Z., Lomax, M.E. and O'Neill, P., 2008. Interplay of two major repair pathways in the processing of complex double-strand DNA breaks. *DNA Repair* **7:** 1372-1383.

Dodson, M.L. and Lloyd, R.S., 2002. Mechanistic comparisons among base excision repair glycolsylases. *Free Radic. Biol. Med.* **32:** 678-682.

Dusinska, M., Dzupinkova, Z., Wsolova, L., Harrington, V. and Collins, A.R., 2006. Possible involvement of XPA in repair of oxidative DNA damage deduced from analysis of

damage, repair and genotype in a human population study. *Mutagenesis* **21:** 205-211.

Duthie, S. and Dobson, V.L., 1999. Dietary flavonoids protect human colonocyte DNA from oxidative attack. *Eur. J. Nutr.* **38:** 28-34.

Englander, E.W. and Ma, H.X., 2006. Differential modulation of base excision repair activities during brain ontogeny: Implications for repair of transcribed *DNA. Mech. Ageing Dev.* **127:** 64-69.

ESCODD - European Standard Committee on Oxidative DNA Damage, 2003. Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. *Free Radic. Biol. Med.* **34:** 1089-1099.

Fantini, D., Vascotto, C., Deganuto, M., Bivi, N., Gustincich, S., Marcon, G., Quadrifoglio, F., Damante, G., Bhakat, K.K., Mitra, S. and Tell, G., 2008. *Free Radic. Res* **42:** 20-29.

Fishel, M.L., Vasko, M.R. and Kelley, M.R., 2007. DNA repair in neurons: So if they don't divide what's to repair? *Mutat. Res.* **614:** 24-36.

Gabbita, S.P., Lovell, M.A. and Markesbery, W.R., 1998. Increased nuclear DNA oxidation in the brain in Alzheimer's disease. *J. Neurochem.* **71:** 2034-2040.

Gaivão, I., Piasek, A., Brevik, A., Shaposhnikov, S. and Collins, A.R., 2008. Comet assay-based methods for measuring DNA repair in vitro; estimates of inter- and intra-individual variation. *Cell Biol. Toxicol.* **25:** 45-52.

Gedik, C.M., Boyle, S.P., Wood, S.G., Vaughan, N.J. and Collins, A.R., 2002. Oxidative stress in humans: validation of biomarkers of DNA damage. *Carcinogenesis* **23:** 1441-1446.

Gedik, C.M. and Collins, A.R., 2004. Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study. *FASEB J.* **18:** 82-104.

Greene, L.A. and Tischler, A.S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **73:** 2424-2428.

Guan, S., Bao, Y.-M., Jiang, B. and An, L.-J., 2006. Protective effect of protocatechuic acid from Alpinia oxyphylla on hydrogen peroxide-induced oxidative PC12 cell death. *Eur. J. Pharmacol.* **538:** 73-79.

Hazra, T.K., Das, A., Das, S., Choudhury, S., Kow, Y.W. and Roy, R., 2007. Oxidative DNA damage repair in mammalian cells: A new perspective. *DNA Repair* **6:** 470-480.

Hill, J.W., Hazra, T.K., Izumi, T. and Mitra, S., 2001. Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair. *Nucleic Acids Res.* **29:** 430-438.

Jung, J.Y., Han, C.R., Jeong, Y.J., Kim, H.J., Lim, H.S., Lee, K.H., Park, H.O., Oh, W.M., Kim, S.H. and Kim, W.J., 2007. Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. *Neurosci. Lett.* **411:** 222-227.

Kruman, I.I., 2004. Why do neurons enter the cell cycle? *Cell Cycle* **3:** 769-773.

Le Page, F., Klungland, A., Barnes, D.E., Sarasin, A. and Boiteux, S., 2000. Transcription coupled repair of 8-oxoguanine in murine cells: The Ogg1 protein is required for repair in nontranscribed sequences but not in transcribed sequences. *Proc. Natl. Acad. Sci. U. S. A.* **97:** 8397-8402.

Lima, C.F., Fernandes-Ferreira, M. and Pereira-Wilson, C., 2006. Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. *Life Sci.* **79:** 2056-2068.

Loft, S. and Moller, P., 2006. Oxidative DNA damage and human cancer: Need for cohort studies. *Antioxid. Redox Signal.* **8:** 1021-1031.

Martin, L.J., 2008. DNA damage and repair: Relevance to mechanisms of neurodegeneration. *J. Neuropathol. Exp. Neurol.* **67:** 377-387.

Moller, P., Vogel, U., Pedersen, A., Dragsted, L.O., Sandstrom, B. and Loft, S., 2003. No effect of 600 grams fruit and vegetables per day on oxidative DNA damage and repair in healthy nonsmokers. *Cancer Epidemiol. Biomarkers Prev.* **12:** 1016-1022.

Neri, S., Gardini, A., Facchini, A., Olivieri, F., Franceschi, C., Ravaglia, G. and Mariani, E., 2005. Mismatch repair system and aging: Microsatellite instability in peripheral blood cells from differently aged participants. *J. Gerontol. A Biol. Sci. Med. Sci.* **60:** 285-292.

Palomba, L., Sestili, P. and Cantoni, O., 2001. *tert*-butylhydroperoxide induces peroxynitrite-dependent mitochondrial permeability transition leading PC12 cells to necrosis. *J. Neurosci. Res.* **65:** 387-395.

Perron, N.R., Hodges, J.N., Jenkins, M. and Brumaghim, J.L., 2008. Predicting How Polyphenol Antioxidants Prevent DNA Damage by Binding to Iron. *Inorg. Chem.* **47:** 6153-6161.

Pias, E.K. and Aw, T.Y., 2002. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC-12 cells. *Cell Death Differ.* **9:** 1007-1016.

Piga, R., Saito, Y., Chen, Z., Yoshida, Y. and Niki, E., 2005. Characterization of monochloramine toxicity on PC12 cells and protective effect of tocopherol via antioxidative function. *Arch. Biochem. Biophys.* **436:** 101-109.

Powell, C.L., Swenberg, J.A. and Rusyn, I., 2005. Expression of base excision DNA repair genes as a biomarker of oxidative DNA damage. *Cancer Lett.* **229:** 1-11.

Ramos, A.A., Lima, C.F., Pereira, M.L., Fernandes-Ferreira, M. and Pereira-Wilson, C., 2008. Antigenotoxic effects of quercetin, rutin and ursolic acid on HepG2 cells: Evaluation by the comet assay. *Toxicol. Lett.* **177:** 66-73.

Rao, K.S., 2007. DNA repair in aging rat neurons. *Neurosci.* 145, 1330-1340.

Rolseth, V., Rundén-Pran, E., Luna, L., McMurray, C., Bjorás, M. and Ottersen, O.P., 2008. Widespread distribution of DNA glycosylases removing oxidative DNA lesions in human and rodent brains. *DNA Repair* **7:** 1578-1588.

Saitoh, T., Shinmura, K., Yamaguchi, S., Tani, M., Seki, S., Murakami, H., Nojima, Y. and Yokota, J., 2001. Enhancement of OGG1 protein AP lyase activity by increase of APEX protein. *DNA Repair* **486:** 31-40.

Sasaki, N., Toda, T., Kaneko, T., Baba, N. and Matsuo, M., 2003. Protective effects of flavonoids on the cytotoxicity of linoleic acid hydroperoxide toward rat pheochromocytoma PC12 cells. *Chem. Biol. Interact.* **145:** 101-116.

Sestili, P., Diamantini, G., Bedini, A., Cerioni, L., Tommasini, I., Tarzia, G. and Cantoni, O., 2002. Plant-derived phenolic compounds prevent the DNA single-strand breakage and cytotoxicity induced by *tert*-butylhydroperoxide via an iron-chelating mechanism. *Biochem. J.* **364:** 121-128.

Silva, J.P., Areias, F.M., Proença, M.F. and Coutinho, O.P., 2006. Oxidative stress protection by newly synthesized nitrogen compounds with pharmacological potential. *Life Sci.* **78:** 1256-1267.

Silva, J.P., Gomes, A.C. and Coutinho, O.P., 2008a. Oxidative DNA damage protection and repair by polyphenolic compounds in PC12 cells. *Eur. J. Pharmacol.* **601:** 50-60.

Silva, J.P., Proença, M.F. and Coutinho, O.P., 2008b. Protective role of new nitrogen compounds on ROS/RNS-mediated damage to PC12 cells. *Free Radic. Res.* **42:** 57-69.

Spencer, J.P.E., El Mohsen, M.M. and Rice-Evans, C., 2004. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch. Biochem. Biophys.* **423:** 148-161.

Tomasetti, M., Alleva, R. and Collins, A.R., 2001. In vivo supplementation with coenzyme Q(10) enhances the recovery of human lymphocytes from oxidative DNA damage. *FASEB J.* **15:** 1425-1451.

Tudek, B., 2007. Base excision repair modulation as a risk factor for human cancers. *Mol. Asp. Med.* **28:** 258-275.

Vidal, A.E., Hickson, I.D., Boiteux, S. and Radicella, J.P., 2001. Mechanism of stimulation of the DNA glycosylase activity of hOGG1 by the major human AP endonuclease: bypass of the AP lyase activity step. *Nucleic Acids Res.* **29:** 1285-1292.

Willcox, J.K., Ash, S.L. and Catignani, G.L., 2004. Antioxidants and Prevention of Chronic Disease. *Crit. Rev. Food Sci. Nutr.* **44:** 275-295.

Wentrup, C., 1984. Reactive molecules: the neutral reactive intermediates in organic chemistry. John Wiley and Sons, New York, **Chapter 2:** 23-161.

Youn, C.K., Kim, S.H., Lee, D.Y., Song, S.H., Chang, I.Y., Hyun, J.W., Chung, M.H. and You, H.J., 2005. Cadmium Down-regulates Human *OGG1* through Suppression of Sp1 Activity. *J. Biol. Chem.* **280:** 25185-25195.

CHAPTER: 4

OXIDATIVE DNA DAMAGE PROTECTION AND REPAIR BY POLYPHENOLIC COMPOUNDS IN PC12 CELLS

Silva, J.P., Gomes, A.C. and Coutinho, O.P., 2008. Oxidative DNA damage protection and repair by polyphenolic compounds in PC12 cells. *Eur. J. Pharmacol.* **601:** 50-60.

Abstract

Biological systems are frequently exposed to excessive reactive oxygen species (ROS), causing a disturbance in the cells natural antioxidant defence systems and resulting in damage to all biomolecules, including nucleic acids. In fact, oxidative DNA damage is described as the type of damage most likely to occur in neuronal cells. In this study, three polyphenolic compounds, luteolin, quercetin and rosmarinic acid, were investigated for their protective effects against oxidative DNA damage induced in PC12 cells, a neuronal cell model. Although luteolin and quercetin prevented the formation of strand breaks to a greater extent than rosmarinic acid, this last one presented the highest capacity to repair strand breaks formation. In addition, rosmarinic acid was the only compound tested that increased the repair of oxidised nucleotidic bases induced with the photosensitiser compound Ro 19-8022. The activity of repair enzymes was indicated by the *in vitro* base excision repair assay, using a cell-free extract obtained from cells previously treated with the compounds to incise DNA. The protective effect of rosmarinic acid was further confirmed by the increased expression of *OGG1* repair gene, observed through *real time* RT-PCR. The data obtained is indicative that rosmarinic acid seems to act on the intracellular mechanisms responsible for DNA repair, rather than by a direct effect on ROS scavenging, as deducted from the effects observed for luteolin and quercetin. Therefore, these results suggest the importance of these polyphenols, and in particular rosmarinic acid, as protectors of oxidative stress-induced DNA damage that commonly occurs in several pathological conditions, such as neurodegenerative diseases.

4.1. Introduction

In living cells, when the formation of intracellular reactive oxygen species (ROS) exceeds the cells' antioxidant capacity, oxidative stress can arise, resulting in damage to cellular macromolecules such as proteins, lipids and DNA (Nordberg and Arner, 2001; Valko *et al.*, 2007). DNA is a particularly sensitive cellular target because of the potential to create cumulative mutations that can disrupt cellular homeostasis. In this case, ROS can lead to the formation of single and double-strand breaks, as well as induce chemical and structural modifications to purine and pyrimidine bases, and also to 2'-deoxyribose (Powell *et al.*, 2005; Hazra *et al.*, 2007).

Oxidative DNA damage has been considered as an important promoter of cancer, besides being implicated in the normal process of aging (Bjelland and Seeberg, 2003). In addition, according to some authors, it is regarded as the type of damage most likely to occur in neuronal cells (Fishel *et al.*, 2007). However, this kind of DNA damage is predominantly corrected by the base excision repair (BER) pathway (Coppede *et al.*, 2007), although certain types of oxidative lesions also appear to be repaired by nucleotide excision repair (NER) (Dusinska *et al.*, 2006) and mismatch repair (MMR) (Neri *et al.*, 2005). BER is a generic mode of repair, whose first steps are the recognition of damaged bases by specific DNA glycosylases, hydrolysis of the glycosidic bond between base and deoxyribose and incision of the affected DNA strand by an apurinic/apyrimidinic (AP) endonuclease at the resulting abasic site, thus creating a DNA single-strand break (Burkle, 2006; Collins and Gaivão, 2007). The most important enzymes involved in DNA repair are 8-oxoguanine DNA glycosylase 1 (OGG1) and AP endonuclease 1 (APE1). In mammals, OGG1 is responsible for the removal of 8-oxoguanine, a lesion that arises through the incorporation, during DNA replication, of 8-oxo-dGTP formed from oxidation of dGTP by ROS. This enzyme also has an AP lyase activity, which is slow and limits the overall rate of repair (Alamo *et al.*, 1998; Dodson and Lloyd, 2002). APE1, by its turn, is an AP endonuclease that bypasses the AP lyase activity of OGG1, enhancing OGG1 turnover, thus having an important role in the regulation of base excision repair of oxidative DNA damage (Hill *et al.*, 2001; Vidal *et al.*, 2001).

Antioxidant activity, as well as interaction with several enzymes and synergy with other antioxidants, has been recognised to polyphenolic compounds (Horvathova *et al.*, 2005), which are secondary plant metabolites with numerous

other biological activities (Rice-Evans *et al.*, 1996; Hollman and Katan, 1999; Skibola and Smith, 2000). In the present study, attention was given to the flavonoids quercetin and luteolin, and to the phenolic acid, rosmarinic acid (Fig. 4.1). Quercetin is one of the most abundant natural flavonoids and can be found in onion, tea and apple, for example (Scalbert *et al.*, 2005). Luteolin, which differs from quercetin by having one less hydroxyl group in the C-ring of its molecular structure (Fig. 4.1), is found in high celery, green pepper and chamomile (Gutierrez-Venegas *et al.*, 2006). Both quercetin and luteolin, which by their chemical nature are antioxidants, have been associated to the prevention of cancer, diabetes, osteoporosis, as well as cardiovascular and neurodegenerative diseases, among others (Hollman and Katan, 1999; Aherne and O'Brien, 2000; Scalbert *et al.*, 2005). Rosmarinic acid is present in many plants, such as rosemary (Petersen and Simmonds, 2003) and its therapeutical value has been attributed to its antioxidant, anti-inflammatory, anti-bacterial and anti-viral properties (Petersen and Simmonds, 2003; Chlopcíková *et al.*, 2004; Kim *et al.*, 2005; Iuvone *et al.*, 2006). Neuroprotective effects have also been described for all of these compounds (Gelinas and Martinoli, 2002; Sasaki *et al.*, 2003; Iuvone *et al.*, 2006), suggesting their potential protective role in neurodegenerative diseases. Moreover, studies using some of these compounds have demonstrated their ability to protect different cell types against oxidative DNA damage (Noroozi *et al.*, 1998; Duthie and Dobson, 1999; Horvathova *et al.*, 2005; Lima *et al.*, 2006). However, whether they act by enhancing DNA repair or simply by preventing oxidative DNA damage is still unknown.

In this work, we evaluated the potential of these polyphenolic compounds to protect PC12 cells against oxidative DNA damage, by using the Comet assay. In addition, by addressing specific repair enzymes, we aimed to further characterise that protection and explore the involvement of those compounds in DNA repair mechanisms. The PC12 cell model was used as a simple model in which a correlation between oxidative stress and neurodegeneration, characteristic of Parkinson's or Alzheimer diseases, has been established by several authors (Piga *et al.*, 2005; Guan *et al.*, 2006; Jung *et al.*, 2007).

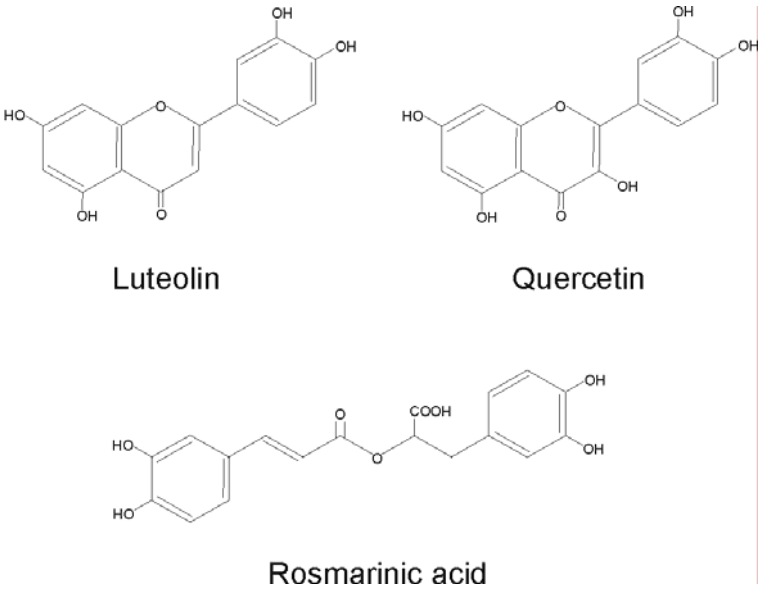


Figure 4.1 – Chemical structures of the polyphenolic compounds used in this study: luteolin, quercetin and rosmarinic acid.

4.2. Materials and Methods

4.2.1. Chemicals

Quercetin and rosmarinic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Luteolin was purchased from Extrasynthese (Genay, France). The polyphenolic compounds were dissolved in DMSO, aliquoted and maintained frozen at -20ºC until usage. Each aliquot was thawed only once.

RPMI-1640 cell culture medium, dimethyl sulfoxide (DMSO), EDTA, trypsin, *tert*-butyl hydroperoxide were also purchased from Sigma–Aldrich (St. Louis, MO, USA).

Foetal bovine serum (FBS) was from BioChrom KG (Berlin, Germany); horse serum donor herd was purchased from Gibco (Paisley, UK). Ro 19-8022 (used to induce specific DNA damage) was kindly provided by Hoffman La-Roche (Basel, Switzerland). Primers specific for 18S, *OGG1* and APE1 genes were synthesised by STAB-VIDA (Oeiras, Portugal). Qiagen RNeasy total RNA isolation kit was purchased to Qiagen (Hilden, Germany). Superscript Reverse Transcriptase III kit was obtained from Invitrogen (USA). Power SYBR Green master mix was acquired from Applied Biosciences (Cheshire, UK).

4.2.2. PC12 cell culture and treatment conditions

PC12, a neuronal cell line established from a rat adrenal pheochromocytoma (Greene and Tischler, 1976) was used in this study. When grown in serum-containing medium, these cells divide and resemble precursors of adrenal chromaffin cells and sympathetic neurons, being able to release dopamine (Sasaki *et al.*, 2003). In addition, they are an user-friendly cell model with some advantages over primary cultured neuronal cells, including the homogeneity of the cell population (Colognato *et al.*, 2006; Silva *et al.*, 2008). Cells were cultured in suspension in 75 cm^2 flasks, in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated foetal bovine serum and 1% (v/v) of an antibiotic/antimycotic solution. Cultures were maintained in a humidified incubator containing 95% air and 5% CO_2, and passed twice a week. Before each assay, the cell aggregates were carefully disrupted by gently pipetting and the separated cells plated in poly-D-lysine-coated multiwells, at a density of 2.5×10^5 cells/cm^2, for the MTT assay, and at a density of 5×10^5 cells/well for the other studies. After plating, cells were left for adhesion overnight. The polyphenols were either pre-incubated for the indicated periods of time or added simultaneously with the deleterious stimuli.

L929 cells, used as substrates in the *in vitro* base excision repair assay, were routinely grown in 75 cm^2 tissue culture flasks in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) of an antibiotic/antimycotic solution containing 10,000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml.

4.2.3. Analysis of cell survival

Cell viability was evaluated by the MTT reduction test, as previously described (Silva *et al.*, 2006). A volume of 0.5 ml MTT (final concentration 0.5 mg/ml, in Krebs medium, pH = 7.4), prepared just before usage and maintained in the dark, was added to the PC12 cells, at a density of 2.5×10^5 cells/cm^2. Absorbance was read at 570 nm in a multiplate reader (Spectramax 340PC). The survival of PC12 cells was expressed as the percentage of OD towards control cells, containing the same amount of the compounds' solvent, DMSO.

4.2.4. Single cell gel electrophoresis (Comet assay)

The protection against oxidative DNA damage conferred by the polyphenols herein studied was assessed using the Comet assay, in which the strand breaks present in the DNA of nucleoids, obtained after lysis of gel-embedded cells, migrate towards the anode during an electrophoresis in alkali conditions, yielding an image that, after staining with a fluorescent dye, looks like a comet, hence its name. This method was performed as previously described (Tice *et al.*, 2000), with the following modifications. Cells were plated at a density of 7.5×10^5 cells per well and left to adhere overnight. Oxidative DNA damage was induced in the presence of either 200 μM *t*-BHP (for strand breaks assessment) or 0.6 μM Ro 19-8022, in order to measure oxidised purines. Cells were then trypsinised, resuspended in phosphate buffered saline (PBS) and counted. About 50,000 cells were centrifuged at ~5,000 rpm, for 1 min, in an Eppendorf 5415C centrifuge. Supernatants were discarded and pellets mixed with 100 μl of low melting point agarose 0.5% (w/v) in PBS, at 37°C, and spread on slides previously coated with normal melting point agarose for 10 min, at 4ºC. The slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 v/v added at the time of buffer preparation) at 4°C for a minimum of 2 h. Slides were then rinsed with distilled water and immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13), in an horizontal electrophoresis tank, at 4°C for 40 min, to allow alkaline unwinding. Electrophoresis was carried out at 4°C, under alkaline conditions for 20 min at 1 V/cm. Finally, slides were neutralised by washing three times (5 min each) with 0.4 M Tris, pH 7.5, and fixed with absolute ethanol (two washes).

Comets were stained with ethidium bromide (10 μg/ml in PBS) and analyzed under a fluorescence microscope. Comet quantification was performed through either of two ways: visual scoring, a method in which comets are classified into one of five classes of damage (from 0 to 4) in 100 nucleoids (range of score: 0 – 400); and/or through a computer-assisted image analysis (TriTek CometScore™ Freeware v1.5), by measuring the percentage of DNA in the tail. The method identifies DNA strand breaks.

Cells repair capacity (RC) was determined by using the following formula:

$$RC = 100 \times [(D_0X - D_tX) / D_0X],$$

where D_0X represent DNA damage before the recovery period in the condition X and D_tX represent DNA damage after a recovery period, for the same condition. The increase in cells' repair capacity induced by the polyphenolic compounds was obtained by subtracting the percentage of repair observed in the presence of the deleterious stimulus alone, to the percentage of repair in the presence of the compounds and the deleterious stimulus.

4.2.5. Measurement of oxidised purines

Occurrence of oxidised bases were measured by using the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG), which recognises oxidised purines, creating breaks at those sites (Duthie and Dobson, 1999).

Cells were treated with the photosensitiser compound Ro 19-8022 (a kind gift from Hoffmann La-Roche, Basel, Switzerland). A stock of a 1 mM concentration was prepared in 70 % ethanol and stored in small aliquots at -20ºC. The working solutions were prepared immediately before use by diluting this stock solution with PBS (132 mM NaCl, 4 mM KCl, 1.2 mM NaH_2PO_4, 1.4 mM $MgCl_2$, 10 mM HEPES, 6 mM glucose, 1 mM $CaCl_2$ pH 7.4). The experiment was carried out in the dark and all solutions were kept on ice. Cells, plated and treated as previously described, were rinsed with PBS and 3 ml of the diluted Ro 19-8022 solution was added to each well. The plates were irradiated on ice at a distance of 33 cm from a 500 W halogen lamp, for 5 min. Control cells were incubated in the presence of PBS alone. After washing the cells with PBS to remove traces of Ro 19-8022, the Comet assay was performed as described above, with a small change: following lysis the slides were washed 3 times (5 min each) in cold enzyme reaction buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8, 4 ºC), blotted dry and incubated with the repair enzyme (FPG), or buffer, for 30 min, at 37 ºC. The amount of oxidised purines (FPG-sensitive sites) was then determined by subtracting the amount of strand breaks (samples incubated with buffer alone) to the total amount of breaks obtained after incubation with FPG.

4.2.6. In vitro *base excision repair assay*

The base excision repair (BER) assay measures the ability of a cell-free extract to recognise the damage in the DNA of substrate nucleoids and incise the DNA

containing specific damage, in this case 8-oxoguanine (8-oxoGua). The increase in the amount of strand breaks produced reflects the DNA repair activity of the cell extract (Collins, 2004).

L929 fibroblasts were used as substrates. This allows an easier measurement of comets damage due to the greater size of these cells and their lack of a tendency to form aggregates when compared to PC12 cells. Briefly, L929 cells in a 75 cm^2 flask near confluence were treated with the photosensitiser Ro 19-8022 plus visible light (5 min irradiation on ice at 33 cm from a 500 W halogen lamp). Cells were then washed with PBS, trypsinised and collected in 5 ml of culture medium. After a centrifugation at 400 *g* for 5 min, at 4ºC, in a Sigma 2K15 microcentrifuge, the pellet was suspended in freezing medium (culture medium with 20 % fetal bovine serum and 10% DMSO) at a density of 3 × 10^6 cells/ml. Cells were then aliquoted and stored at -80ºC.

For the extract preparation, PC12 cells, at a density of 5 × 10^6 cells/ml, were incubated with the polyphenols for either 1 or 24 h. Cells were then washed with PBS, trypsinised and collected in PBS to microcentrifuge tubes. After centrifuging at 800 *g* for 5 min, at 4ºC, in a Sigma 2K15 microcentrifuge, the pellets were resuspended in ice cold PBS. Cells were divided into 1 ml aliquots and centrifuged at 14,000 *g* for 5 min, at 4ºC. Supernatants were discarded and the dry pellets flash frozen in liquid nitrogen, to be stored at -80ºC. On the day of the experiment, one of these aliquots was thawed and the pellet resuspended in 65 µl of lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10 % glycerol, pH 7.8 with KOH, supplemented with 0.25 % Triton X-100). The mixture was vortexed for 5 s, followed by a 5 min incubation on ice, and then centrifuged at 14,000 *g* for 5 min, at 4ºC. Finally, 55 µl of the supernatant were removed and combined with 220 µl of cold enzyme reaction buffer.

An aliquot of Ro- and light-treated substrate L929 cells was thawed, washed twice in cold PBS and spinned for 5 min at 800 *g*, 4ºC. The pellets were suspended in 100 µl of PBS and 35 µl of this suspension was mixed with 1.5 ml of 1 % low-melting point agarose at 37ºC. These cells were then embedded in agarose and the procedure then followed according to the standard Comet assay, with a slight modification: after lysis, the slides were washed three times (for 5 min each) in cold enzyme reaction buffer and incubated with 35 µl of treated or non-treated extract for 20 min. Control slides incubated with either FPG (positive control) or buffer alone (negative control) were performed.

4.2.7. Quantification of rOGG1 and rAPE1 expression

Total RNA was isolated from PC12 cells treated in the same conditions as for the base excision repair assay using a Qiagen RNeasy total RNA isolation kit (Qiagen, USA), following standard protocol. RNA purity was confirmed by determining the OD260/OD280 nm absorption ratio. 1 µg of total RNA was reversed transcribed with the Superscript Reverse Transcriptase III kit (Invitrogen, USA), by using 50 ng/µl of random hexamers and 10 mM of a dNTP mix according to the manufacturer's instructions. cDNA integrity was verified by gel electrophoresis after PCR amplification of Gapdh, using sequence-specific primers.

PCR was performed using 18S specific primers (forward: 5'- AAG TCC CTG CCC TTT GTA CAC A -3'; reverse: 5'- GCC TCA CTA AAC CAT CCA ATC G -3') as an internal reference. Specific primer pairs for r*OGG1* (forward: 5'- ACT TAT CAT GGC TTC CCA AAC C -3'; reverse: 5'- CAA CTT CCT CAG GTG GGT CTC T -3') and rAPE1 (forward: 5'- GCG GCA GCG GAA GAC -3'; reverse: 5'- GCC TCC TTC TCA GTT TTC TTT GCT -3') were described in Englander and Ma, 2006. Primers were manufactured by STAB-VIDA, Portugal.

Real-time RT-PCR was performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, USA) using 1 µl of the cDNA preparation, which was added to a reaction mixture containing 12.5 µl Power SYBR Green master mix (Applied Biosciences, Cheshire, UK), 1 µl of each primer (25 pmol/µl) and autoclaved water to a final volume of 25 µl per well. The plates were covered, centrifuged and placed in the thermal cycler. The PCR conditions used were: 50ºC for 2 min, 95ºC for 10 min and 40 cycles (95ºC, 15 s; 60ºC, 60 s).

The expression of *OGG1* and APE1 mRNA in samples was determined from a standard curve constructed from serial dilutions of cDNA obtained from unstimulated PC12 cells. Target genes' transcript levels were all normalised to 18S mRNA levels. The average of at least two replicates for each of three independent experiments was used.

4.2.8. Statistical analysis

Data are expressed as the mean ± S.E.M., of the indicated number of experiments. The significance of the differences between the means observed was

evaluated using the unpaired two-tailed Student's *t*-test. A difference of $p \leq 0.05$ was considered significant.

4.3. Results

4.3.1. Polyphenols toxicity to PC12 cells

The beneficial effects of polyphenolic compounds like the ones herein tested in cells under oxidative stress are well recognised (Sasaki *et al.*, 2002; Horvathova *et al.*, 2005; Iuvone *et al.*, 2006). Nevertheless, in specific conditions, some of them can also induce harmful effects. For example, quercetin was described as becoming toxic as a result of its own protective activity (Leung *et al.*, 2005; Hur *et al.*, 2007; Boots *et al.*, 2007). We therefore evaluated the cytotoxicity of quercetin, luteolin and rosmarinic acid in our biological model. As depicted in figure 4.2, for the concentrations tested, none of the compounds showed a statistical significant decrease in cell viability, even after an overnight incubation.

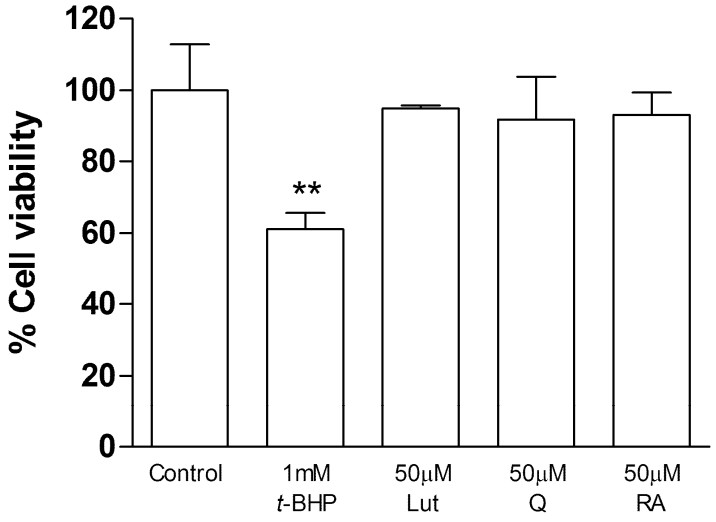


Figure 4.2 – Cell viability assessed by the MTT reduction test. PC12 cells were incubated overnight with increasing concentrations of the polyphenolic compounds. Control cells were incubated in the presence of 1% DMSO. Each bar represents the mean ± S.E.M., considering the results obtained in at least three independent experiments. Cell damage induced by 1 mM *t*-BHP was used as a positive control. ** $P \leq 0.01$, compared with the control.

4.3.2. Determination of t-BHP-induced damage to PC12 cells

The extent of oxidative DNA damage was evaluated by the Comet assay, by incubating PC12 cells for one hour with increasing concentrations of the thiol-oxidizing agent, *t*-BHP, a widely accepted inducer of oxidative stress (Ahmed-Choudhury *et al.*, 1998; Palomba *et al.*, 2001; Pias and Aw, 2002). The dose-response curves to *t*-BHP on DNA damage (Figs. 4.3A and 4.3B) obtained by comet quantification assessed both by visual scoring (A) and computer-assisted image analysis (B), indicate a concentration-dependent effect in DNA damage. The results showed a good correlation between the semi-quantitative method of visual scoring and the % of DNA in the tail assessed by computerised image analysis (Fig. 4.3C), which is in accordance with other authors (Collins *et al.*, 1997). Despite a considerable amount of DNA damage being observed for concentrations above 200 μM, cell viability (assessed by the MTT assay) was significantly decreased at concentrations of 500 and 1000 μM (Fig. 4.3D). Therefore, for subsequent induction of oxidative DNA damage, PC12 cells were incubated with 200 μM *t*-BHP for 1 h.

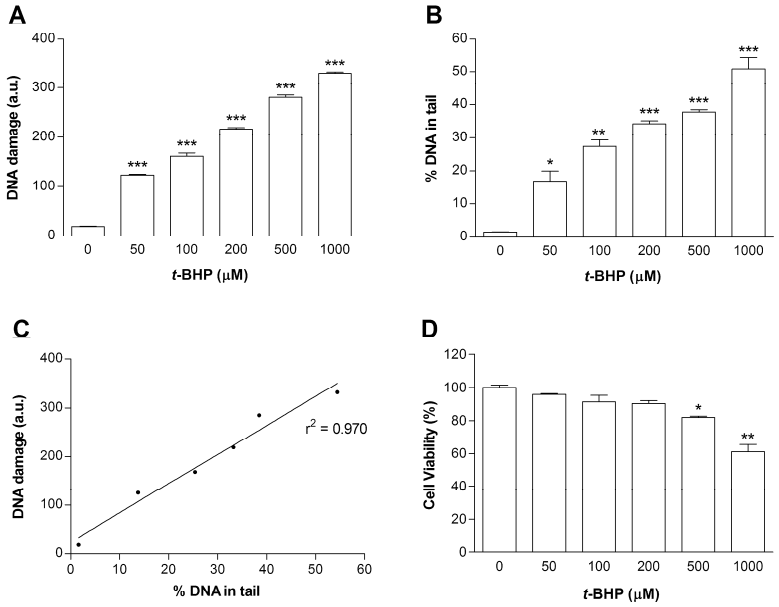


Figure 4.3 – *t*-BHP-induced damage in PC12 cells. Cells were incubated with different concentrations of *t*-BHP for 1 h and DNA damage assessed by the Comet assay. Comets were quantified either by visual scoring (A) or by computer-assisted image analysis (B). C) Correlation coefficient between the semi-quantitative method and the computer assisted parameter. D) Cell viability assessed by MTT reduction test. Each bar represents the mean ± S.E.M., for at least three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$, compared with the control.

4.3.3. Evaluation of the protective effect of the compounds against DNA strand breaks formation

The interaction between ROS and DNA can lead to the oxidation of this biomolecule, resulting in several types of oxidative DNA damage, including strand breaks and oxidised bases (Saitoh *et al.*, 2001). We firstly studied the protective effects of the compounds against the formation of strand breaks, induced by *t*-BHP. The polyphenolic compounds were either pre-incubated for a period of 3 h, or incubated simultaneously with the oxidative stimulus. As it can be observed in Fig. 4.4, all the compounds significantly decreased the *t*-BHP-induced formation of DNA strand breaks. Among them, luteolin showed the highest protective effect, with a reduction in DNA damage of about 71 % for either incubation conditions.. No differences could be observed between pre-incubating or not the cells with the luteolin or rosmarinic acid. For quercetin, the protective effect was significantly increased when added simultaneously with *t*-BHP (28.8 and 54.1 % with or without pre-incubation, respectively). The results relative to the simultaneous incubation of the cells with the compounds and *t*-BHP are clearly demonstrated by the comets representative images for each condition (Fig. 4.4, A-E).

We then investigated the ability of these compounds in the stimulation of strand breaks repair. In this way, after cell incubation in the presence of the polyphenolic compounds and 200 μM *t*-BHP for 1 h, culture media was replaced and cells given a 1 h recovery period, to allow the repair of DNA damage. After this recovering period, and in the presence of the deleterious stimulus alone, cells had repaired 45.3% of strand breaks (Fig. 4.5). When cells were co-incubated with the polyphenols, the strand breaks repair capacity was significantly increased.

There seems to be an inverse correlation between the compounds capacity to protect the cells against the formation of strand breaks and their ability to increase the cells' repair capacity of this kind of damage. In fact, despite having a weaker protective effect against the formation of strand breaks, rosmarinic acid induced the highest increase in repair capacity (32.4% above cells own repair), followed by quercetin and luteolin, which increased repair capacity by 24.7 % and 13.8%, respectively. These results reflect the ability of these compounds to protect against DNA strand breaks formation and, more importantly, to enhance the repair of this type of damage, suggesting an involvement on specific mechanisms of DNA repair.

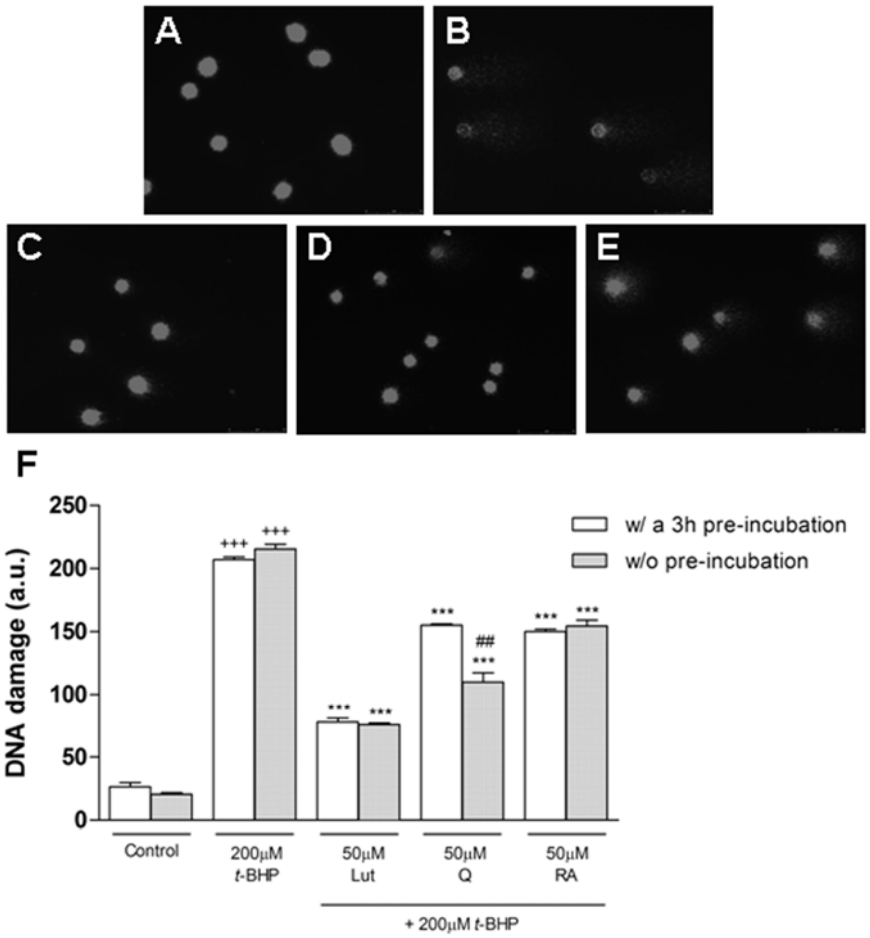


Figure 4.4 – Compounds protection against *t*-BHP-induced formation of DNA strand breaks, evaluated by the Comet assay. A-E) representative images of Comets from control, 200μM *t*-BHP, 50 μM luteolin, 50 μM quercetin and 50 μM rosmarinic acid (without pre-incubation), respectively. F) Quantification of DNA damage by visual scoring. PC12 cells were incubated for 1 h in the presence of 200μM *t*-BHP. Polyphenolic compounds were added to the cells either at the same time or 3 h prior to the addition of the oxidative stimulus. Each bar represents the mean ± S.E.M for at least three independent experiments. $^{+++}$ $P \leq 0.001$, compared to respective control cells; *** $P \leq 0.001$, compared to 200μM *t*-BHP (for each respective incubation condition); $^{\#\#}$ $P \leq 0.01$, compared with the same compound, after a 3 h pre-incubation.

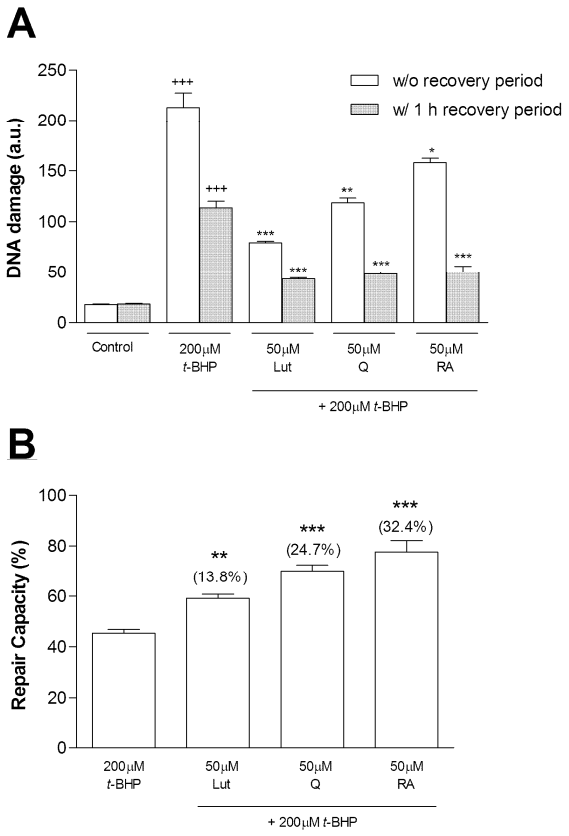


Figure 4.5 – Effects of the compounds on DNA strand breaks repair. A) After 1 h of cells' simultaneous incubation with the compounds and 200μM *t*-BHP, cell culture media was replaced and DNA damage evaluated 1 h later. Comets were visually scored and results expressed in percentage of damage relatively to the maximum obtained before the recovery period. B) Repair capacity was calculated using the formula presented in the Material and Methods section. Values in brackets represent the increase in repair capacity. For each bar is represented the mean ± S.E.M for at least three independent experiments. $^{+++}$ $P \leq 0.001$, compared to respective control cells; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, compared to 200μM *t*-BHP (for each respective incubation condition).

4.3.4. Effects of the polyphenolic compounds on base oxidation

In addition to formation of strand breaks, structure modifications at the level of nucleotidic bases can occur as a consequence of oxidative stress. Oxidation of nucleotidic bases is likely to be as important as DNA strand breaks to overall cellular function and survival (Bjelland and Seeberg, 2003). We therefore investigated the ability of the polyphenols herein studied to help cells overcome the formation of oxidised bases. Since *t*-BHP induced a great amount of strand breaks along with

bases oxidation, making it difficult to distinguish between these two types of damage, we used Ro 19-8022, a photosensitiser compound that induces only the accumulation of oxidised bases, namely 8-oxoGua, without the induction of a significant amount of strand breaks (Angelis *et al.*, 1999; Gedik *et al.*, 2002). Indeed, treatment of PC12 cells with increasing concentrations of Ro 19-8022 (Fig. 4.6) resulted in a gradual increase in the percentage of DNA in the comet tails. From these results, we selected the 0.6 μM Ro 19-8022 concentration as the most adequate for subsequent experiments, since a significant increase in DNA damage could be observed, with few class 4 comets generated. This indicates that the assay had not reached a saturation point, which must be avoided, since it could lead to underestimation of enzyme-sensitive sites (Gedik *et al.*, 2002).

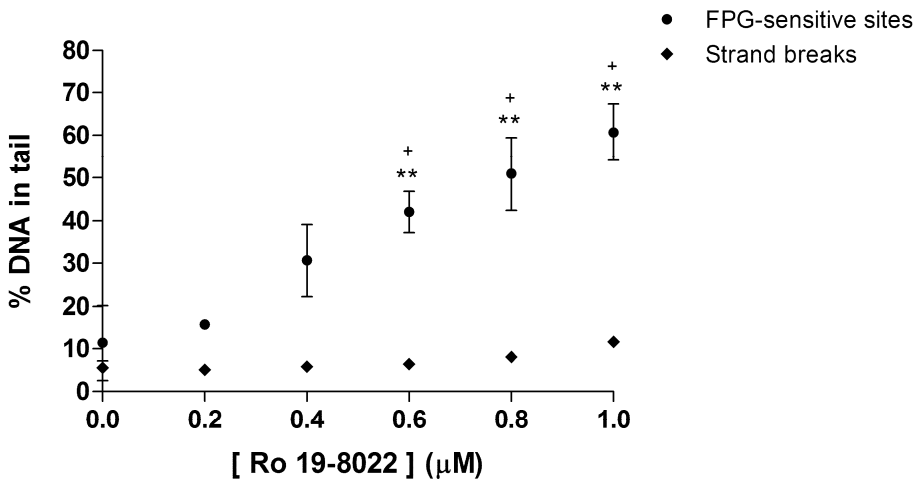


Figure 4.6 – Dose-response curve for cells treatment with Ro 19-8022. PC12 cells were incubated for 5 min, on ice and under a 500 W halogen lamp, in the presence of increasing concentrations of Ro 19-8022. The level of oxidised purines was determined through sample incubation with FPG, for 30 min. FPG-sensitive sites were obtained by subtracting the amount of strand breaks alone to the amount of breaks obtained after incubation with FPG. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. For each condition is represented the mean ± S.E.M for at least three independent experiments. $^{+}$ $P \leq 0.05$, compared to cells in the absence of Ro 19-8022; ** $P \leq 0.01$, relatively to strand breaks for the same condition.

The polyphenolic compounds effects were then evaluated on the formation of oxidised bases induced by 0.6 μM Ro 19-8022. Two pre-incubation periods, 1h and 24 h, were tested. Results in figure 4.7 show that none of the compounds

significantly reduced the amount of oxidised purines, independently of the pre-incubation period used.

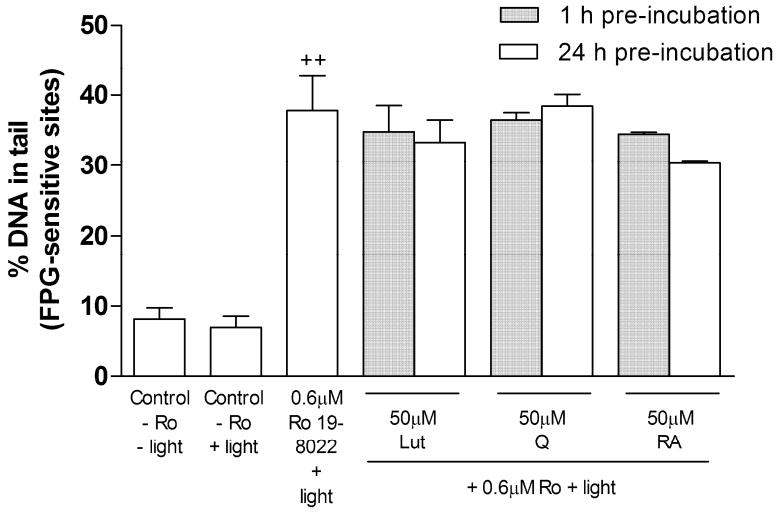


Figure 4.7 – Effect of the polyphenolic compounds on Ro 19-8022-induced purines oxidation. PC12 cells were either incubated for 1 h or 24 h, prior to the treatment with 0.6 μM Ro 19-8022 plus light. The level of oxidized purines was determined through sample incubation with FPG, for 30 min. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. Each bar represents the mean ± S.E.M for at least three independent experiments. $^{++}$ $P \leq 0.01$, compared to cells in the absence of Ro 19-8022.

We also investigated the effects of the polyphenolic compounds after a recovery period after the incubation with Ro 19-8022, since time can be decisive for the observation of a possible protective effect. In this way, after Ro 19-8022 treatment, culture media was replaced and cells allowed to recover for a period of 6 h. The selection of this time period was based on results from Fig. 4.8, which show that, after a 6 h recovery, cells had already repaired about 51 % of oxidised bases and the assay had not reached saturation. These results are in agreement with previous reports by other authors using different cell lines, and can be attributed to the longer period of time required by oxidised bases to be repaired, when compared to strand breaks (Collins and Horvathova, 2001).

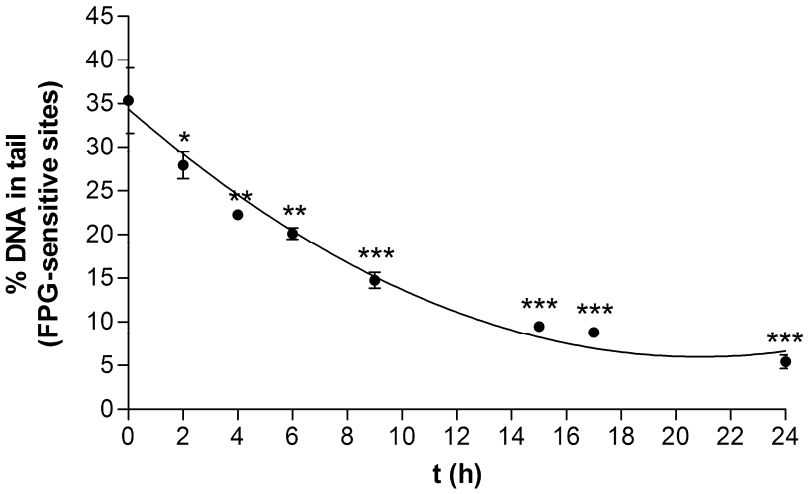


Figure 4.8 – DNA repair of Ro-induced oxidised purines. PC12 cells were treated with Ro 19-8022 plus light for 5 min, on ice. Culture media was then replaced to allow damage repair. At defined time-points, cells were collected and assayed for the level of oxidised purines, by incubating samples with FPG. Percentage of damage in the comet tails was quantified by computer-assisted image analysis. For each condition is represented the mean ± S.E.M for at least three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to cells in the absence of Ro 19-8022.

As presented in Fig. 4.9A, after a 6 h recovery period following a 24 h incubation of the cells in the presence of the polyphenolic compounds, the levels of DNA in the comet tails was not significantly altered by the presence of the polyphenols. Nevertheless, after calculating the repair capacity induced by each compound, we observed that rosmarinic acid significantly increased the cells capacity to repair oxidised purines in about 9.4 % (Fig. 4.9B). On the other hand, neither luteolin nor quercetin were able to significantly increase the cells' intrinsic repair capacity, although for luteolin there seems to exist a trend to do so.

The lack of effect obtained for quercetin had been previously reported in lymphocytes, using hydrogen peroxide as an inducer of base oxidation (Duthie and Dobson, 1999). Our results clearly indicate that, among the polyphenolic compounds tested, only rosmarinic acid seems to affect the repair of oxidised bases.

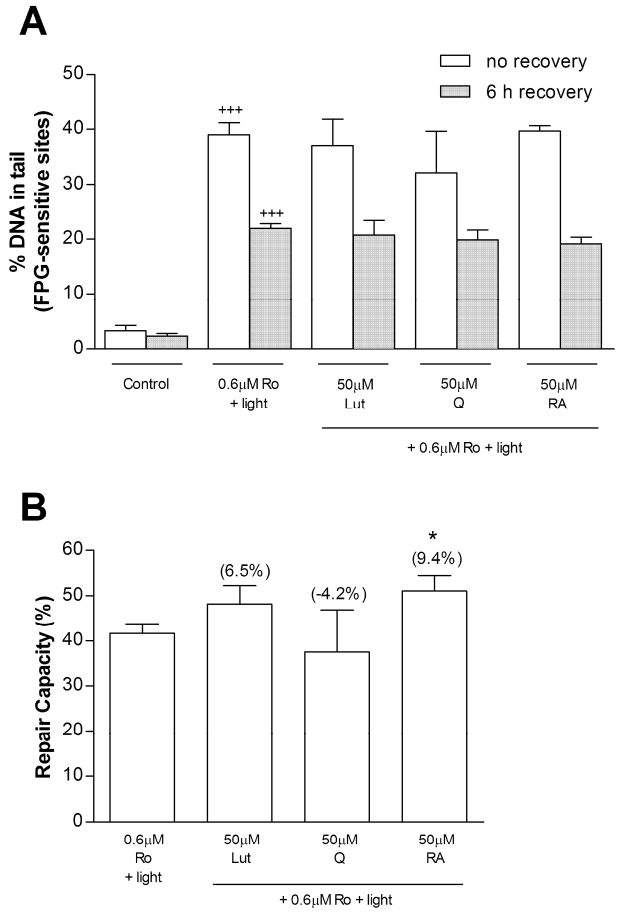


Figure 4.9 – Effects of the compounds on purine oxidation repair. A) After a 24 h incubation in the presence of the compounds, cells were treated with Ro 19-8022 plus light for 5 min, on ice. Culture media was then replaced and DNA damage evaluated by the Comet assay 6 h later. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. B) Repair capacity calculated according to the formula presented in Material and Methods. Values in brackets represent the increase in repair capacity. For each bar is represented the mean ± S.E.M. for at least three independent experiments. $^{+++}$ $P \leq 0.001$, compared to respective control cells; * $P \leq 0.05$, compared to 0.6 μM Ro 19-8022 + light.

4.3.5. In vitro *base excision repair assay*

The capacity for incision activity by repair enzymes at oxidised purines in DNA was monitored in cell-free extracts, obtained from PC12 cells previously incubated in the presence of the polyphenols for 24 h. Damage-containing substrates were obtained by treating L929 cells with 1 μM Ro 19-8022, for 5 min on ice, under a 500

W halogen lamp. This concentration was selected based on a dose-response curve of DNA damage against Ro 19-8022 (Fig. 4.10).

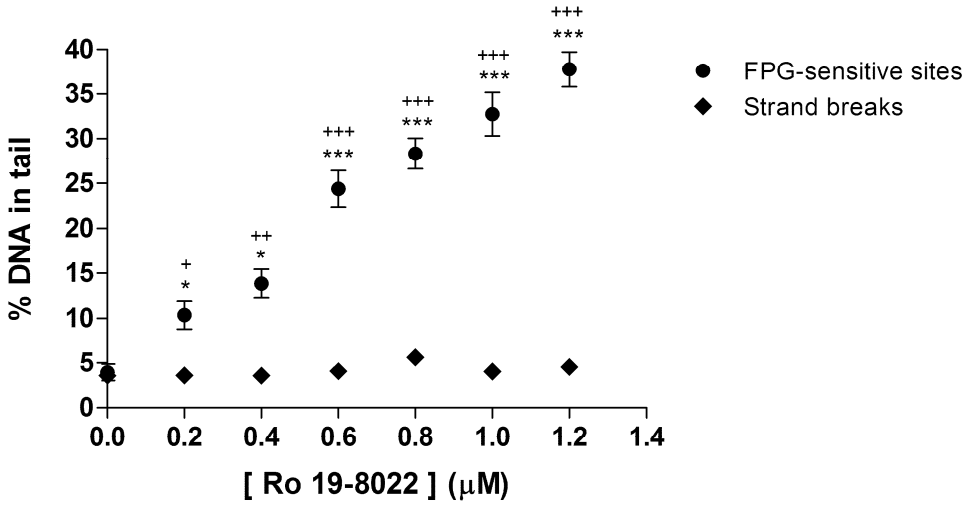


Figure 4.10 – Dose-response curve of Ro 19-8022-induced DNA damage. L929 cells were incubated in the same conditions indicated in Fig. 6. Levels of oxidized purines were determined and quantified as previously described. For each condition is represented the mean ± S.E.M for at least three independent experiments. $^{+}$ $P \leq 0.05$, $^{++}$ $P \leq 0.01$, $^{+++}$ $P \leq 0.001$ compared to cells in the absence of Ro 19-8022; * $P \leq 0.05$, *** $P \leq 0.001$, relatively to strand breaks for the same condition.

Results in Figure 4.11 show that after a 20 min incubation of the substrate with a cell-free extract, L929 suffer an increase in the number of DNA breaks relatively to the control (24.84 ± 0.47 % *vs* 6.12 ± 0.97 %, $p \leq 0.001$), as previously observed by (Collins *et al.*, 2003). Since substrate cells were treated with Ro 19-8022 and light, these breaks reflect only the amount of oxidised nucleotidic bases. It is also evident in the same figure that only rosmarinic acid led to an improvement of PC12 cells' capacity to repair this kind of damage, as demonstrated by an increase of 6.96 % of DNA in the tail for the rosmarinic acid-treated extract. This is equivalent to a 37.1 % increase in DNA repair capacity relatively to the non-treated extract. In the presence of extracts treated with either luteolin or quercetin, DNA repair was unaffected. The increase in incision activity induced by the rosmarinic acid-treated extract, as well as the lack of effect observed for luteolin and quercetin, are clearly demonstrated in the representative images of BER assay (Fig. 4.11A-E).

These data suggest a possible involvement of rosmarinic acid in the regulation of gene expression of DNA repair enzymes.

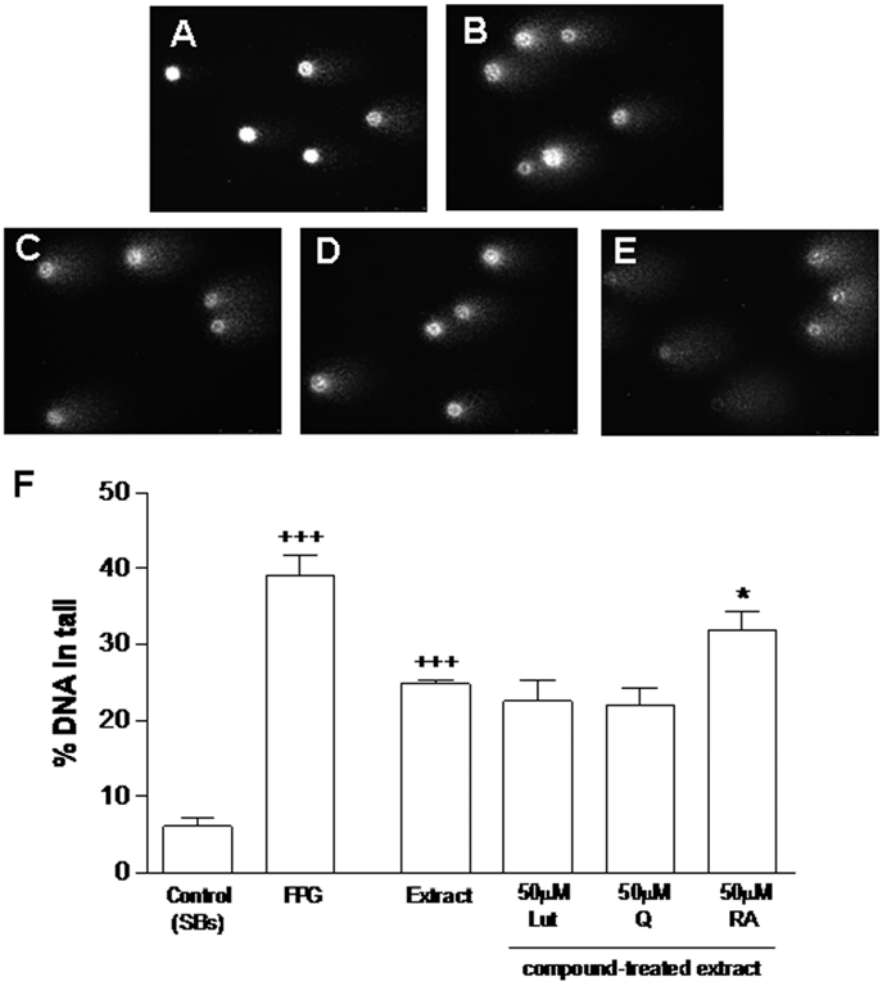


Figure 4.11 – Effect of the compounds on the *in vitro* BER assay. A-E) representative images of Comets (purines only) from control (buffer alone), extract from non-treated cells, extract from 50 μM luteolin-treated cells, extract from 50 μM quercetin-treated cells and extract from 50 μM rosmarinic acid-treated cells, respectively. F) Quantification of the percentage of DNA in the comets tails was done after treatment of damage-containing substrate cells with a cell-free extract obtained from PC12 cells incubated with the polyphenols for 24 h. The repair enzyme FPG was used as a positive control. Levels of oxidized purines were determined and quantified as previously described. For each condition is represented the mean ± S.E.M for at least three independent experiments. $^{+++}$ $P \leq$ 0.001compared to control cells; * $P \leq 0.05$, relatively to non treated extract. SBs – Strand Breaks.

4.3.6. Expression of DNA repair genes

We tried to elucidate the molecular mechanisms underlying the results observed with rosmarinic acid, namely by investigating a direct effect of this compound on the expression of the DNA repair genes *OGG1* and APE1, which play an important role in DNA repair, using *real time* RT-PCR. Treatment of PC12 cells with rosmarinic acid alone results in a significant decrease in the expression of APE1, (Figure 4.12, white bars). However, in the presence of Ro 19-8022, rosmarinic acid seems to significantly enhance the expression of the repair gene *OGG1*. Such results confirm the action of rosmarinic acid at the level of DNA repair, which is discussed further on.

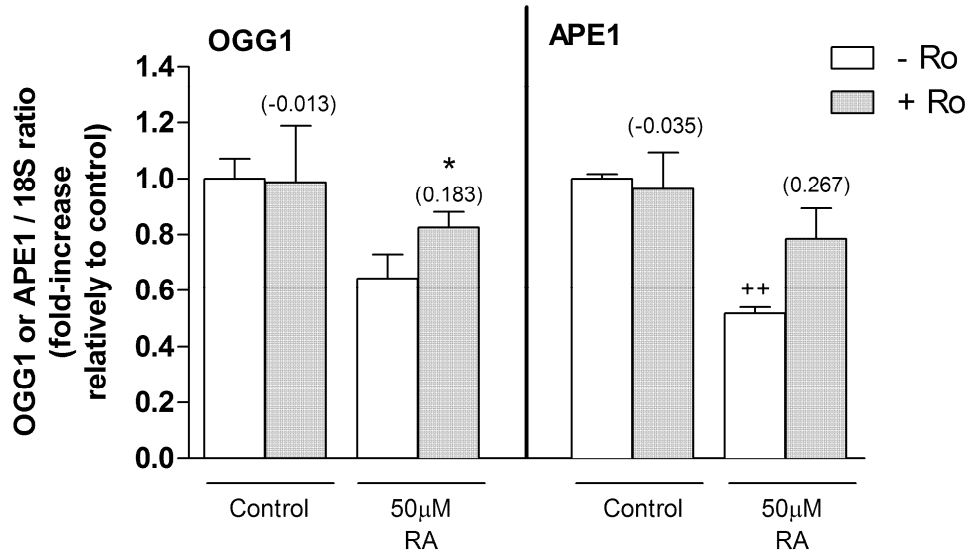


Figure 4.12 – Effects of rosmarinic acid on the expression of repair genes *OGG1* and *APE1*. PC12 cells were incubated for 24 h in the presence of rosmarinic acid, prior to the exposure to Ro 19-8022 plus visible light. Levels of genes expression was determined by *real time* RT-PCR and results expressed as the increase in the expression of each gene relatively to the control. Values in brackets represent the difference in the fold-increase expression between Ro-treated and non-treated conditions. Each bar represents the mean ± S.E.M for at least two independent experiments. $^{++}$ $P \leq 0.01$, compared to control cells in the absence of Ro 19-8022; * $P \leq 0.05$, compared to cells treated with rosmarinic acid alone.

4.4. Discussion

Oxidative DNA damage is generally regarded as a dynamic steady-state: an equilibrium is maintained between input of damage (*i.e.* endogenous and/or exogenous free radicals attack), which can be attenuated by antioxidant defences, and output from this damage (*i.e.* DNA repair). A change in input or output will lead to an increase or decrease in the level of damage, until a new equilibrium is attained. The implication is that DNA repair, in normal physiological conditions, maintains oxidative damage at a level that is tolerable in terms of genetic stability (Tomasetti *et al.*, 2001; Collins and Gaivão, 2007).

In this work, three polyphenolic compounds (luteolin, quercetin and rosmarinic acid) were investigated for their protective effects on oxidative DNA damage in a neuronal cell model, with a particular focus on the mechanisms by which they may be acting. Their antioxidant properties have already been well characterised in the literature (Noroozi *et al.*, 1998; Petersen and Simmonds, 2003) and, in addition, some authors have described their protective effects against oxidative DNA damage induced in cancer cell lines, in a clear association with their anti-carcinogenic properties (Duthie and Dobson, 1999; Lima *et al.*, 2006). In neuronal cells, it is described the cumulative effect of DNA damage in human brain over time (especially in mitochondrial DNA), which is supposed to play a critical role in aging and in the pathogenesis of several neurodegenerative diseases (Bjelland and Seeberg, 2003; Fishel *et al.*, 2007; Coppede *et al.*, 2007).

To date, no studies have reported the effects of these polyphenols on oxidative DNA damage in neuronal models. Additionally, it remains unknown whether these compounds exert their protective effects against oxidative DNA damage by enhancing DNA repair or simply by preventing the oxidation of DNA due to their antioxidant properties.

Based on the fact that, in our cell model, a 50 μM concentration of the polyphenolic compounds did not present any toxicity to cells, and after selecting the best conditions to obtain a significant amount of DNA damage without inducing a significant decrease in cell viability, we proceeded to investigate the compounds protection against *t*-BHP-induced oxidative DNA damage. It should be noted that the use of such concentrations has been also reported in other works with neuronal cell models (Sasaki *et al.*, 2003; Park *et al.*, 2007; Okawara *et al.*, 2007) and for some authors were considered low (Horvathova *et al.*, 2005). As expected from previous reports, regarding the protective effects of these polyphenolic compounds on

oxidative DNA damage (Noroozi *et al.*, 1998; Duthie and Dobson, 1999; Lima *et al.*, 2006), all of them were also able to decrease *t*-BHP-induced DNA strand breaks formation in our cell model. Among them, luteolin proved to be the most efficient one, which is in accordance with previous studies reporting the high antioxidant activity for this compound, observed in different cells, such as skin fibroblasts or macrophages (Filipe *et al.*, 2005; Harris *et al.*, 2006). Although no differences in protection were observed when luteolin or rosmarinic acid were added simultaneously or 3 h prior to the addition of *t*-BHP, quercetin presented an ameliorated effect when simply co-incubated with the deleterious stimulus. A similar effect has been reported in human lymphocytes and was attributed to the metabolization of this flavonoid into a less active compound (Duthie and Dobson, 1999).

The ability of the compounds to increase strand breaks repair varies in the inverse order of that observed for the prevention of strand breaks formation. In fact, although all the compounds tested were able to enhance the repair of strand breaks in PC12 cells, rosmarinic acid induced the highest repair capacity for this kind of damage. Since both luteolin and quercetin are known to possess high antiradical scavenging activities (Noroozi *et al.*, 1998; Choi *et al.*, 2003; Horvathova *et al.*, 2005), and based on the fact that most of their protective effects against DNA damage occurred during the 1 h incubation with *t*-BHP, we are led to believe that these effects are mainly due to their antioxidant properties. In addition, features such as their high lipophilicities (Areias *et al.*, 2001) and abilities to chelate metal ions (Mira *et al.*, 2002) may also be responsible for their protective effects. Rosmarinic acid, by its turn, has a weaker ROS scavenging activity and a lower lipophilicity when compared to luteolin and quercetin, thus the results obtained suggest its involvement in the intracellular pathways related to DNA repair.

The hypothesis that rosmarinic acid could be acting on those intracellular pathways, in opposition to the more direct effect of luteolin and quercetin, was further studied by investigating the involvement of these polyphenolic compounds on the protection against base oxidation. It should be taken into account that the effect of antioxidants on recovery from oxidative DNA damage may be justified by at least two different explanations: 1) by stimulating the activity of repair enzymes or 2) through a direct protection against oxidation (Tomasetti *et al.*, 2001). In the conditions tested, none of the polyphenols was able to decrease the amount of oxidised purines induced by the photosensitiser compound, independently of the time used in the pre-treatment. Nevertheless, rosmarinic acid significantly increased

the PC12 cell capacity to repair oxidised purines. For luteolin, a trend to increase the repair of oxidised purines was observed, though this increase was not statistically significant.

Since Ro 19-8022 can induce a great amount of DNA damage in a short period of time, the antioxidant properties of the polyphenols tested may not be sufficient to exert a protective effect against DNA damage induced by this agent. Instead, a protective effect in this case, to be detected, may be more easily attributed to an involvement of a compound in the mechanisms responsible for Ro 19-8022 toxicity. Considering this, the results obtained are indicative that only rosmarinic acid may be acting on the intracellular pathways leading to repair of DNA damage.

In order to better re-create *in vitro* the conditions for DNA repair occurring *in vivo*, an alternative approach for the assessment of repair capacity of a sample extract, provided with a DNA substrate carrying specific type of damage, was used (Tomasetti *et al.*, 2001). We observed that only rosmarinic acid was able to increase the PC12 cells' capacity to repair oxidised bases, as indicated by the greater amount of breaks formed in the presence of extract pre-treated with this compound, relatively to the non-treated extract. This effect on DNA repair appears to be mediated through change in gene expression, namely an increase in *OGG1* mRNA levels. However, in the presence of rosmarinic acid alone the levels of repair genes expression decreased. This might be explained considering the dynamic steady state of oxidative DNA damage, previously referred. Based on this theory (Collins and Gaivão, 2007) we may assume that, in the presence of rosmarinic acid, basal damage input to DNA induced by the deleterious stimulus will be lower than in the absence of the compound. In this situation, a smaller amount of oxidised nucleotidic bases might be expected. So, repair enzymes might not be recruited to a great extent, which would explain the lower expression of repair genes. In addition, the lack of a significant effect of rosmarinic acid on APE1 expression may be attributed to a later recruitment of this enzyme during the repair process, as also described elsewhere (Hill *et al.*, 2001). It should be further noted that DNA repair enzymes, such as OGG1, are considered housekeeping genes (Loft and Moller, 2006). In this way, an effect at the level of base oxidation repair, if present, should be subtle, since the intracellular pathways that are involved in the repair of oxidative DNA damage are vital to cells and are therefore tightly regulated. This fact also justifies the small variation obtained in expression of these genes with or without the deleterious stimulus.

Extracts pre-treated with either luteolin or quercetin were unable to increase the DNA repair capacity, confirming that these compounds cannot act against the oxidation of nucleotidic bases induced by Ro 19-8022.

In this study, we have shown some of the underlying mechanisms associated to the protective effects on oxidative DNA damage in PC12 cells for three polyphenolic compounds: luteolin, quercetin and rosmarinic acid. While the action of both luteolin and quercetin seems to be associated with a direct effect on ROS scavenging, with major implications on the prevention of *t*-BHP-induced strand breakage (in accordance with results reported in other cell models), rosmarinic acid may rather exert an indirect effect, by mediating intracellular mechanisms responsible for DNA repair. However, it should be taken into account that this study intended to be the first step in the evaluation of the protective effects of these polyphenols on oxidative DNA damage induced to neuronal cells. Before any correlations can be made between the protection observed *in vitro*, in this cell model, and what happens *in vivo*, bioavailability studies should be performed. Some polyphenols do not pass easily through the blood brain barrier and therefore are likely present, in the central nervous system, at low concentrations. This has been reported for both quercetin and rosmarinic acid (Youdim *et al.*, 2004; Li *et al.*, 2007) but not for luteolin which seems to have a good ability to cross the barrier (Hendriks *et al.*, 2004). Nevertheless, a study with quercetin, showed that a combination of this flavonoid with phospholipids like lecitin is a way of increasing its ability to cross the blood brain barrier and exert its protective effect in the brain (Dajas *et al.*, 2003). So a role for these compounds in neuroprotection should not be ruled out.

In conclusion, our results strongly suggest that the polyphenols tested, which are present in the human diet, and in particular rosmarinic acid, should be considered as valuable protectors against oxidative stress-induced DNA damage that commonly occurs in several pathological conditions, namely in neurodegenerative diseases.

4.5. References

Aherne, S.A. and O'Brien, N.M., 2000. Lack of effect of the flavonoids, myricetin, quercetin, and rutin, on repair of H_2O_2-induced DNA single-strand breaks in Caco-2, HepG2, and V79 cells. *Nutr. Cancer* **38:** 106-115.

Ahmed-Choudhury, J., Orsler, D.J. and Coleman, R., 1998. Hepatobiliary effects of tertiary-butylhydroperoxide (*t*BOOH) in isolated rat hepatocyte couplets. *Toxicol. Appl. Pharmacol.* **152**: 270-275.

Alamo, M.J.P., Jurado, J., Francastel, E. and Laval, F., 1998. Rat 7,8-dihydro-8-oxoguanine DNA glycosylase: substrate specificity, kinetics and cleavage mechanism at an apurinic site. *Nucleic Acids Res.* **26:** 5199-5202.

Angelis, K.J., Dusinská, M. and Collins, A.R., 1999. Single cell gel electrophoresis: Detection of DNA damage at different levels of sensitivity. *Electrophoresis* **20:** 2133-2138.

Areias, F.M., Rego, C., Oliveira, C. and Seabra, R.M., 2001. Antioxidant effect of flavonoids after ascorbate/Fe^{2+}-induced oxidative stress in cultured retinal cells. *Biochem. Pharmacol.* **62:** 111-118.

Bjelland, S. and Seeberg, E., 2003. Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutat. Res.* **531:** 37-80.

Boots, A.W., Li, H., Schins, R.P.F., Duffin, R., Heemskerk, J.W.M., Bast, A. and Haenen, G.R.M.M., 2007. The quercetin paradox. *Toxicol. Appl. Pharmacol.* **222:** 89-96.

Burkle, A., 2006. DNA repair and PARP in aging. *Free Radic. Res.* **40:** 1295-1302.

Chlopcíková, S., Psotova, J., Miketová, P., Sousek, J., Lichnovsky, V. and Simanek, V., 2004. Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes part II. caffeic, chlorogenic and rosmarinic Acids. *Phytother. Res.* **18:** 408-413.

Choi, Y.J., Kang, J.S., Park, J.H.Y., Lee, Y.J., Choi, J.S. and Kang, Y.H., 2003. Polyphenolic flavonoids differ in their antiapoptotic efficacy in hydrogen peroxide-treated human vascular endothelial cells. *J. Nutr.* **133:** 985-991.

Collins, A.R., Dusinska, M., Franklin, M., Somorovska, M., Petrovska, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslova, K. and Vaughan, N., 1997. Comet assay in human biomonitoring studies: Reliability, validation, and applications. *Environ. Mol. Mutagen.* **30:** 139-146.

Collins, A.R., 2004. The comet assay for DNA damage and repair - Principles, applications, and limitations. *Mol. Biotechnol.* **26:** 249-261.

Collins, A.R. and Gaivão, I., 2007. DNA base excision repair as a biomarker in molecular epidemiology studies. *Mol. Aspects Med.* **28:** 307-322.

Collins, A.R., Harrington, V., Drew, J. and Melvin, R., 2003. Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis* **24:** 511-515.

Collins, A.R. and Horvathova, E., 2001. Oxidative DNA damage, antioxidants and DNA repair: applications of the comet assay. *Biochem. Soc. Trans.* **29:** 337-341.

Colognato, R., Laurenza, I., Fontana, I., Coppede, F., Siciliano, G., Coecke, S., Aruoma, O. I., Benzi, L. and Migliore, L., 2006. Modulation of hydrogen peroxide-induced DNA damage, MAPKs activation and cell death in PC12 by ergothioneine. *Clin. Nutr.* **25:** 135-145.

Coppede, F., Mancuso, M., Lo Gerfo, A., Manca, M. L., Petrozzi, L., Migliore, L., Siciliano, G. and Murri, L., 2007. A Ser326Cys polymorphism in the DNA repair gene hOGG1 is not associated with sporadic Alzheimer's disease. *Neurosci. Lett.* **414:** 282-285.

Dajas, F., Rivera-Megret, F., Blasina, F., Arredondo, F., bin-Carriquiry, J.A., Costa, G., Echeverry, C., Lafon, L., Heizen, H., Ferreira, M. and Morquio, A., 2003. Neuroprotection by flavonoids. *Braz. J. Med. Biol. Res.* **36:** 1613-1620.

Dodson, M.L. and Lloyd, R.S., 2002. Mechanistic comparisons among base excision repair glycolsylases. *Free Rad. Biol. Med.* **32:** 678-682.

Dusinska, M., Dzupinkova, Z., Wsolova, L., Harrington, V. and Collins, A.R., 2006. Possible involvement of XPA in repair of oxidative DNA damage deduced from analysis of damage, repair and genotype in a human population study. *Mutagenesis* **21:** 205-211.

Duthie, S. and Dobson, V.L., 1999. Dietary flavonoids protect human colonocyte DNA from oxidative attack. *Eur. J. Nutr.* **38:** 28-34.

Filipe, P., Silva, J.N., Haigle, J., Freitas, J.P., Fernandes, A., Santus, R. and Morliere, P., 2005. Contrasting action of flavonoids on phototoxic effects induced in human skin fibroblasts by UVA alone or UVA plus cyamemazine, a phototoxic neuroleptic. *Photochem. Photobiol. Sci.* **4:** 420-428.

Fishel, M.L., Vasko, M.R. and Kelley, M.R., 2007. DNA repair in neurons: So if they don't divide what's to repair? *Mutat. Res.* **614:** 24-36.

Gedik, C.M., Boyle, S.P., Wood, S.G., Vaughan, N.J. and Collins, A.R., 2002. Oxidative stress in humans: validation of biomarkers of DNA damage. *Carcinogenesis* **23:** 1441-1446.

Gelinas, S. and Martinoli, M.G., 2002. Neuroprotective effect of estradiol and phytoestrogens on MPP^+-induced cytotoxicity in neuronal PC12 cells. *J. Neurosci. Res.* **70:** 90-96.

Greene, L.A. and Tischler, A.S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **73:** 2424-2428.

Guan, S., Bao, Y.-M., Jiang, B. and An, L.-J., 2006. Protective effect of protocatechuic acid from Alpinia oxyphylla on hydrogen peroxide-induced oxidative PC12 cell death. *Eur. J. Pharmacol.* **538:** 73-79.

Gutierrez-Venegas, G., Kawasaki-Cardenas, P., Arroyo-Cruz, S.R. and Maldonado-Frias, S., 2006. Luteolin inhibits lipopolysaccharide actions on human gingival fibroblasts. *Eur. J.Pharmacol.* **541:** 95-105.

Harris, G.K., Qian, Y., Leonard, S.S., Sbarra, D.C. and Shi, X.L., 2006. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E-2 formation in RAW 264.7 cells. *J. Nutr.* **136:** 1517-1521.

Hazra, T.K., Das, A., Das, S., Choudhury, S., Kow, Y.W. and Roy, R., 2007. Oxidative DNA damage repair in mammalian cells: A new perspective. *DNA Repair* **6:** 470-480.

Hendriks, J.J.A., Alblas, J., van der Pol, S.M., van Tol, E.A., Dijkstra, C.D. and de Vries, H.E., 2004. Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. *J. Exp.Med.* **200:** 1667-1672.

Hill, J.W., Hazra, T.K., Izumi, T. and Mitra, S., 2001. Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair. *Nucleic Acids Res.* **29:** 430-438.

Hollman, P.C. and Katan, M.B., 1999. Dietary flavonoids: intake, health effects and bioavailability. *Food Chem. Toxicol.* **36:** 937-942.

Horvathova, K., Chalupa, I., Sebova, L., Tothova, D. and Vachalkova, A., 2005. Protective effect of quercetin and luteolin in human melanoma HMB-2 cells. *Mutat. Res.* **565:** 105-112.

Hur, Y.G., Suh, C.H., Kim, S. and Won, J., 2007. Rosmarinic acid induces apoptosis of activated t cells from rheumatoid arthritis patients via mitochondrial pathway. *J. Clin. Immunol.* **27:** 36-45.

Iuvone, T., De Filippis, D., Esposito, G., D'Amico, A. and Izzo, A.A., 2006. The spice sage and its active ingredient rosmarinic acid protect PC12 cells from amyloid-beta peptide-induced neurotoxicity. *J. Pharmacol. Exp. Ther.* **317:** 1143-1149.

Jung, J.Y., Han, C.R., Jeong, Y.J., Kim, H.J., Lim, H.S., Lee, K.H., Park, H.O., Oh, W.M., Kim, S.H. and Kim, W.J., 2007. Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. *Neurosci. Lett.* **411:** 222-227.

Kim, D.S., Kim, H.R., Woo, E.R., Hong, S.T., Chae, H.J. and Chae, S.W., 2005. Inhibitory effects of rosmarinic acid on adriamycin-induced apoptosis in H9c2 cardiac muscle cells by inhibiting reactive oxygen species and the activations of c-Jun N-terminal kinase and extracellular signal-regulated kinase. *Biochem. Pharmacol.* **70:** 1066-1078.

Leung, H.W.C., Wu, C.H., Lin, C.H. and Lee, H.Z., 2005. Luteolin induced DNA damage leading to human lung squamous carcinoma CH27 cell apoptosis. *Eur. J. Pharmacol.* **508:** 77-83.

Li, X., Yu, C., Lu, Y., Gu, Y., Lu, J., Xu, W., Xuan, L. and Wang, Y., 2007. Pharmacokinetics, tissue distribution, metabolism, and excretion of depside salts from Salvia miltiorrhiza in rats. *Drug Metabol. Dispos.* **35:** 234-239.

Lima, C.F., Fernandes-Ferreira, M. and Pereira-Wilson, C., 2006. Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. *Life Sci.* **79:** 2056-2068.

Loft, S. and Moller, P., 2006. Oxidative DNA damage and human cancer: Need for cohort studies. *Antioxid. Redox Signal.* **8:** 1021-1031.

Mira, L., Fernandez, M.T., Santos, M., Rocha, R., Florencio, M.H. and Jennings, K.R., 2002. Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radic. Res.* **36:** 1199-1208.

Neri, S., Gardini, A., Facchini, A., Olivieri, F., Franceschi, C., Ravaglia, G. and Mariani, E., 2005. Mismatch repair system and aging: Microsatellite instability in peripheral blood cells from differently aged participants. *J. Gerontol. A Biol. Sci. Med. Sci.* **60:** 285-292.

Nordberg, J. and Arner, E.S., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* **31:** 1287-1312.

Noroozi, M., Angerson, W.J. and Lean, M.E.J., 1998. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am. J. Clin. Nutr.* **67:** 1210-1218.

Okawara, M., Katsuki, H., Kurimoto, E., Shibata, H., Kume, T. and Akaike, A., 2007. Resveratrol protects dopaminergic neurons in midbrain slice culture from multiple insults. *Biochem. Pharmacol.* **73:** 550-560.

Palomba, L., Sestili, P. and Cantoni, O., 2001. *tert*-butylhydroperoxide induces peroxynitrite-dependent mitochondrial permeability transition leading PC12 cells to necrosis. *J. Neurosci. Res.* **65:** 387-395.

Park, B.C., Lee, Y.S., Park, H.J., Kwak, M.K., Yoo, B.K., Kim, J.Y. and Kim, J.A., 2007. Protective effects of fustin, a flavonoid from *Rhus verniciflua* Stokes, on 6-hydroxydopamine-induced neuronal cell death. *Exp. Mol. Med.* **39:** 316-326.

Petersen, M. and Simmonds, M.S., 2003. Rosmarinic acid. *Phytochem.* **62:** 121-125.

Pias, E.K. and Aw, T.Y., 2002. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC-12 cells. *Cell Death Diff.* **9:** 1007-1016.

Piga, R., Saito, Y., Chen, Z., Yoshida, Y. and Niki, E., 2005. Characterization of monochloramine toxicity on PC12 cells and protective effect of tocopherol via antioxidative function. *Arch. Biochem. Biophys.* **436:** 101-109.

Powell, C.L., Swenberg, J.A. and Rusyn, I., 2005. Expression of base excision DNA repair genes as a biomarker of oxidative DNA damage. *Cancer Lett.* **229:** 1-11.

Rice-Evans, C., Miller, N.J. and Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **20:** 933-956.

Saitoh, T., Shinmura, K., Yamaguchi, S., Tani, M., Seki, S., Murakami, H., Nojima, Y. and Yokota, J., 2001. Enhancement of OGG1 protein AP lyase activity by increase of APEX protein. *DNA Repair* **486:** 31-40.

Sasaki, N., Toda, T., Kaneko, T., Baba, N. and Matsuo, M., 2003. Protective effects of flavonoids on the cytotoxicity of linoleic acid hydroperoxide toward rat pheochromocytoma PC12 cells. Chemico-Biological Interactions 145: 101-116.

Sasaki, N., Toda, T., Kaneko, T., Baba, N. and Matsuo, M., 2003. Protective effects of flavonoids on the cytotoxicity of linoleic acid hydroperoxide toward rat pheochromocytoma PC12 cells. *Chem. Biol. Interact.* **145:** 101-116.

Scalbert, A., Manach, C., Morand, C., Remesy, C. and Jimenez, L., 2005. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **45:** 287-306.

Silva, J.P., Areias, F.M., Proença, M.F. and Coutinho, O.P., 2006. Oxidative stress protection by newly synthesized nitrogen compounds with pharmacological potential. *Life Sci.* **78:** 1256-1267.

Silva, J.P., Proença, M.F. and Coutinho, O.P., 2008. Protective role of new nitrogen compounds on ROS/RNS-mediated damage to PC12 cells. *Free Radic. Res.* **42:** 57-69.

Skibola, C.F. and Smith, M.T., 2000. Potential health impacts of excessive flavonoid intake. *Free Radic. Biol. Med.* **29:** 375-383.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C. and Sasaki, Y.F., 2000. Single cell gel/Comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.* **35:** 206-221.

Tomasetti, M., Alleva, R. and Collins, A.R., 2001. In vivo supplementation with coenzyme Q(10) enhances the recovery of human lymphocytes from oxidative DNA damage. *FASEB J.* **15:** 1425-1451.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39:** 44-84.

Vidal, A.E., Hickson, I.D., Boiteux, S. and Radicella, J.P., 2001. Mechanism of stimulation of the DNA glycosylase activity of hOGG1 by the major human AP endonuclease: bypass of the AP lyase activity step. *Nucleic Acids Res.* **29:** 1285-1292.

Youdim, K.A., Qaiser, M.Z., Begley, D.J., Rice-Evans, C.A. and Abbott, N.J., 2004. Flavonoid permeability across an in situ model of the blood-brain barrier. *Free Radic. Biol. Med.* **36:** 592-604.

CHAPTER: 5

NITROGEN COMPOUNDS PREVENT H9C2 MYOBLAST OXIDATIVE STRESS-INDUCED MITOCHONDRIAL DYSFUNCTION AND CELL DEATH

Silva, J.P., Sardão, V.A., Coutinho, O.P and Oliveira, P.J. Nitrogen compounds prevent H9c2 myoblast oxidative stress-induced mitochondrial dysfunction and cell death. *Submitted*.

Abstract

Oxidative stress has been connected to various forms of cardiovascular diseases. Previously, we have been investigating the potential of new nitrogen-containing synthetic compounds using a neuronal cell model and different oxidative stress conditions in order to elucidate their potential in the amelioration of neurodegenerative diseases. Here, we evaluated the protective role of four of those new synthetic compounds (FMA4, FMA7, FMA762 and FMA796) against oxidative damage induced to H9c2 cardiomyoblasts by *tert*-butylhydroperoxide (*t*-BHP), the hypothesis being that the test compounds will be able to prevent oxidative damage to the cell line tested. FMA762 and FMA796 decreased *t*-BHP-induced cell death, as measured both by sulforhodamine B assay and nuclear chromatin condensation counting. In addition, the two mentioned compounds inhibit intracellular signalling mechanisms leading to apoptotic cell death, namely those mediated by mitochondria, which was confirmed by their ability to overcome *t*-BHP-induced morphological changes in the mitochondrial network, loss of mitochondrial membrane potential, increased expression of the pro-apoptotic proteins p53, Bax and AIF, and activation of caspases-3 and -9. Moreover, our results indicate that the compounds' ROS scavenging ability plays a crucial role in this protection profile, as a significant decrease in *t*-BHP-induced oxidative stress was observed in their presence. The data obtained indicates that some of the test compounds may clearly prove valuable in a clinical context by ameliorating cardiovascular pathologies where oxidative stress in involved.

5.1. Introduction

Cardiovascular diseases have been established as one of the leading causes of death worldwide (Wilcox *et al.*, 2004; Sgobbo *et al.*, 2007). Oxidative stress, an event that occurs when the amount of reactive oxygen species (ROS) formed intracellularly, surpasses the cells' natural antioxidant defence systems (Halliwell, 2001; Piga *et al.*, 2005), has been connected to various forms of cardiovascular diseases, including atherosclerosis, ischemia-reperfusion injury, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure (Lefer and Granger, 2000; Giordano, 2005; Sam *et al.*, 2005). In support of the connection between oxidative stress and cardiac pathologies, it has been demonstrated that the administration of exogenous antioxidants results in the protection against oxidative myocardial injury (Nakamura *et al.*, 2002; Kaiserova *et al.*, 2007).

The mitochondrial respiratory chain on the inner mitochondrial membrane has been recognised as the major intracellular source of ROS (Zhao *et al.*, 2004). In fact, electron transfer along the mitochondrial electron transport complexes is associated with single electron reduction of molecular oxygen to form superoxide anions. In addition, it has been estimated that as much as 1–2 % of the overall oxygen consumption can result in the formation of free radicals (Sharma and Morgan, 2001; Sgobbo *et al.*, 2007). Since cardiac tissue is very abundant in mitochondria (up to 35 % of the cell volume) and the cardiac muscle is dependent on a continuous supply of oxidative phosphorylation-derived ATP (Wallace, 2007), cardiac cells can be considered to be at a high risk of suffering oxidative damage.

The production of ROS by mitochondria seems to be also involved in the initiation and execution of apoptotic cell death (Sgobbo *et al.*, 2007). Apoptosis, which is an active process of cell death, has been associated with human cardiovascular system impairment, such as in cardiomyocytes during end-stage heart failure or after myocardial infarction, and in vascular smooth muscle cells with atherosclerotic plaques or after arterial injury (Narula *et al.*, 1996; Isomoto *et al.*, 2006). ROS can promote cytochrome c release from mitochondria, by inducing its dissociation from cardiolipin, a phospholipid to which cytochrome c is associated in the inner mitochondrial membrane. Cytochrome c is then released to the cytosol probably by a mechanism involving the mitochondrial permeability transition (MPT), which involves swelling of the mitochondrial matrix and rupture of the outer membrane (Petrosillo *et al.*, 2003). Alternatively, cytochrome c may also be released in a MPT-independent way, since some members of the Bcl-2 family of proteins

(*e.g.* the pro-apoptotic Bax and Bid, and the anti-apoptotic Bcl-2 and Bcl-X_L) can regulate mitochondrial cytochrome c release with consequent downstream activation of caspases, thus playing an important role in the overall regulation of cardiomyocyte apoptosis (Reeve *et al.*, 2007). Therefore, pharmacological agents which would be able to effectively suppress ROS formation and modulate mitochondrial function should deserve a more intense investigation, in view of treating patients with cardiac disease (Kaiserova *et al.*, 2007; Sgobbo *et al.*, 2007).

We have previously described the potential of new nitrogen compounds originated from organic synthesis, against different models in which oxidative stress is involved (Silva *et al.*, 2006; Silva *et al.*, 2008a). According with those studies, four nitrogen compounds (named FMA4, FMA7, FMA762 and FMA796) have previously shown an elevated ROS scavenging activity in a neuronal cell model, which resulted in lipid peroxidation and oxidative DNA damage protection. The new molecules were synthesised based on the knowledge that the hydroxyl groups of the phenol ring (Figure 5.1) are usually responsible for the antioxidant properties of a compound and that nitrogen compounds can easily interact with active centres responsible for different functions in living organisms. Therefore, the association of these two moieties in the same molecule was expected to result in new structures with good potential as antioxidants in living systems (Areias, 2006).

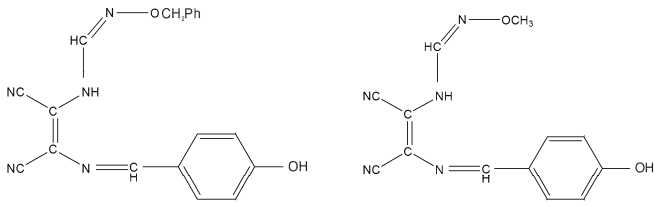
In the present study, the effects of these active four new nitrogen compounds (FMA4, FMA7, FMA762 and FMA796) on oxidative damage and apoptosis of H9c2 cells, a clonal myogenic cell line derived from embryonic rat ventricle was investigated by using the pro-oxidant *tert*-butylhydroperoxide (*t*-BHP) as model oxidant. This cell line had shown electrophysiological and biochemical properties of both skeletal and cardiac tissues, including depolarization in response to acetylcholine and rapid activation of calcium currents through L-type channels. H9c2 cells are considered a proper model to study molecular responses to oxidative damage to the cardiomyocyte (Dangel *et al.*, 1996; L'Ecuyer *et al.*, 2001). The hypothesis behind the present work is that the test compounds will be able to prevent oxidative damage to H9c2 myoblasts, including the prevention of mitochondrial damage.

5.2. Materials and methods

5.2.1. Compounds

The compounds used in this study (Figure 5.1) were prepared in the group of Organic Synthesis, Chemistry Department, University of Minho, from the reaction of an appropriate phenolic aldehyde with a substituted amidine, following an experimental procedure adapted from previous work (Booth *et al.*, 1999; Areias, 2006). The compounds were provided as a yellowish powder, which was reconstituted in DMSO, aliquoted and maintained frozen at -80ºC until use. Each aliquot was thawed only once.

Foetal bovine serum (FBS) was obtained from BioChrom KG (Berlin, Germany); Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium, dimethyl sulfoxide (DMSO), EDTA, trypsin, *tert*-butyl hydroperoxide, sulforhodamine, phenazine methosulfate (PMS), reduced nicotinamide adenine dinucleotide (NADH), NitroBlue Tetrazolium (NBT) and Hoechst 33342 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tetramethyl rodhamine methyl ester (TMRM), Calcein-AM and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) were obtained from Invitrogen (Eugene, OR, USA).



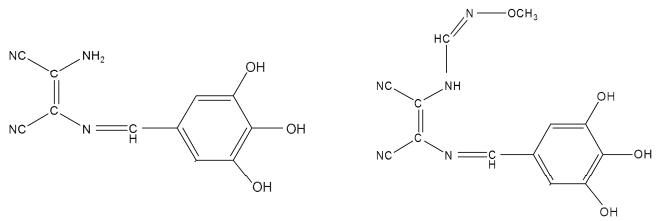


Figure 5.1 – Schematic structure of the newly synthesized nitrogen compounds. A phenolic unit is linked to an amidine function through a linear chain containing a nitrogen atom in three carbon atoms. FMA762 and FMA796 differ mainly from FMA4 and FMA7 by the presence of three hydroxyl groups within the phenol ring, instead of one. The compounds were prepared by F. M. Areias, Group of Organic Chemistry, University of Minho.

5.2.2. H9c2 cell culture

H9c2 cell line was originally derived from embryonic rat heart tissue using selective serial passages (Kimes and Brandt, 1976) and was purchased from America Tissue Type Collection (Manassas, VA, U.S.A.). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1.5 g/L sodium bicarbonate, 10% foetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin in 75 cm^2 tissue culture flasks, and maintained at 37°C, in a humidified incubator containing 5% CO_2. Cells were fed every 2 – 3 days, and sub-cultured once they reached 70 – 80% confluence, by treatment with a 0.05% trypsin/EDTA solution. To prevent loss of differentiation potential, cells were not allowed to become confluent. Cells were seeded at a density of 35 000 cells per ml, either in 24 well plates (final volume of 1 ml/well) for sulforhodamine B assays, or in 100 mm diameter tissue culture dishes for western blots. For experiments using fluorescence microscopy, cells were seeded as described above, but in 6-well plates containing glass coverslips (final volume of 2 ml medium/well). Cells were treated with both the compounds in study and *t*-BHP, for either 3 h or 6 h, as mentioned in the legends of figures.

5.2.3. Sulfhorodamine B (SRB) assay

The effects of the nitrogen compounds on cell proliferation *per se* and on the protection against *t*-BHP-induced cell death was evaluated by the sulforhodamine B assay, according to (Papazisis *et al.*, 1997), with slight modifications (Sardão *et al.*, 2007). H9c2 cells were seeded in 24-well plates and treated with the nitrogen compounds in different time points, in the presence or absence of *t*-BHP. Following this treatment, the incubation medium was removed and cells were washed with PBS and fixed in ice cold methanol, containing 1 % acetic acid, for at least 1 h. Cells were then incubated with 0.5 % (w/v) sulforhodamine B dissolved in 1 % acetic acid for 1 h at 37ºC. Unbound dye was removed by washing several times with 1 % acetic acid. Bound SRB was then solubilised with 10 mM Tris base solution, pH 10. After shaking the plates to dissolve the SRB, 200 µl from each well were transferred to 96-well plates and the absorbance read at 540 nm, against a blank containing 10 mM Tris alone. Results were expressed relatively to t = 0 h in the presence of the

vehicle alone (DMSO), which was considered as 100 % of cell proliferation/cell viability.

5.2.4. Chromatin condensation detection

The morphology of cells nuclei was observed by using the cell-permeable DNA dye Hoechst 33342. Cells presenting nuclei homogeneously stained with the dye were considered to be normal, whereas the ones presenting chromatin condensation were considered as apoptotic.

In brief, after being treated with the compounds in the presence of *t*-BHP, cells were carefully washed with PBS, fixed with 2 ml of ice cold absolute methanol and stained with 1 µg/ml of Hoechst 33342 for 30 minutes, at 37ºC, in the dark. Nuclear morphological changes were detected in a fluorescence microscope (Zeiss Axioskop 2 plus) with an UV filter. Two hundred cells from several randomly chosen fields were counted and the number of apoptotic cells expressed as a percentage of the total number of cells.

5.2.5. Triple labelling of H9c2 cells with TMRM, Hoechst 33342 and calcein-AM

H9c2 cellular and mitochondrial morphological changes in were analyzed by fluorescence microscopy, according to Sardão and collaborators (Sardão *et al.*, 2007), with slight modifications. Briefly, cells adhered to coverslips placed in the bottom of 6-well plates and treated with the compounds in the presence of *t*-BHP, were incubated with 100 nM tetramethyl rodhamine methyl ester (TMRM), 1µg/ml Hoechst 33342 and 300 nM Calcein-AM for 30 minutes, at 37ºC, in the dark. Coverslips were then carefully removed and cells observed under a fluorescence microscope.

5.2.6. Measurement of mitochondrial membrane potential ($\Delta\psi$)

Monitorization of the mitochondrial $\Delta\psi$ was performed by loading cells with TMRM, a membrane-permeable cationic fluorophore that accumulates electrophoretically inside mitochondria, proportionally to their $\Delta\psi$ (Ehrenberg *et al.*, 1988). Thirty minutes before the end of the 3 h incubation period with 50 µM *t*-BHP

and the nitrogen compounds, TMRM was added to the cell culture medium, to a final concentration of 100 nM. Cells were then visualised under a fluorescence microscope, using the adequate filter setting. Cell mean fluorescence intensity and the standard deviation associated to it were quantified using ImageJ 1.40g software (National Institutes of Health, USA). Standard deviation is a more accurate parameter for quantification, since an initial slight depolarization may cause the release of TMRM from mitochondria and subsequent accumulation in the cytosol, resulting in practically no alterations in the mean cell fluorescence value. As a test control, cells were subjected to complete depolarization by treatment with 50 µM carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP).

5.2.7. Preparation of total protein extracts and nuclear fractionation

To obtain total protein extracts, after treatment with the compounds in the presence of *t*-BHP, H9c2 cells were harvested by trypsinization and centrifuged twice at 1,000 ×*g*, for 5 min. Floating cells were also collected and combined with the adhered ones. Cellular pellets were then resuspended in 50 µl of lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM Sucrose, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA) supplemented with 2 mM dithiothreitol (DTT), 100 µM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (containing 1µg/ml of leupeptin, antipain, chymostatin and pepstatin A). Afterwards, pellets were disrupted with ~30 passages through a 27-gauge needle. The cell suspension was then rapidly frozen/thawed three times in liquid nitrogen and kept at -80°C until use.

For the preparation of nuclear fractions, cells were harvested as described above and resuspended in homogenization buffer (250 mM sucrose, 20 mM HEPES/KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA) supplemented with 1 mM DTT, 100 µM PMSF and protease inhibitor cocktail (containing 1µg/ml of leupeptin, antipain, chymostatin and pepstatin A). Cells were then homogenised in a Potter–Elvehjem homogeniser with a Teflon pestle and the homogenate was centrifuged at 220 ×*g*, for 5 min, 4ºC. The pellet was resuspended in 50 µl of a 250 mM sucrose/ 10 mM $MgCl_2$ buffer and the resulting suspension was carefully poured over 500 µl of another buffer containing 350 mM sucrose/ 0.5 mM $MgCl_2$, in order to form a gradient. These gradients were then centrifuged at ~1,271 ×*g*, for 5 min, at 4ºC and the pellets resuspended in 50 µl of the 350 mM sucrose/ 0.5 mM $MgCl_2$ buffer.

The amount of protein in each sample was determined by the Bradford method, using bovine serum albumin (BSA) as standard.

5.2.8. Determination of caspase-like activity

The activities of caspases 3 and 9 were assessed spectrophotometrically by determining the cleavage of the respective colorimetric substrate, as described in (Serafim *et al.*, 2008). Briefly, after treating cells with the compounds for 3 h, total protein extracts were obtained according to the procedure described above, and a sample volume corresponding to 25 µg and 50 µg of protein for caspase-3 and caspase-9, respectively, was added to the reaction buffer (0.1% CHAPS, 10% Sucrose, 25 mM HEPES, pH 7.5, supplemented with 10 mM DTT). The enzymatic reactions, in a final volume of 200 µl, were initiated by the addition of the colorimetric substrates Ac–DEVD–*p*NA, for caspase-3-like protease activity, and Ac–LEHD–*p*NA, for caspase-9-like protease activity, to a final concentration of 100 µM. After 2 h incubation at 37ºC, in the dark, 100 µl from each sample were transferred to a 96-well plate and the reaction product (nM / µg protein) measured spectrophotometrically at 405 nm. A calibration with known concentrations of *p*-nitroanilide(*p*NA) was done.

5.2.9. Western-blot analysis

Protein expression was determined by western-blot. Equal amounts of protein (50 µg) were denatured in sample loading buffer, separated in 8-15% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked for 2 h, at room temperature, in TPBS (PBS and 0.1% Tween 20, pH 7.4), containing 5% skim milk. Blots were then incubated overnight, at 4ºC, with goat anti-p53 (1:200, Santa Cruz Biotechnology, CA, USA), rabbit anti-Bax (1:8,000, Cell Signaling Technology, Beverly, MA, USA), rabbit anti-AIF (1:1,000, Cell Signaling Technology) or mouse anti-β-actin (1:10,000, Sigma-Aldrich, St. Louis, MO, USA). All primary antibodies were diluted in TPBS containing 1% bovine serum albumin (BSA). After three washes, membranes were incubated for 1 h in the presence of the horseradish peroxidase-conjugated anti-goat immunoglobulin (IgG) for p53

(1:5,000, DAKO Cytomation, Denmark), anti-mouse immunoglobulin (IgG) for β-actin (1:10,000, Amersham) and anti-rabbit immunoglobulin (IgG) for the other proteins (1:10,000, Amersham). Membranes were then reacted with an enhanced chemifluorescence system (Amersham). The bands were visualised by using a molecular imager Chemi-Doc XRS system (Bio-Rad, Hercules, CA, USA) and the images were analyzed with the Quantity One software (Bio-Rad). Band intensity for p53, Bax and AIF was normalized against the loading control β-actin.

5.2.10. Detection of intracellular reactive oxygen species

Intracellular ROS formation was assessed by using the non-fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), which permeates cell membrane and becomes oxidised in the presence of ROS, yielding the fluorescent 2,7'-dichlorofluorescein (DCF), by a method previously described (Sardão *et al.*, 2007), slightly modified. Briefly, after a 3 h treatment with *t*-BHP and the nitrogen compounds, cells were incubated with 7.5 μM CM-H_2DCFDA for 30 min, at 37ºC, in the dark. Cells in coverslips were then observed by fluorescence microscopy using a fluorescein filter in a Leica DM 4000B microscope. The intracellular mean fluorescence intensity was quantified using the ImageJ 1.40g software (National Institutes of Health, USA).

5.2.11. Evaluation of superoxide radical scavenging activity

The superoxide radical scavenging activity of the compounds was determined in a cell free system, using the phenazine methosulfate (PMS)/NADH non-enzymatic assay, according to a method previously described (Valentão *et al.*, 2001), which is based on the reduction, at 560 nm, of Nitroblue Tetrazolium (NBT) to the blue chromogen formazan by $O_2^{\bullet-}$. Briefly, increasing concentrations of the compounds (1 – 100 μM) were added, in a 96-well plate, to a reaction mixture consisting of 166 μM NADH and 43 μM NBT. Reactions were then initiated by the addition of 2.7 μM PMS, to a final volume of 300 μl, and followed spectrophotometrically for 2 min, at room temperature. All reagents were dissolved in 19 mM phosphate buffer, pH 7.4.

IC_{50} values were determined from the dose-response curves obtained for each compound, as the concentrations required to inhibit the reduction of NBT in 50 %.

5.2.12. Statistical analysis

Data are expressed as the mean ± S.E.M., of the indicated number of experiments. The significance of the differences between the means observed was evaluated using the unpaired two-tailed Student's *t*-test or the one-way ANOVA followed by the Student-Newman-Keuls post-hoc test. A difference of $p \leq 0.05$ was considered significant.

5.3. Results

5.3.1. Effects of the compounds on cell proliferation

The sulforhodamine B assay was used to assess the effect of the compounds *per se* on cell proliferation in a time and dose-dependent manner. As it can be observed in Figure 5.2, at the concentrations tested, none of the compounds *per se* seems to affect cellular proliferation, at least up to 96 h in culture. Despite the absence of statistical significance, a 40 μM concentration of FMA762 and FMA796 caused a small inhibition of cell growth, hence those concentrations were not used in subsequent assays. The next step was to verify if any of the test compounds could afford protection against the loss of cell mass caused by *t*-BHP-induced toxicity to H9c2 cells. Data in Figure 5.3 indicate that after a 6 h treatment with *t*-BHP, a decrease of about 55 % in cell mass occurs, in comparison to control cells. However, this decrease was prevented in the presence of the compounds, except for FMA4. The protection observed with FMA762 and FMA796, besides more powerful, is attained for a lower concentration (5 μM, instead of 40 μM). This superior protective effect of FMA762 and FMA796 against *t*-BHP-induced toxicity is in accordance with our previous results, obtained in PC12 cells (Silva *et al.*, 2008a).

5.3.2. Chromatin condensation

Chromatin condensation is a hallmark of apoptosis. To verify if the protection against the cytotoxicity induced by *t*-BHP involves apoptosis inhibition, as measured by chromatin condensation, H9c2 cells were stained with the fluorescent probe Hoechst 33342, which binds to DNA.

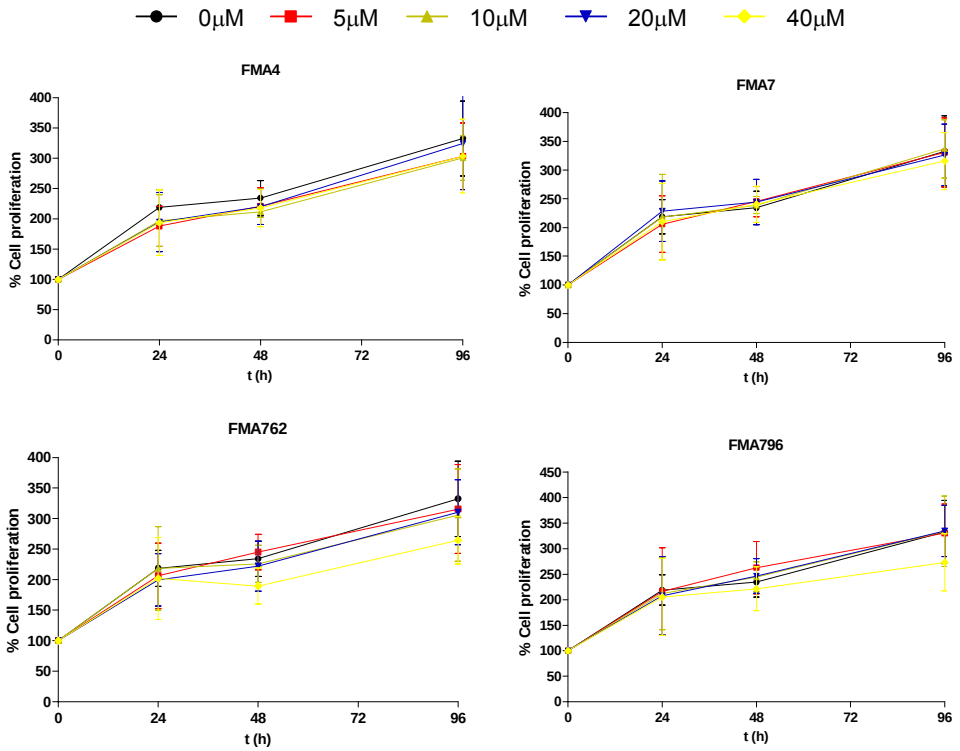


Figure 5.2 – Cellular proliferation assessed with the sulforhodamine B assay. H9c2 cells proliferation was followed up to 96 h, in the presence of different compounds concentrations, as indicated. The percentage of cellular proliferation was calculated relatively to t = 0 h. Proliferation in control cells (no test compounds) was assessed in the presence of 0.1 % DMSO. For each concentration the mean ± S.D. for at least three independent experiments is represented. No statistically significant differences were found between the different concentrations tested and the respective controls for each time point.

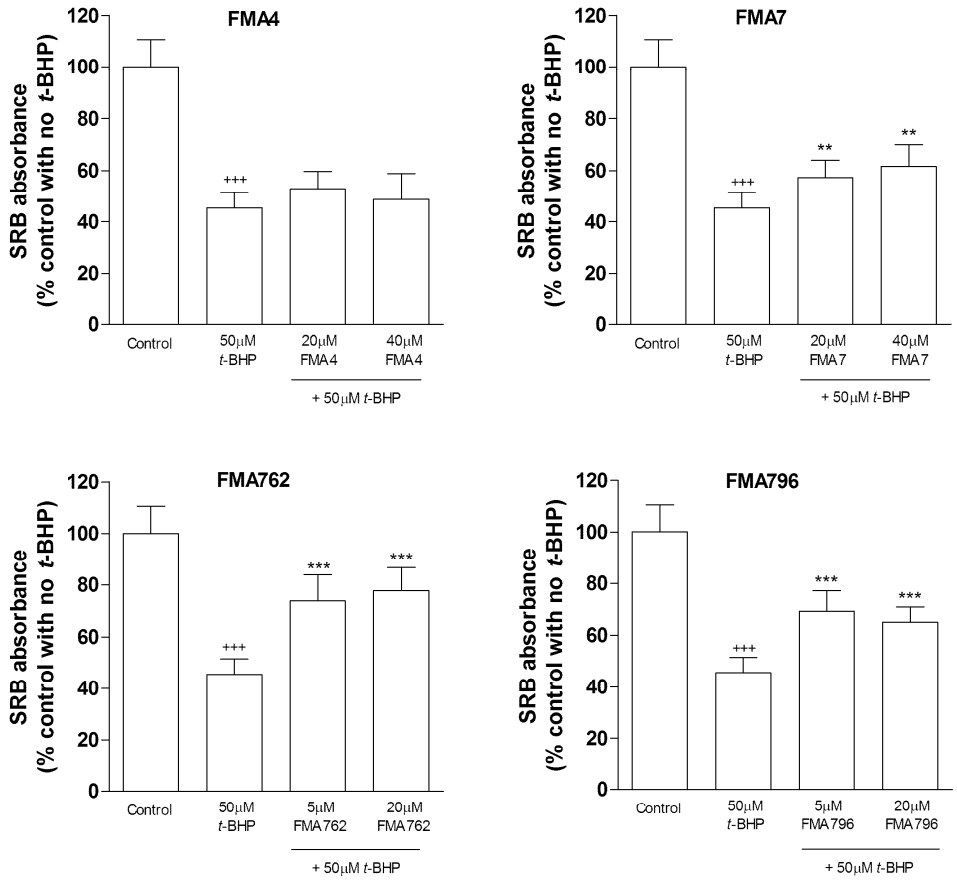


Figure 5.3 – Effects of the compounds on the reduction of cell viability induced by 50μM *t*-BHP, evaluated by the sulforhodamine B assay. H9c2 cells were incubated for 6 h in the presence of the nitrogen compounds and *t*-BHP. Results are presented in terms of percentage of cell viability, determined relatively to the control containing only 0.1 % DMSO, which was considered as representing 100 % of viability. For each bar, the mean ± S.E.M. for at least three independent experiments is represented. $^{+++}$ $p \leq 0.001$, compared to control cells (DMSO); ** $p \leq 0.01$, *** $p \leq 0.001$, as compared to 50 μM *t*-BHP.

Figure 5.4 shows that the treatment with *t*-BHP leads to a statistically significant increase in the number of nuclei with condensed chromatin, which occurs in a time-dependent manner. When the nitrogen compounds were co-incubated with tBHP, decreased numbers of apoptotic cells occurred. Co-incubation with FMA762 and FMA796 resulted in almost a complete inhibition in the formation of apoptotic nuclei (Fig. 5.4). The data suggests that the protection afforded by the test compounds against loss of cells (Fig. 5.3) also results from inhibition of apoptosis.

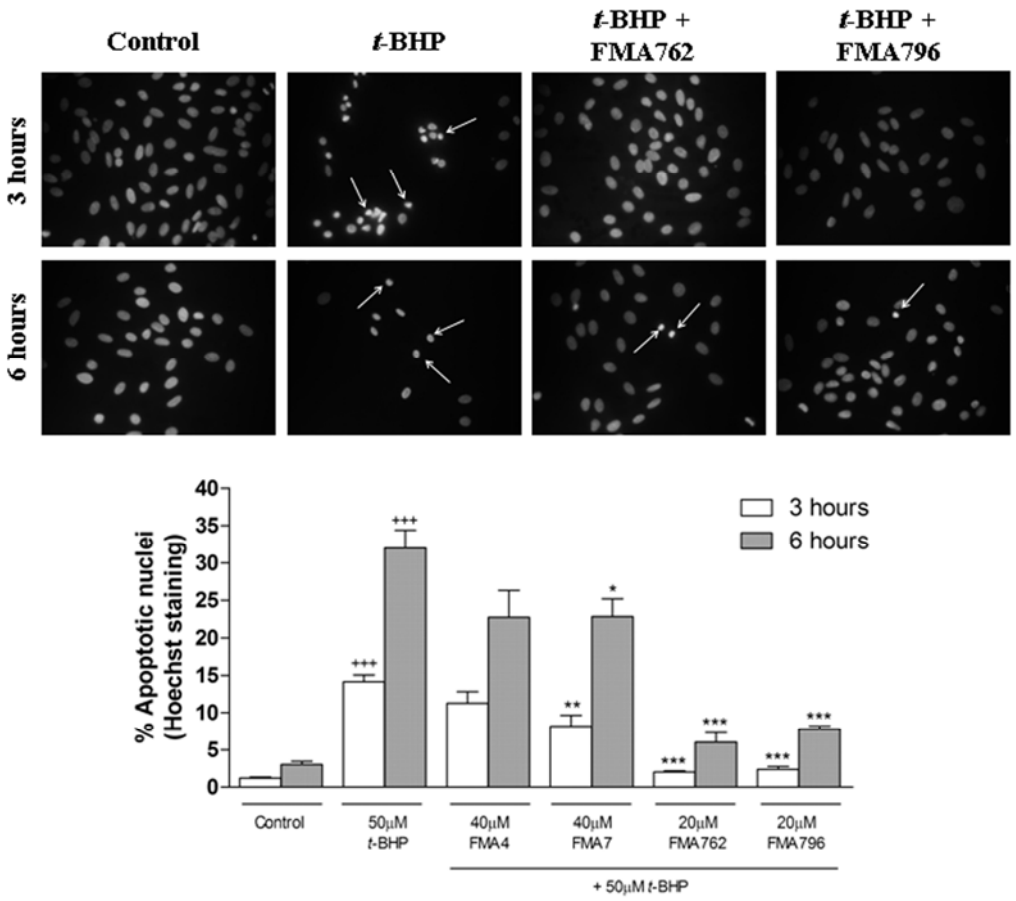


Figure 5.4 – Quantification of apoptotic nuclei after labelling cells with the fluorescent dye Hoechst 33342. Top panel: Representative images, obtained by fluorescence microscopy of Hoechst-labelled nuclei of H9c2 cells after treatment with *t*-BHP and the nitrogen compounds (in this case, only FMA762 and FMA796 are represented) for 3 and 6 hours. Arrows indicate nuclei showing condensed chromatin, characteristic of apoptosis. Bottom panel: The number of apoptotic nuclei were counted and expressed as the percentage of total cells counted (approximately 200 cells per coverslip). For each bar the mean ± S.E.M. for five independent experiments is represented. $^{+++}$ $p \leq 0.001$, compared to respective control; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, compared to 50 μM *t*-BHP (for the respective time period).

5.3.3. Vital imaging of H9c2 cells labelled with TMRM, Hoechst 33342 and calcein-AM

In order to identify possible morphological alterations induced by *t*-BHP, a triple labelling with tetramethyl rodhamine methyl ester (TMRM), Calcein-AM and Hoechst 33342 was used. TMRM is a cationic, mitochondrial selective probe that is accumulated by that organelle in a membrane potential ($\Delta\psi$)-dependent manner. Calcein-AM readily passes through the cell membrane of viable cells because of its high hydrophobicity. After it permeates into the cytoplasm, it is hydrolysed by esterases to calcein, which remains inside the cells and emits a green fluorescence. Thus, the accumulation of this probe inside the cells is indicative of membrane integrity, since damaged cell membrane in necrotic cells prevents calcein from accumulating intracellularly. Hoechst, as mentioned above, binds to DNA and is commonly used to visualize nuclei, allowing the observation of chromatin condensation in apoptotic cells or other nuclear alterations.

After the results obtained in Figure 5.4, 3 h of incubation with *t*-BHP were chosen in order to detect alterations that may precede widespread cell death. Representative images in Figure 5.5 show that control (untreated cells) exhibit polarized mitochondria distributed throughout the cytoplasm (A and B), are strongly labelled by calcein (C) and show well-defined nuclei, with normal chromatin distribution (D). On the other hand, cells treated with *t*-BHP displayed several morphological changes, which include a much smaller size, resulting from rounding up (A) and loss of the mitochondrial polarization (B). Some cells presented other features, such as a weak calcein labelling (C), condensed chromatin (D) or notorious membrane blebbing (A and C). The morphological alterations are indicative of the induction of apoptotic and/or necrotic events induced by *t*-BHP in accordance with the literature (Sardão *et al.*, 2007). The results also indicate that, among the nitrogen compounds tested, only FMA762 and FMA796 are able to prevent almost completely *t*-BHP-induced alterations of H9c2 cells. In fact, no evidence of necrotic cells could be found in the cell populations treated with FMA762 or FMA796 plus *t*-BHP. For FMA4, and especially FMA7, a slight protective effect was also observed, although not as powerful as FMA762 or FMA796.

These results are indicative of a superior protective effect of FMA762 and FMA796, against *t*-BHP-induced morphological changes and constitute a stronger evidence of the compounds' role in the apoptotic cascade of events.

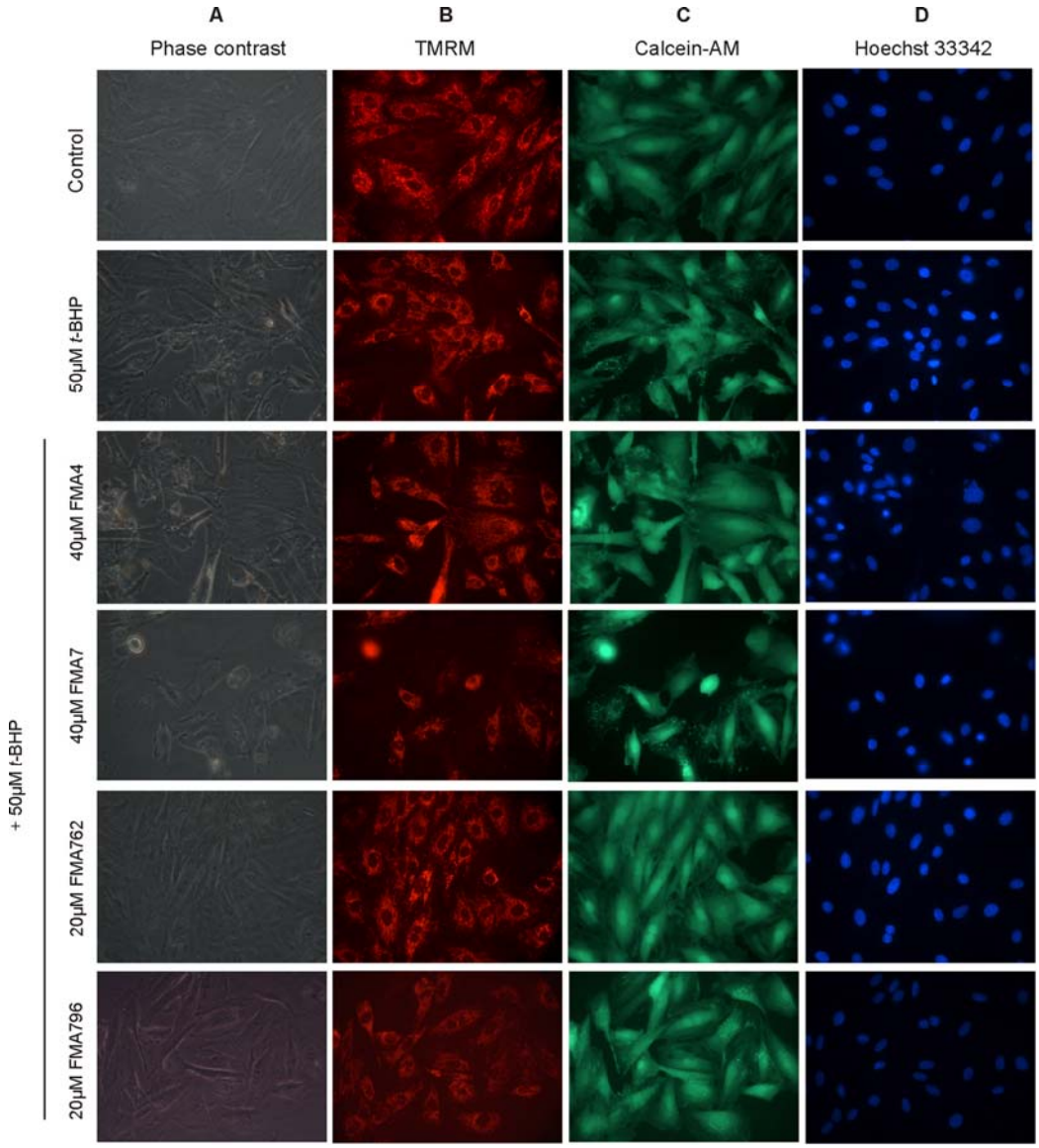


Figure 5.5 – Protection against *t*-BHP- induced alterations in cellular and mitochondrial morphology. H9c2 cells were incubated for 3 h in the presence of 50 μM *t*-BHP with or without the nitrogen compounds. Cells were then labelled with 100 nM TMRM (red), 300 nM Calcein-AM (green) and 1 μg/ml Hoechst 33342 (blue). Images are representative of five independent experiments.

5.3.4. Determination of the compounds' effects on mitochondrial membrane potential

Changes in mitochondrial membrane potential ($\Delta\psi$) were assessed after loading the cells with the membrane-permeable cationic fluorophore TMRM. In order to

reduce the risk of generating artefacts in the determination of fluorescence intensity due to probe release to the cytosol, two quantification parameters were used: cell mean fluorescence and fluorescence standard deviation. Results in Figure 5.6 (bottom panel) show that in the presence of *t*-BHP there is a decrease in both parameters relatively to control cells, which is indicative of the depolarization of mitochondria. The simultaneous incubation of *t*-BHP with the compounds FMA762 and FMA796 significantly prevented mitochondrial depolarization, as demonstrated by the values obtained in these conditions, which are similar to the control. In addition, the nitrogen compounds, by themselves, did not cause any changes in the mitochondrial $\Delta\psi$ as measured by the two values. The effects obtained with CCCP, a protonophore that induces the collapse of mitochondrial membrane potential (Lim *et al.*, 2001), validated the assay. The representative images on the top panel of the same figure illustrate the observations described.

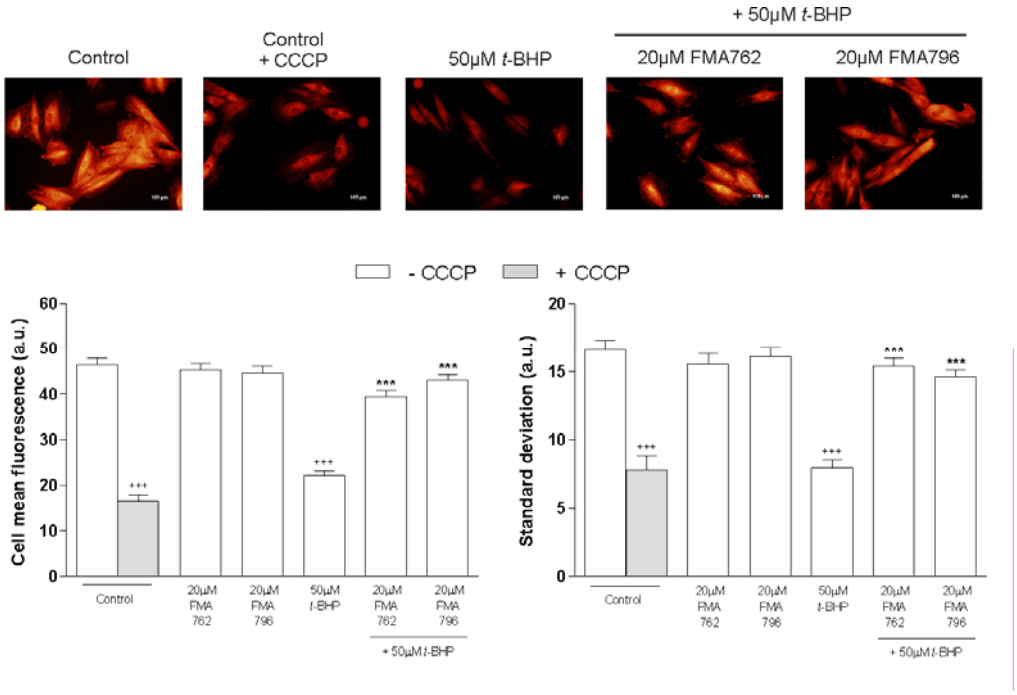


Figure 5.6 – Effects of the compounds on mitochondrial membrane depolarization. Cells were loaded with 2 μM TMRM 30 minutes prior to the end of the 3 h incubation in the presence of t-BHP and of the nitrogen compounds. CCCP was used to induce the complete depolarization of the inner mitochondrial membrane. Top panel: Images representative of each condition. Bottom panel: quantification of mitochondrial membrane potential, using two different parameters: cell mean fluorescence intensity and standard deviation. Each bar represents the mean ± S.E.M for at least four independent experiments. $^{+++}$ $p \leq 0.001$, compared to control cells in the absence of CCCP; *** $p \leq 0.001$, compared to 50 μM t-BHP.

Results in this figure indicate the involvement of mitochondria depolarization in myoblast cell death induced by *t*-BHP and the protection afforded by the compounds in study.

5.3.5. Assessment of caspases -3 and -9 activities

The loss of mitochondrial membrane potential, as well as cell blebbing and redistribution of lipids in the outer plasma membrane are some of the phenotypic characteristics of apoptotic cell death that can be mediated by caspases activation (Beere, 2005).

As depicted in Figure 5.7A, *t*-BHP induced the activation of caspase-3, an effector caspase, as indicated by the almost 3-fold increase in the cleavage of its substrate. caspase-3 activation seems to be mediated by the apoptotic mitochondrial apoptotic pathway, since caspase-9 activation was also observed (Fig. 5.7B). Confirming previous data, FMA762 and FMA796 were able to prevent the activation of both caspases tested in a statistically significant manner, which is indicative of a protective role for the nitrogen compounds against *t*-BHP-induced apoptotic cell death, particularly by an involvement in the mitochondrial cascade.

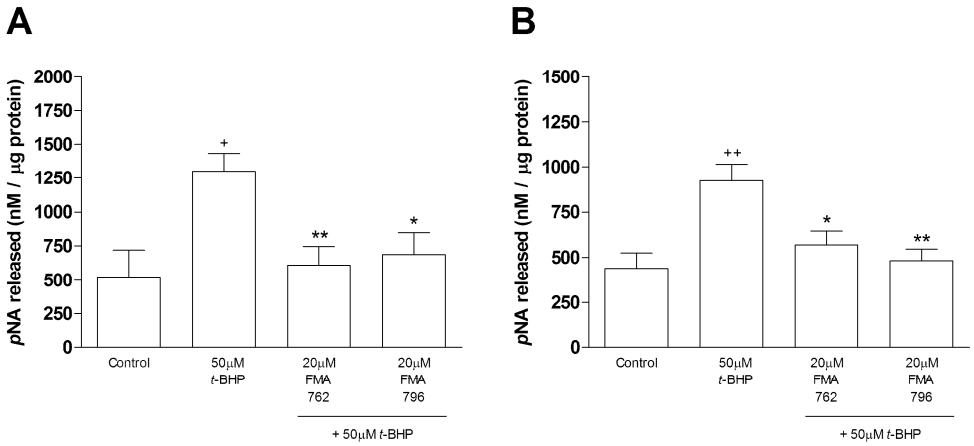


Figure 5.7 – Activities of caspases -3 (A) and -9 (B). H9c2 cells were incubated in the presence of both nitrogen compounds and *t*-BHP for 3 hours. Each bar represents the mean ± S.E.M for at least three independent experiments. $^{+}$ $p \leq 0.05$, compared to control cells; * $p \leq 0.05$, ** $p \leq 0.01$, in comparison with 50 µM *t*-BHP.

5.3.6. Expression of pro-apoptotic proteins

Given the protective effects of the compounds on caspases activation (Fig. 5.7) and on apoptotic-like morphological changes induced by *t*-BHP in H9c2 cells, namely at the mitochondrial and nuclear levels (Fig. 5.5), we tried to identify more specific targets, especially proteins known to activate apoptotic pathways, such as p53, Bax and AIF.

As observed in Figure 5.8A, *t*-BHP induces an increase in the expression of the transcription factor p53, known to lead to the expression of proteins that prevent cell division and cause apoptosis (Gartel, 2008). These results are also in accordance with data obtained with doxorubicin-induced myocyte cell death (L'Ecuyer *et al.*, 2006; Sardão *et al.*, 2009). This activation of p53 was significantly reversed in the presence of FMA762 and FMA796. Likewise, the increase in Bax expression observed in the presence of *t*-BHP (Fig. 5.8B) was also prevented by the action of the nitrogen compounds. Bax acts downstream of p53 and is responsible for the permeabilization of the outer mitochondrial membrane, leading to the release of pro-apoptotic proteins (Cui *et al.*, 2002). Therefore, these results confirm that the nitrogen compounds may prevent DNA damage by the pro-oxidants, increased expression of p53 and its targets and subsequently the activation of the apoptotic mitochondrial pathway.

Apoptosis may also be induced by a caspase-independent pathway, namely by the translocation of the apoptosis-inducing factor (AIF) from mitochondria to the nucleus, where it interacts with nucleic acids, causing chromatin condensation and DNA fragmentation (Bahi *et al.*, 2006; Lorenzo and Susin, 2007). As it is shown in Fig. 5.8C, *t*-BHP led to an increase in the expression of AIF in the nuclear extracts of H9c2 cells, which was once again significantly prevented by FMA762 and FMA796.

5.3.7. Measurement of intracellular reactive oxygen species

Our previous results with the same compounds in a neuronal cell model have indicated their important ROS/RNS scavenging activity. *t*-BHP is a pro-oxidant agent widely used as an inducer of oxidative stress (Ahmed-Choudhury *et al.*, 1998; Pias and Aw, 2002). So, the effect of the compounds on *t*-BHP-induced oxidative stress

on H9c2 cells was assessed by fluorescence microscopy, through the detection of oxidized dichlorofluorescein (DCF) form.

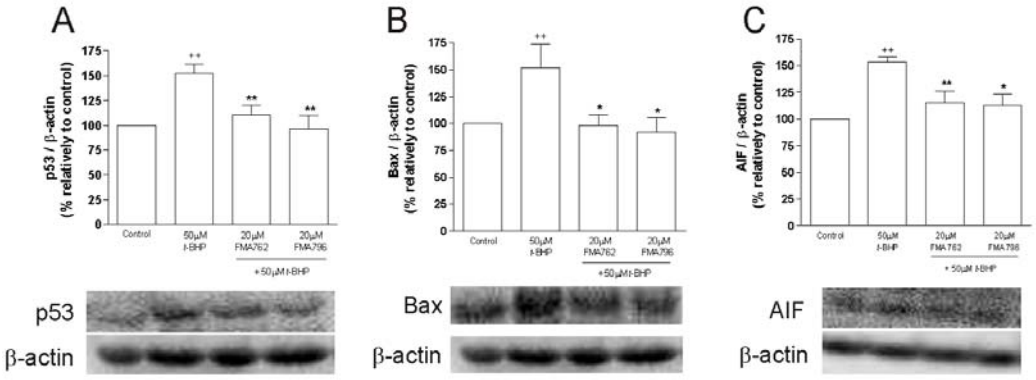


Figure 5.8 – Effects of the test compounds on the expression of the pro-apoptotic proteins (A) p53, (B) Bax and (C) apoptosis-inducing factor (AIF). Cells were treated with *t*-BHP and the nitrogen compounds either for 6 h (Bax and p53) or 15 h (AIF). For the analysis of Bax and p53 expression, total extracts were used, while extracts of the nuclear fractions were prepared to assess AIF expression. p53, Bax and AIF were identified as protein bands of 53, 20 and 57 KDa, respectively, and their expression calculated relatively to β-actin. Each bar represents the mean ± S.E.M for at least five independent experiments. $^{++}$ $p \leq 0.01$, compared to control; * $p \leq 0.05$, ** $p \leq 0.01$ compared with 50 μM *t*-BHP.

As expected, cell treatment with 50 μM *t*-BHP induces an increase in intracellular oxidative stress, as demonstrated by the increase in the cell mean fluorescence after 3 h incubation with the oxidant (Figure 5.9). The fluorescence increase is attenuated in the presence of three of the four compounds studied, namely FMA7, FMA762 and FMA796. Once again, in the case of FMA762 and FMA796, oxidative stress was reduced to values similar to the control for the concentration (20 μM) at which they proved protection in all the end-points tested previously. These results are in agreement with previous data obtained in PC12 cells (Silva *et al.*, 2008a).

In a cell-free system, the nitrogen compounds confirmed their antiradical activity, namely regarding their superoxide anion ($O_2^{\bullet -}$) scavenging ability evaluated through the use of a PMS/NADH system to generate this radical.

As it can be observed in Figure 5.10, FMA762 and FMA796 show a significant superoxide radical scavenging activity, with IC_{50} values of 7.01 ± 0.80 μM e 15.44 ± 0.68 μM, respectively. These low values were somewhat expected, since we have

previously reported the ability of these compounds to scavenge the DPPH radical, with IC_{50} values of 3.7 μM and 3.4 μM, respectively (Silva *et al.*, 2008a).

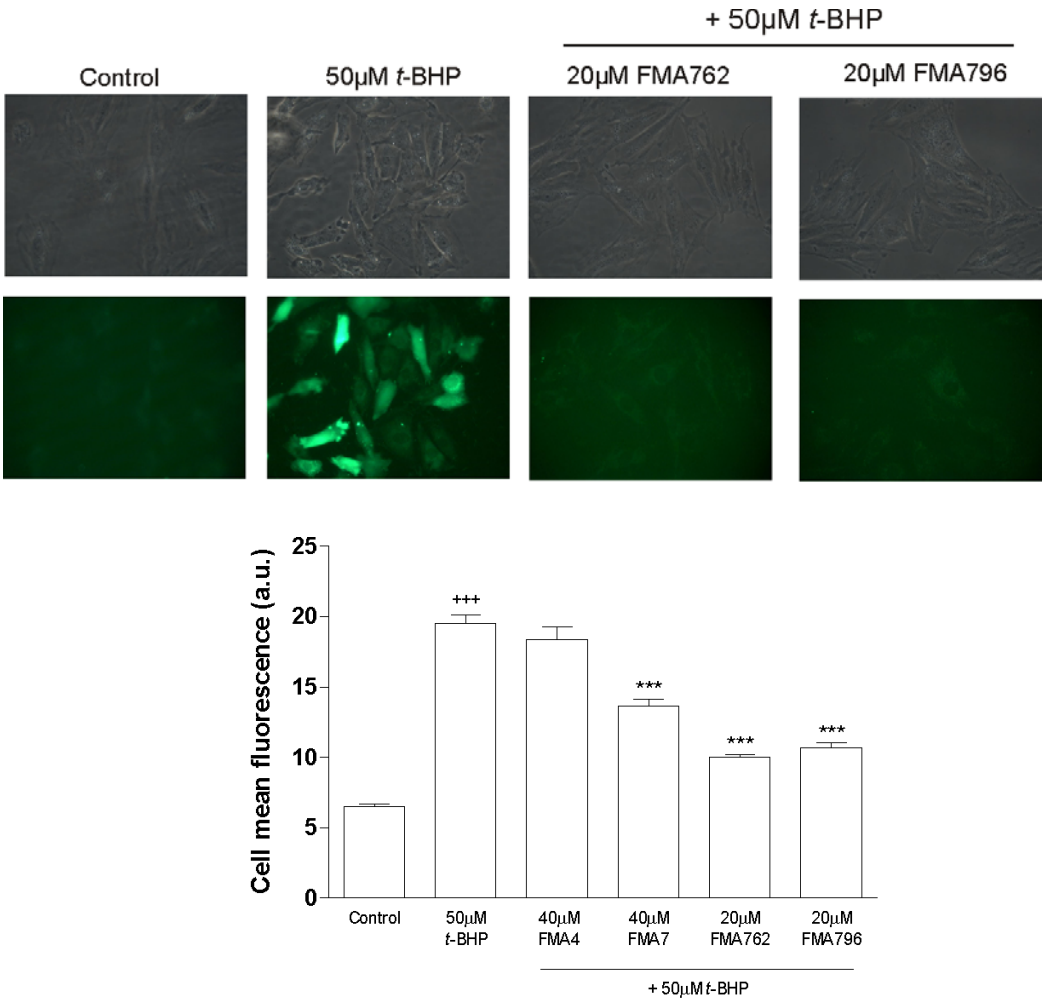


Figure 5.9 – Effects of the compounds on *t*-BHP-induced intracellular oxidative stress. H9c2 cells were incubated in the presence of *t*-BHP and the nitrogen compounds for 3 hours. Increase in intracellular oxidative stress was detected by oxidation of the fluorescent probe dichlorofluorescein. Top panel: Representative images, obtained by fluorescence microscopy, of cells treated with *t*-BHP and the nitrogen compounds FMA762 and FMA796. Bottom panel: Quantification of DCF fluorescence intensity. At least five fields per sample were analyzed in each experiment. For each bar is represented the mean ± S.E.M. for five independent experiments. $^{+++}$ $p \leq 0.001$, compared with control, *** $p \leq 0.001$, relatively to *t*-BHP.

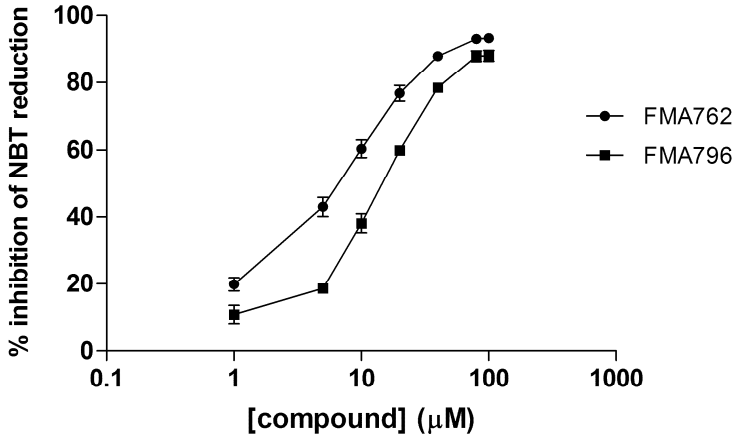


Figure 5.10 – Effect of the nitrogen compounds on NBT reduction induced by superoxide radical generated in a NADH/PMS system. The IC_{50} values were calculated from the represented curves, as the concentration needed to inhibit the reduction of NBT by 50%. For each concentration the mean ± S.E.M. is represented, considering the results obtained in at least three different experiments.

5.4. Discussion

Oxidative stress has been reported to be involved in several cardiovascular diseases, including atherosclerosis, ischemia/reperfusion injury, hypertension, congestive heart failure and other cardiomyopathies (Valko *et al.*, 2007). In fact, additionally to the modifications induced by ROS to phospholipids and proteins, abnormalities to the myocyte function, including a reduction in the sarcolemmal Ca^{2+}-pump, inhibiting Ca^{2+} sequestration from the cytoplasm in cardiomyocytes and Na^{+}-K^{+} ATPase activities may occur (Dhalla *et al.*, 2000). So, if correct localization and accumulation are granted, the use of antioxidants may prove beneficial to prevent and/or treat cardiac diseases, as these agents render heart resistance against excessive ROS (Dhalla *et al.*, 2000; Kaiserova *et al.*, 2007; Han *et al.*, 2008).

Recently, we have been characterizing the antioxidant potential of novel nitrogen compounds against events where neuronal oxidative stress is involved, trying to find possible action targets for neurodegeneration prevention (Silva *et al.*, 2006; Silva *et al.*, 2008a). In this particular study, we evaluated their ability to protect against oxidative injury induced by *t*-BHP to a model of cardiomyocytes, the H9c2 cell line, the hypothesis being that the test compounds may also prove useful to protect this

cardiac-like cell line against oxidative aggressions. This can be regarded as another possibly usefulness of the same compounds for improvement of cardiac diseases where oxidative stress is involved. The thiol-oxidizing agent *t*-BHP is metabolised intracellularly, generating *tert*-butoxyl radicals, and has been commonly used as an inducer of oxidative stress in several cell models (Pias and Aw, 2002; Alia *et al.*, 2005), including cardiomyocytes (Xie *et al.*, 2003; Przygodzki *et al.*, 2006; Sardão *et al.*, 2007). Depending on the cell line used, results obtained have also shown that it may induce characteristics of cell apoptosis, such as cytochrome c release from mitochondria, increased expression of p53, mitochondrial membrane permeabilization and caspases activation (Sonee *et al.*, 2003; Zhao *et al.*, 2004).

In the present study, we observed that none of the nitrogen compounds interfered with the normal H9c2 cell proliferation, as assessed by sulforhodamine B binding to basic amino acids of cellular proteins, at least up to 96 h. Based on the proliferation curves obtained, the concentrations of compounds to be used in the following experiments were chosen, namely 20 and 40 μM for FMA4 and FMA7, and 5 and 20 μM for FMA762 and FMA796. The lower concentrations tested for each compound correspond to the IC_{50} values previously obtained through the DPPH discolouration assay. Moreover, all the concentrations chosen had previously shown high levels of protection against oxidative damage induced to PC12 cells (Silva *et al.*, 2006; Silva *et al.*, 2008a).

At the concentrations mentioned above and after a 6 h incubation period, all the compounds, with the exception of FMA4, led to a decrease in *t*-BHP cytotoxicity. Cell death induced by *t*-BHP was shown to be associated with an increase in the number of apoptotic cells, as demonstrated by the presence of nuclear chromatin condensation. These results previously observed by others (Sardão *et al.*, 2007) were further supported by the analysis of apoptotic typical morphological changes (Beere, 2005) occurring after incubation with *t*-BHP, which included membrane blebbing and breakdown of the mitochondrial network. Apoptotic cells, which accounted for the majority of cells undergoing cell death, still accumulated green calcein fluorescence (due to the maintenance of plasma membrane integrity), but showed a morphologically altered mitochondrial network, with loss of mitochondrial membrane potential (decrease in TMRM fluorescence). The data also confirms that H9c2 cells respond to *t*-BHP by both apoptosis and necrosis, the difference being in the levels of intracellular energy maintained after the oxidative insult. Necrotic cells showed a loss of membrane integrity and of mitochondrial membrane potential. Interestingly, some of the necrotic cells showed smaller nuclei when compared to

non-treated cells, which can be indicative of secondary necrosis phenomenon, in which massive apoptosis overwhelms the available cell defence capacity, ensuing a transition to a necrotic process in the absence of a phagocytic process (Silva *et al.*, 2008b).

The morphological changes induced by *t*-BHP to H9c2 cells did not seem to be reverted by incubating cells with FMA4, and FMA7, which appeared to only have a slight protective effect, since apoptosis and necrosis still occurred. On the other hand, in the presence of FMA762 and FMA796 only a few apoptotic cells were identified and no necrotic cells could be vizualised, indicating the higher protective action of these two compounds.

Mitochondria are recognised as the major sites of ROS production in the cell, since about 2% of total oxygen escapes the electron transport chain, leading to the formation of superoxide radicals and subsequently other reactive species, such as hydrogen peroxide and hydroxyl radicals (Sharma and Morgan, 2001; Somayajulu *et al.*, 2005). As a result, mitochondria also become the primary targets for oxidative stress-induced damage (Cadenas and Davies, 2000).

Mitochondrial membrane depolarization has been described as one of the events that occurs as a response to oxidant stimuli (Orrenius *et al.*, 2007). In agreement with that, we observed a loss of mitochondrial $\Delta\psi$ in the presence of *t*-BHP, as indicated by a decrease in TMRM mean fluorescence inside mitochondria. The evaluation of the fluorescence intensity standard deviation confirms this observation. In fact, both mean fluorescence intensity and fluorescent intensity standard deviation shows that FMA762 and FMA796 were able to prevent *t*-BHP-induced mitochondrial depolarization. These results assume great relevance given the importance of mitochondria in the regulation of intracellular pathways leading to cell death. In fact, the depolarization resulting from mitochondrial $\Delta\psi$ disruption leads to an increase in the permeability of the outer mitochondrial membrane, which causes the release of several pro-apoptotic factors (*e.g.* cytochrome *c*, AIF, among others) from mitochondria (Haidara *et al.*, 2002).

Apoptosis is an active cell death process that has been recognised as essential to the pathogenesis of cardiovascular diseases (Reeve *et al.*, 2005). It can be initiated by two different signalling pathways: the mitochondria-mediated (intrinsic) pathway and the death receptor (extrinsic) pathway (Dong *et al.*, 2005; de Bruin and Medema, 2008). Mitochondrial damage, including loss of mitochondrial $\Delta\psi$, results in the activation of caspase-9. This and other initiator caspases then lead to the activation of downstream effector caspases, such as caspase-3 (Isomoto *et al.*,

2006). In this study, the tested nitrogen compounds, namely FMA762 and FMA796, proved their effectiveness in preventing the activation of both caspases 3 and 9. The results demonstrate the ability of the compounds to act on the intracellular signalling cascade leading to apoptotic cell death, most likely by preventing mitochondrial disruption.

It is generally accepted that the tumour suppressor protein p53 is activated by DNA damage, leading to an increase in the expression of genes that prevent cell division and cause apoptosis. This may result in the activation of pro-apoptotic Bcl-2 family members, such as Bax, causing the permeabilization of the outer mitochondrial membrane and subsequent activation of the above mentioned mitochondrial-mediated apoptotic pathway (Cui *et al.*, 2002; L'Ecuyer *et al.*, 2006). The results presented show that FMA762 and FMA796 decreased *t*-BHP-induced increase in p53 and Bax protein levels, indicating that their protective effects may precede mitochondria depolarization and subsequent activation of caspases.

Additionally, we determined a possible action of the compounds on the caspase-independent pathway, mediated by the apoptosis inducing factor (AIF). The AIF is a mitochondrial flavoprotein oxidoreductase that is translocated to the cytosol and then to the nucleus, where it interacts with nucleic acids, causing chromatin condensation and DNA fragmentation (Bahi *et al.*, 2006; Lorenzo and Susin, 2007). Here, we observed an increased AIF expression in nuclear extracts from *t*-BHP-treated cells, which was reverted once again by FMA762 and FMA796. This allowed us to conclude that, in addition to caspase-dependent apoptotic pathways, the nitrogen compounds in study may also act on a mitochondria-mediated caspase-independent cell death.

The protective role of the compounds on all these events may be mediated by their ROS scavenging activity. In fact they were able to significantly protect H9c2 cells against intracellular oxidative stress induced by *t*-BHP, as expected. We obtained additional evidence for their ability to scavenge the superoxide anion ($O_2^{\bullet -}$) in a cell-free system. Although this radical is not regarded as highly reactive in a cellular context due to its inability to cross lipid membranes, it is formed during many metabolic processes, such as during the electron transport in the mitochondria respiratory chain or by the action of intracellular oxidases, lipoxygenases and ciclooxygenases (Spiteller, 2001). In addition, two molecules of superoxide anion can easily yield more reactive species, such as hydrogen peroxide and molecular oxygen, by the action of superoxide dismutase (Nordberg and Arner, 2001; Valko *et al.*, 2006). Therefore, the significant superoxide scavenging activity demonstrated by

the new compounds, namely FMA762 and FMA796 (indicated by their low IC_{50} values), is of great relevance. The results confirm our previous data (Silva *et al.*, 2008a) on the ability of the compounds to scavenge the DPPH and the hydroxyl radicals, at the same concentration range. In that particular study, these compounds proved their ability to protect PC12 cells against the intracellular formation of both reactive oxygen and nitrogen species (Silva *et al.*, 2008a), which were correlated with ROS-induced lipid peroxidation and oxidative DNA damage (Silva *et al.*, 2006; Silva *et al.*, 2008a).

We conclude that the new nitrogen compounds in study clearly protect H9c2 cells against oxidative stress-induced damage. FMA762 and FMA796, which differ from the other two (FMA4 and FMA7) by the presence of three hydroxyl groups within the phenol ring, revealed a superior profile of protection, since they were able to protect cells to a higher extent and at lower concentrations. In addition, FMA762 and FMA796 seem to be able to interfere in the regulation of intracellular signalling mechanisms leading to apoptotic cell death, namely those mediated by mitochondria. This was confirmed by their ability to overcome *t*-BHP-induced morphological changes in the mitochondrial network, loss of mitochondrial membrane potential, caspases activation and increased expression of pro-apoptotic proteins leading to induction of both caspase-dependent and –independent pathways. The results suggest that the biological effects of the test compounds correlate well with their antioxidant effects in a cell free system.

The overall results obtained with these compounds are very promising in the context of the development of novel antioxidant agents capable of protecting the cardiovascular system against oxidative aggressions. The novel results in the present cardiomyocyte model add up to the data collected in previous models for neurodegenerative diseases, which increases their clinical relevance even further.

5.5. References

Ahmed-Choudhury, J., Orsler, D.J. and Coleman, R., 1998. Hepatobiliary effects of *tertiary*-butylhydroperoxide (*t*BOOH) in isolated rat hepatocyte couplets. *Toxicol. Appl. Pharmacol.* **152**: 270-275.

Alia, M., Ramos, S., Mateos, R., Bravo, L. and Goya, L., 2005. Response of the antioxidant defense system to *tert*-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *J. Biochem. Mol. Toxicol.* **19**: 119-128.

Areias, F.M. 2006. Novos compostos heterocíclicos de azoto com unidades fenólicas: síntese e actividade biológica. *Ph.D. Thesis*. University of Minho, Braga, Portugal. 259p. Available online at: http://hdl.handle.net/1822/5941.

Bahi, N., Zhang, J.S., Llovera, M., Ballester, M., Comella, J.X. and Sanchis, D., 2006. Switch from caspase-dependent to caspase-independent death during heart development - Essential role of endonuclease G in ischemia-induced DNA processing of differentiated cardiomyocytes. *J. Biol. Chem.* **281**: 22943-22952.

Beere, H.M., 2005. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J. Clin. Invest.* **115**: 2633-2639.

Booth, B.L., Costa, F.A.T., Mahmood, Z., Pritchard, R.G. and Proenca, M.F., 1999. Synthesis of (Z)-N-(2-amino-1,2-dicyanovinyl)formamide O-alkyloximes and a study of their cyclization in the presence of base. *J. Chem. Soc. [Perkin 1]* 1853-1858.

Cadenas, E. and Davies, K.J.A., 2000. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic. Biol. Med.* **29**: 222-230.

Cui, H., Schroering, A. and Ding, H.F., 2002. p53 Mediates DNA damaging drug-induced Apoptosis through a Caspase-9-dependent Pathway in SH-SY5Y Neuroblastoma Cells. *Mol. Cancer Ther.* **1**: 679-686.

Dangel, V., Giray, J., Ratge, D. and Wisser, H., 1996. Regulation of beta-adrenoceptor density and mRNA levels in the rat heart cell-line H9c2. *Biochem. J.* **317**: 925-931.

de Bruin, E.C. and Medema, J.P., 2008. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treat. Rev.* **34**: 737-749.

Dhalla, N.S., Elmoselhi, A.B., Hata, T. and Makino, N., 2000. Status of myocardial antioxidants in ischemia-reperfusion injury. *Cardiovasc. Res.* **47**: 446-456.

Dong, C., Li, Q., Lyu, S.C., Krensky, A.M. and Clayberger, C., 2005. A novel apoptosis pathway activated by the carboxyl terminus of p21. *Blood* **105**: 1187-1194.

Ehrenberg, B., Montana, V., Wei, M.D., Wuskell, J.P. and Loew, L.M., 1988. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys. J.* **53**: 785-794.

Gartel, A.L., 2008. Transcriptional inhibitors, p53 and apoptosis. *Biochim. Biophys. Acta* **1786**: 83-86.

Giordano, F.J., 2005. Oxygen, oxidative stress, hypoxia, and heart failure. *J. Clin. Invest.* **115**: 500-508.

Haidara, K., Morel, I., Abaléa, V., Barré, M.G. and Denizeau, F., 2002. Mechanism of tert-butylhydroperoxide induced apoptosis in rat hepatocytes: involvement of mitochondria and endoplasmic reticulum. *Biochim. Biophys. Acta* **1542**: 173-185.

Halliwell, B., 2001. Role of free radicals in the neurodegenerative diseases - Therapeutic implications for antioxidant treatment. *Drugs & Aging* **18**: 685-716.

Han, X., Ren, D., Fan, P., Shen, T. and Lou, H., 2008. Protective effects of naringenin-7-O-glucoside on doxorubicin-induced apoptosis in H9C2 cells. *Eur. J. Pharmacol.* **581**: 47-53.

Isomoto, S., Kawakami, A., Arakaki, T., Yamashita, S., Yano, K. and Ono, K., 2006. Effects of antiarrhythmic drugs on apoptotic pathways in H9c2 cardiac cells. *J. Pharmacol. Sci.* **101**: 318-324.

Kaiserova, H., Simunek, T., van der Vijgh, W.J.F., Bast, A. and Kvasnickova, E., 2007. Flavonoids as protectors against doxorubicin cardiotoxicity: Role of iron chelation, antioxidant activity and inhibition of carbonyl reductase. *Biochim. Biophys. Acta* **1772**: 1065-1074.

Kimes, B.W. and Brandt, B.L., 1976. Properties of a clonal muscle cell line from rat heart. *Exp. Cell Res.* **98**: 367-381.

L'Ecuyer, T., Horenstein, M.S., Thomas, R. and Heide, R.V., 2001. Anthracycline-induced cardiac injury using a cardiac cell line: potential for gene therapy studies. *Mol. Gen. Metabol.* **74**: 370-379.

L'Ecuyer, T., Sanjeev, S., Thomas, R., Novak, R., Das, L., Campbell, W. and van der Heide, R., 2006. DNA damage is an early event in doxorubicin-induced cardiac myocyte death. *Am. J. Physiol.* **291**: H1273-H1280.

Lefer, D.J. and Granger, D.N., 2000. Oxidative stress and cardiac disease. *Am. J. Med.* **109**: 315-323.

Lim, M.L.R., Minamikawa, T. and Nagley, P., 2001. The protonophore CCCP induces mitochondrial permeability transition without cytochrome c release in human osteosarcoma cells. *FEBS Lett.* **503**: 69-74.

Lorenzo, H.K. and Susin, S.A., 2007. Therapeutic potential of AIF-mediated caspase-independent programmed cell death. *Drug Resist. Updat.* **10**: 235-255.

Nakamura, K., Kusano, K., Nakamura, Y., Kakishita, M., Ohta, K., Nagase, S., Yamamoto, M., Miyaji, K., Saito, H., Morita, H., Emori, T., Matsubara, H., Toyokuni, S. and Ohe, T., 2002. Carvedilol decreases elevated oxidative stress in human failing myocardium. *Circulation* **105**: 2867-2871.

Narula, J., Haider, N., Virmani, R., DiSalvo, T.G., Kolodgie, F.D., Hajjar, R.J., Schmidt, U., Semigran, M.J., Dec, G. and Khaw, B.A., 1996. Apoptosis in myocytes in end-stage heart failure. *N. Engl. J. Med.* **335**: 1182-1189.

Nordberg, J. and Arner, E.S., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* **31**: 1287-1312.

Orrenius, S., Gogvadze, V. and Zhivotovsky, B., 2007. Mitochondrial Oxidative Stress: Implications for Cell Death. *Ann. Rev. Pharmacol. Toxicol.* **47**: 143-183.

Papazisis, K.T., Geromichalos, G.D., Dimitriadis, K.A. and Kortsaris, A.H., 1997. Optimization of the sulforhodamine B colorimetric assay. *J. Immunol. Methods* **208**: 151-158.

Petrosillo, G., Ruggiero, F.M. and Paradies, G., 2003. Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. *FASEB J.* **17**: 2202-2208.

Pias, E.K. and Aw, T.Y., 2002. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC-12 cells. *Cell Death Differ.* **9**: 1007-1016.

Piga, R., Saito, Y., Chen, Z., Yoshida, Y. and Niki, E., 2005. Characterization of monochloramine toxicity on PC12 cells and protective effect of tocopherol via antioxidative function. *Arch. Biochem. Biophys.* **436**: 101-109.

Przygodzki, T., Lapshina, E., Zavodnik, I., Sokal, A. and Bryszewska, M., 2006. 2,3-butanedione monoxime does not protect cardiomyocytes under oxidative stress. *Cell Biochem. Funct.* **24**: 413-418.

Reeve, J.L.V., Duffy, A.M., O'Brien, T. and Samali, A., 2005. Don't lose heart - therapeutic value of apoptosis prevention in the treatment of cardiovascular disease. *J. Cell. Mol. Med.* **9**: 609-622.

Reeve, J.L.V., Szegezdi, E., Logue, S.E., Chonghaile, T.N., O'Brien, T., Ritter, T. and Samali, A., 2007. Distinct mechanisms of cardiomyocyte apoptosis induced by doxorubicin and hypoxia converge on mitochondria and are inhibited by Bcl-XL. *J. Cell. Mol. Med.* **11**: 509-520.

Sam, F., Kerstetter, D.L., Pimental, D.R., Mulukutla, S., Tabaee, A., Bristow, M.R., Colucci, W.S. and Sawyer, D.B., 2005. Increased reactive oxygen species production and functional alterations in antioxidant enzymes in human failing myocardium. *J. Card. Fail.* **11**: 473-480.

Sardão, V.A., Oliveira, P.J., Holy, J., Oliveira, C.R. and Wallace, K.B., 2009. Doxorubicin-induced mitochondrial dysfunction is secondary to nuclear p53 activation in H9c2 cardiomyoblasts. *Cancer Chemother. Pharmacol.* d.o.i.:10.1007/s00280-009-0932-x.

Sardão, V.A., Oliveira, P.J., Holy, J., Oliveira, C.R. and Wallace, K.B., 2007. Vital imaging of H9c2 myoblasts exposed to tert-butylhydroperoxide - characterization of morphological features of cell death. *BMC Cell Biol.* **8**: 11-27.

Serafim, T.L., Matos, J.A.C., Sardão, V.A., Pereira, G.C., Branco, A.F., Pereira, S.L., Parke, D., Perkins, E.L., Moreno, A.J.M., Holy, J. and Oliveira, P.J., 2008. Sanguinarine cytotoxicity on mouse melanoma K1735-M2 cells - Nuclear vs. mitochondrial effects. *Biochem. Pharmacol.* **76**: 1459-1475.

Sgobbo, P., Pacelli, C., Grattagliano, I., Villani, G. and Cocco, T., 2007. Carvedilol inhibits mitochondrial complex I and induces resistance to H_2O_2-mediated oxidative insult in H9C2 myocardial cells. *Biochim. Biophys. Acta* **1767**: 222-232.

Sharma, P. and Morgan, P., 2001. Ascorbate reduces superoxide production and improves mitochondrial respiratory chain function in human Fibroblasts with electron transport chain deficiencies. *Mitochondrion* **1**: 191-198.

Silva, J.P., Areias, F.M., Proença, M.F. and Coutinho, O.P., 2006. Oxidative stress protection by newly synthesized nitrogen compounds with pharmacological potential. *Life Sci.* **78**: 1256-1267.

Silva, J.P., Proença, M.F. and Coutinho, O.P., 2008a. Protective role of new nitrogen compounds on ROS/RNS-mediated damage to PC12 cells. *Free Radic. Res.* **42**: 57-69.

Silva, M.T., do Vale, A. and dos Santos, N.M.N., 2008b. Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. *Apoptosis* **13**: 463-482.

Somayajulu, M., McCarthy, S., Hung, M., Sikorska, M., Borowy-Borowski, H. and Pandey, S., 2005. Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. *Neurobiol. Dis.* **18**: 618-627.

Sonee, M., Martens, J.R., Evers, M.R. and Mukherjee, S.K., 2003. The effect of *tertiary* butylhydroperoxide and nicotinamide on human cortical neurons. *Neurotoxicol.* **24**: 443-448.

Spiteller, G., 2001. Lipid peroxidation in aging and age-dependent diseases. *Exp. Gerontol.* **36**: 1425-1457.

Valentão, P., Fernandes, E., Carvalho, F., Andrade, P.B., Seabra, R.M. and Bastos, M.L., 2001. Antioxidant activity of *Centaurium erythraea* infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. *J. Agric. Food Chem.* **49**: 3476-3479.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39**: 44-84.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem.-Biol. Int.* **160**: 1-40.

Wallace, K.B., 2007. Adriamycin-induced interference with cardiac mitochondrial calcium homeostasis. *Cardiovasc. Toxicol.* **7**: 101-107.

Wilcox, J.K., Ash, S.L. and Catignani, G.L., 2004. Antioxidants and Prevention of Chronic Disease. *Crit.Rev. in Food Sci. Nutr.* **44**: 275-295.

Xie, G.H., Rah, S.Y., Yi, K.S., Han, M.K., Chae, S.W., Im, M.J. and Kim, U.H., 2003. Increase of intracellular Ca^{2+} during ischemia/reperfusion injury of heart is mediated by cyclic ADP-ribose. *Biochem. Biophys. Res. Comm.* **307**: 713-718.

Zhao, K., Zhao, G.M., Wu, D., Soong, Y., Birk, A.V., Schiller, P.W. and Szeto, H.H., 2004. Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. *J. Biol. Chem.* **279**: 34682-34690.

CHAPTER: 6

FINAL REMARKS

6.1. General Discussion

The work herein presented, which is part of a larger project aiming the development of drugs useful for the improvement of human health, intended to study the protective role of four new nitrogen compounds, from organic synthesis, against different oxidative stress conditions.

Oxidative stress, a condition commonly defined as an imbalance between oxidants and the cells' antioxidant defence systems in favour of the oxidants (Sies, 1997; Nordberg and Arner, 2001), is known to cause damage to all biomolecules, such as lipids, proteins and nucleic acids (Kohen and Nyska, 2002). This oxidative damage can ultimately lead to cell death, and is associated to several pathologies, including neurodegenerative and cardiovascular diseases, among others (Halliwell, 2001; Valko *et al.*, 2007). Therefore, antioxidants mainly derived from plants and vegetables have been used in the treatment of those diseases (Masella *et al.*, 2005). However, mostly due to some drawbacks presented by natural compounds, research on the development of new synthetic structures capable of acting beyond and/or potentiate the cells' natural defence mechanisms has dramatically increased in the last few years.

The synthesis of novel nitrogenated structures, composed of an amidine unit linked to a phenol ring, has been one of the main research interests of the Group of Organic Synthesis of the Department of Chemistry of the University of Minho. The association of the amidine and phenolic moieties in the same structure was expected to yield molecules with high antioxidant capacities. The first study demonstrating the biological relevance of those compounds was done in our lab with two of them, FMA4 and FMA7, and gave strong indications relatively to their potential as antioxidants (Silva *et al.*, 2006). Moreover, the continuous efforts to improve the antioxidant potential of these molecules resulted in the synthesis of several other structures, from which two of them, FMA762 and FMA796, emerged as promising regarding their antioxidant capacities (Areias, 2006).

So, in this work we continued our previous studies (Silva *et al.*, 2006), trying to elucidate the pharmacological potential of those novel nitrogenated structures, namely FMA4, FMA7, FMA762 and FMA796, using different mammalian cell models, in conditions where oxidative stress is involved.

Our first objective was to demonstrate the real antioxidant properties of these new nitrogen compounds (**Chapter 2**). Since oxidative stress is commonly associated with neurodegenerative diseases, we used PC12 cells as a biological model. These cells have been described to resemble precursors of adrenal chromaffin cells and sympathetic neurons, and have been reported as a suitable model for the study of neurodegenerative diseases (Pereira *et al.*, 1999; Piga *et al.*, 2005; Jung *et al.*, 2007).

Initial experiments in cell-free systems, namely by the use of DPPH discolouration and 2-deoxy-D-ribose degradation assays, gave the first indication of the compounds' good antiradical activity, which revealed to be superior for FMA762 and FMA796. The free radical scavenging properties of the nitrogen compounds were confirmed by observing their protective effects on intracellular ROS/RNS formation. Indeed, these compounds, in particular FMA762 and FMA796, were able to decrease the amount of reactive species induced either by ascorbate/iron or sodium nitroprusside (a nitric oxide inducer) in PC12 cells. So, a relationship between the compounds' structure, their antiradical activity and their ability to prevent intracellular ROS formation seems to exist. In fact, given that the number and position of hydroxyl groups within a structure may influence the scavenging activity of a compound (Yokozawa *et al.*, 1998; Lebeau *et al.*, 2000), the presence of three hydroxyl groups within the phenol ring of FMA762 and FMA796, instead of just one, as is the case of FMA4 and FMA7, may explain their higher free radical scavenging activity.

The protection profile of the four compounds against intracellular ROS formation also seems to correlate with their ability to prevent lipid peroxidation, which was once again superior for FMA762 and FMA796. This protection was higher than the one obtained for Trolox (the hydrosoluble form of vitamin E), and similar to α-tocopherol (a membrane-bound, highly lipophilic antioxidant), two classical antioxidants used as comparison. Taking into account that this kind of damage occurs mainly because of free radicals formed intracellularly, we may assume that their relative good liposolubility profiles may, at least partially, contribute to their protective role, since enables them to cross the lipid bilayer and prevent those radicals from inducing further damage.

However, none of the compounds was able to attenuate the *t*-BHP-induced decreases in glutathione peroxidase and superoxide dismutase enzymatic activities. These enzymes, which belong to a group of antioxidants regarded as the cells' first line of defence, act on specific ROS, following their formation, and degrade them to

less harmful products, before they even cause damage to biomolecules. Moreover, neither the decrease in GSH nor the increase in GSSG contents were reverted in the presence of the compounds. Glutathione is a low molecular mass agent that is ubiquitously present in all cells, where it displays several functions, ranging from ROS scavenging to maintenance of the cells' redox state.

The results obtained indicate that the nitrogen compounds were not able to potentiate the cells' natural antioxidant defence systems, which would be of particular interest, given the importance of these mechanisms in preventing oxidative damage. Therefore, in this work we demonstrated the ability of the new nitrogen compounds to rescue PC12 against oxidative and nitrosative stress, which seems to be most likely mediated by their ability to scavenge free radicals, rather than to an involvement on the natural antioxidant defence systems.

Following the biochemical characterisation of the antioxidant potential of these new molecules and given their ability to prevent oxidative injury, namely lipid peroxidation and intracellular ROS formation induced to PC12 cells, we intended to address their protective role against a more specific type of damage, like oxidative DNA damage (**Chapter 3**). In fact, neuronal cells, due to their high rate of oxidative metabolism and low levels of antioxidant enzymes, have been reported as being quite susceptible to ROS (Rolseth *et al.*, 2008). Probably related with that, oxidative DNA damage is regarded as the type of damage most likely to occur in neuronal cells, with severe implications in neurodegenerative diseases and in aging (Fishel *et al.*, 2007).

Through the Comet assay, a widely used technique to assess oxidative injury inflicted to DNA (ESCODD, 2003), we observed a protective effect for all the four compounds against *t*-BHP-induced strand-breaks formation, the most common form of DNA damage. Once again, FMA762 and FMA796 proved to be the most effective ones. Furthermore, not only did they prevent the formation of strand-breaks, but also led to an increase of about 20% in the repair of this kind of damage.

Although none of the compounds was able to reduce the amount of oxidized purines induced by the photosensitiser compound Ro 19-8022, at least FMA796 led to an increase in the repair capacity of this type of lesion. The lack of a protective effect against Ro-induced DNA damage suggests that the nitrogen compounds do not act by scavenging the singlet oxygen produced by the photosensitiser compound. Nevertheless, the effects on oxidised nucleotidic bases repair seemed to be mediated by an increase in the activity of DNA repair enzymes, as assessed by

the *in vitro* base excision repair assay, which uses a cell-free extract, obtained from cells previously treated with the compounds, to incise DNA. However, this effect in the repair enzymes' activity was not due to a regulation at the gene expression level, since no significant changes could be observed in the levels of *OGG1* and *APE1* mRNA. Thus, since repair enzymes' activity may be suppressed by elevated levels of ROS, the compounds ROS scavenging ability (demonstrated throughout Chapter 2) could be on the basis of the observed increase in DNA repair. Alternatively, taking into account that additional factors, such as XPG, have been demonstrated to enhance the activity of *OGG1* (Le Page *et al.,* 2000), a compounds' involvement in the regulation of DNA repair at the post-transcriptional level should be considered. Moreover, the hypothesis that the compounds could lead to an increase in OGG1 protein stability or in the availability of an unknown factor, should not be ruled out. Nevertheless, independently of the explanation, increased repair together with the increased antioxidant capacity, demonstrated by these nitrogen compounds in Chapter 2, can account for the significant decreases in endogenous oxidative DNA damage.

So, results obtained in PC12 cells are indicative of the potential of the novel nitrogen compounds as molecules to be used in the treatment of oxidative stress-induced injury, which normally occurs in neurodegenerative diseases. However, it should be noted that this study only represents the first step in the evaluation of the nitrogen compounds' protective effects against oxidative damage. Therefore, care should be taken before extrapolating the results herein obtained *in vitro*, to *in vivo* situations, as some questions still need clarification, namely regarding the ability of these compounds to cross the blood brain barrier. Nevertheless, even if they are unable to do so, a role for these compounds in neuroprotection should not be ruled out, since the ability of a compound to cross the blood brain barrier can be increased by, for example, combining it with some phospholipids, as described by Dajas and colleagues in a study with flavonoids (Dajas *et al.*, 2003).

At the same time, a parallel project ongoing in our lab focused on the properties of compounds from natural origin against oxidative DNA damage. In this context, we evaluated the protective effects of three polyphenolic compounds, with known antioxidant properties - luteolin, quercetin and rosmarinic acid – against that kind of damage (**Chapter 4**).

All the polyphenols tested were able to decrease *t*-BHP-induced strand breaks. Among them, luteolin revealed to be the most efficient one, which is in accordance

with its reported high antioxidant activity (Harris *et al.*, 2006). We used different incubation conditions to enable compounds to permeate the cell membrane. Although luteolin and rosmarinic acid showed no differences in protection whether added to cells simultaneously or three hours prior to the addition of *t*-BHP, quercetin's protective effect was reduced when cells were pre-incubated with this polyphenol. This may be related with the metabolisation of quercetin to a less protective compound, which can also be genotoxic, as previously reported in a lymphocyte model (Duthie and Dobson, 1999). In this way, the results obtained with our nitrogen compounds (Chapter 3), may indeed indicate that they possess some advantages relatively to, at least some, of the polyphenols, since they show no genotoxicity even for long incubation periods.

Regarding the ability of the polyphenols tested to increase the repair of strand breaks, rosmarinic acid was the one that showed the strongest effects at this level. This was even superior to the values obtained for the nitrogen compounds. On the other hand, even though quercetin was able to enhance the repair of strand breaks to an extent similar to the nitrogen compounds, luteolin's capacity to do so was diminished relatively to the synthetic structures tested.

The polyphenols were unable to decrease the amount of oxidised purines induced by Ro 19-8022 (a photosensitiser compound that was used to induce specifically purines oxidation), similarly to the observed for the nitrogen compounds in Chapter 3. Nevertheless, rosmarinic acid led to an increase in the repair of this kind damage, which was similar to the ones observed for the nitrogen compounds (between 6 and 10%). In addition, results obtained with the BER assay indicated a possible regulation of DNA repair enzymes by rosmarinic acid, as demonstrated by an increase of about 7% in the presence of the rosmarinic acid-treated extract (Chapter 4). This increase, although slightly superior, was also similar to the ones observed for the nitrogen compounds, which varied between 2 and 6 % (Chapter 3). On the other hand, extracts (containing the DNA repair enzymes) from cells treated with either luteolin or quercetin were unable to increase the DNA repair capacity, confirming that these compounds cannot act against the oxidation of nucleotidic bases induced by Ro 19-8022. Moreover, the effect observed for rosmarinic acid on the DNA repair enzymes, contrarily to FMA762 and FMA796, seemed to be mediated through changes in genes expression, namely *OGG1*. Thus, these results indicate that rosmarinic acid may regulate the intracellular pathways responsible for DNA repair, while luteolin and quercetin (which showed no ability to repair oxidised bases) may be acting through a (direct) free radical scavenging mechanism.

Given the results obtained with both nitrogen and polyphenolic compounds on oxidative DNA damage, it is possible to state that the nitrogen compounds possess some advantages over some of the polyphenols studied. In fact, although luteolin and quercetin protect PC12 cells against strand breaks formation to a high extent, and even enhance the repair of this kind of damage, they fail to act at the base oxidation repair level. Therefore, contrarily to the nitrogen compounds, their protective action on oxidative DNA damage may be quite limited. Moreover, the genotoxicity of quercetin (suggested by a reduced level of protection when cells are pre-incubated for longer periods in the presence of this flavonoid) may impair its use as a protector of oxidative DNA injury. In contrast, the beneficial effects of the nitrogen compounds seem to be surpassed by rosmarinic acid, since results with this phenolic acid suggest its involvement in the regulation of pathways responsible for DNA repair, namely by enhancing the levels of expression of repair genes. By its turn, a protective role mediated by their ROS scavenging ability may be attributed to the nitrogen compounds.

Although the protective effects observed for both nitrogen compounds and rosmarinic acid on the repair of base oxidation appear to be “low”, it should be noted that they may in fact assume a great biological relevance. In fact, DNA repair involves a complex and well coordinated network of events, which are in a close association with other important mechanisms, such as cell cycle arrest and cell proliferation. Therefore, a fine equilibrium is required for proper activation and inactivation of DNA damage signalling and repair mechanisms, as great variations in these mechanisms could result in severe anomalies to vital cell processes. In this way, “small” increases in events like the expression of DNA repair genes and base oxidation repair should not be underestimated.

In addition to neurodegenerative diseases, oxidative stress is known to be involved in several cardiovascular diseases, including atherosclerosis, ischemia/reperfusion injury, hypertension, congestive heart failure and other cardiomyopathies (*in* Valko *et al.*, 2007). So, with the perspective of broadening the spectrum of action of the nitrogen compounds and finding them other physiological applications, we investigated their protective role on oxidative injury and apoptosis induced by *t*-BHP to H9c2 cells (**Chapter 5**). This cell line has been considered a suitable cardiac cell model (L'Ecuyer *et al.*, 2001).

The nitrogen compounds did not affect H9c2 cells’ proliferation and were able to decrease *t*-BHP-induced toxicity, as evidenced by the sulforhodamine B assay and

by counting the number of apoptotic cells (after Hoechst 33342 labelling). In addition, the compounds (namely FMA762 and FMA796) prevented almost completely both cellular and mitochondrial morphological changes that occurred after cells incubation with *t*-BHP. These alterations included, additionally to chromatin condensation, rounding up of the cells, membrane blebbing and breakdown of the mitochondrial network. In all these events, FMA4 and FMA7 only showed a slight protective effect. Moreover, FMA762 and FMA796 were able to prevent *t*-BHP-induced mitochondria depolarization, which assumes great relevance, given the importance of mitochondria in the regulation of intracellular pathways leading to cell death (Haidara *et al.*, 2002). Indeed, the nitrogen compounds, namely FMA762 and FMA796, presented an effective action in the regulation of apoptotic pathways, since they prevented the activation of both caspases 3 and 9. This preventive effect of caspase-9 activation was also indicative of a possible involvement of the compounds at the mitochondrial level.

The regulatory action of the compounds on the apoptotic cascade was further confirmed by results showing a decrease in *t*-BHP-induced increased expression of pro-apoptotic proteins like p53, Bax and AIF. These data allowed us to conclude that the compounds may act prior to mitochondria permeabilisation, preventing the releasing of some pro-apoptotic factors, which result in the induction of not only caspase-dependent (as demonstrated by their effects on caspase-9 activity) but also of caspase-independent (indicated by the decrease in AIF in nuclear extracts) pathways of cell death. Furthermore, we found evidence that the ROS scavenging activity of the nitrogen compounds may play a crucial role in their protective effects on the events associated with *t*-BHP-induced cell death. In this way, in addition to the previously demonstrated protective role for the nitrogen compounds on a neuronal cell model (**Chapters 2** and **3**), we also demonstrated their potential to act on oxidative stress induced to a cardiomyocyte cell line.

In conclusion, the overall results presented in this work reveal novel synthetic compounds with a strong ability to scavenge reactive oxygen and nitrogen species and therefore act on oxidative stress-mediated injury associated to several pathologies, including neurodegenerative and cardiovascular diseases. These compounds prevent a series of events related to oxidative stress, detailed in **Chapters 2, 3** and **5**, and, to some extent, seem to act on intracellular signalling mechanisms resulting, for example, in an increase in oxidative DNA damage repair

(**Chapter 3**) and in a regulation of the apoptotic cascade, specifically at the mitochondrial level (**Chapter 5**). The events and pathways on which the nitrogen compounds showed to act is summarised in Figure 6.1.

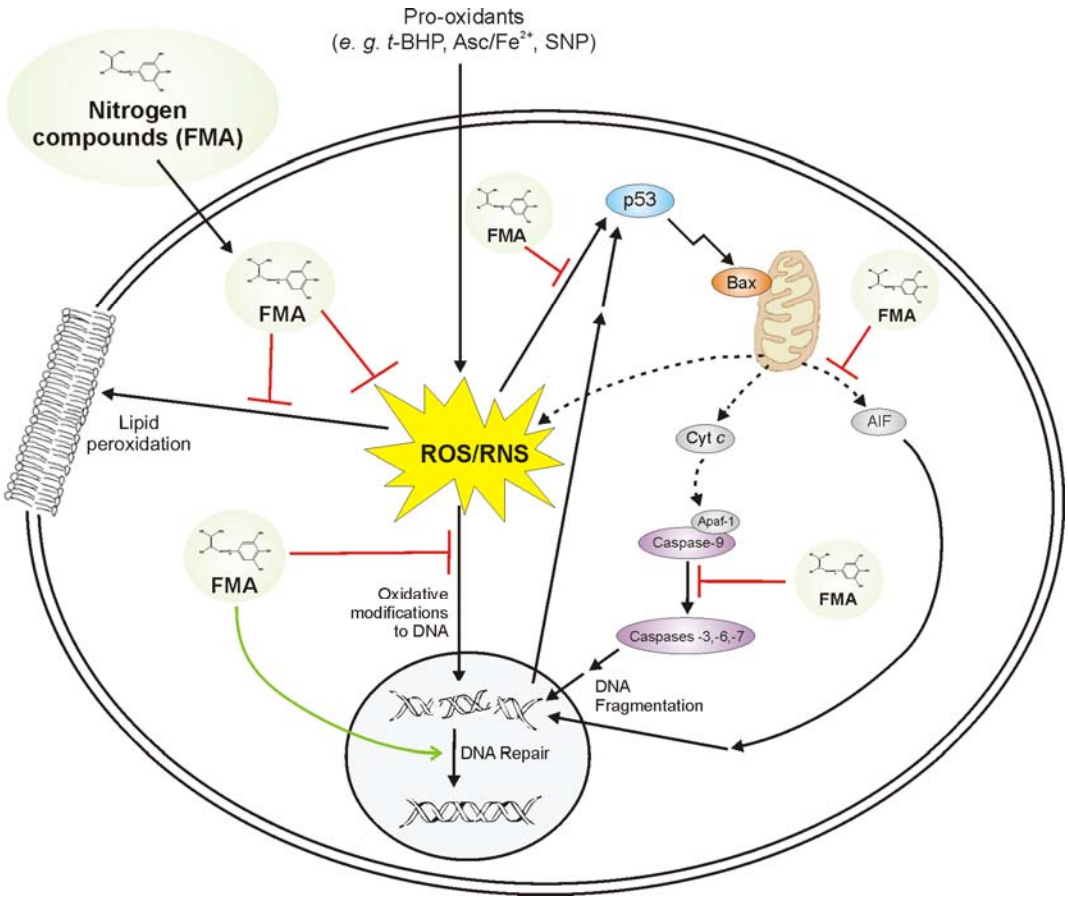


Figure 6.1 – Summary of the events and pathways on which the nitrogen compounds may be acting. These compounds, due to their lipophilicity, cross the lipid membrane and scavenge intracellular ROS and RNS derived from pro-oxidant stimuli and mitochondria (electron transport chain). Thus, they are able to prevent the oxidation of lipids and DNA. In addition, they lead to an increase in DNA repair. Moreover, the nitrogen-containing compounds act on the intracellular pathways leading to apoptotic cell death. In the presence of the compounds, occurs a decrease in the expression of p53 that results in a decreased activation of Bax and subsequently a reduction in the release of pro-apoptotic factors, such as cytochrome *c* and AIF, from the mitochondria. In this way, the compounds prevent the activation of both caspase-dependent and –independent pathways leading to apoptosis.

Moreover, these compounds showed some advantages over the use of some commonly used polyphenols, namely their lack of genotoxicity and their ability to increase the activity of DNA repair enzymes (**Chapter 4**).

The data herein presented, which can be regarded as the first step in the development of these new compounds as drugs useful for clinical practice, may represent a major scientific breakthrough, given the importance of the discovery of new molecules that may be capable of ameliorating several disorders in which oxidative stress is involved.

6.2. Other perspectives

In this work we presented novel molecules with promising antioxidant properties and showed diverse possible applications for their therapeutical use.

We have demonstrated that the novel nitrogen compounds are able to prevent oxidative injury induced to both lipids and DNA (Chapters 2 and 3). Nevertheless, their protective role against protein oxidation, which could be assessed by measuring the protein carbonyl content, should complement the protection profile of the compounds in study. An eventual role at this level would be of great relevance, since the oxidation of proteins may result in conformational changes that may lead to their full or partial inactivation, thus interfering with several cellular processes.

In Chapter 3 we demonstrated the potential of these nitrogen compounds to enhance the repair of oxidative DNA damage. However, the exact mechanisms by which they do so should be clarified, namely whether their ROS scavenging activity is the main responsible for the increase in the activity of DNA repair enzymes, or if their action on the repair of this kind of damage is rather a result of their involvement in the regulation of the expression of known and/or unknown proteins. In this regard, the effects of the compounds on the expression of proteins associated with the cells response to oxidative DNA damage, such as p53 and NF-κB, should be analysed. Furthermore, in order to evaluate their real influence on DNA repair, the nitrogen compounds could be added after damage induction.

Although we have shown that the nitrogen compounds may easily cross the lipid bilayer and act intracellularly, as indicated by their partition coefficients, it would also be interesting to determine their exact concentration once inside the cells, which could be attained by High-Performance Liquid Chromatography.

Regarding the polyphenols tested, the potential of rosmarinic acid to protect neuronal cells against oxidative DNA damage, as well as to induce its repair, can be further explored. In this way, the effect of this phenolic acid on other proteins and genes responsible for the regulation of DNA repair should be clarified, as suggested

for the nitrogen compounds. In addition, given its potential to act at this level, ways of increasing its ability to cross the blood brain barrier should be developed.

Further exploring the nitrogen compounds' involvement on the apoptotic cascade of events leading to cell death (demonstrated in Chapter 5) would allow a better understanding of their action. Calcium, an important pro-apoptotic second messenger, is mainly stored in the endoplasmic reticulum, where some members of the Bcl-2 family, like Bax and Bak are responsible for maintaining its homeostasis. Calcium release from the ER can induce the activation of cytoplasmic calpains and caspase-12, which can lead to the propagation of the apoptotic cascade of events. Intracellular calcium can also be taken up by mitochondria, resulting in a calcium overload, which triggers mitochondria-localised Bax and Bak to induce mitochondrial membrane permeabilisation and subsequently further induce the activation of downstream apoptotic pathways. In this way, the clarification of a possible effect of the compounds on ER-mediated stress could be of particular interest.

Although throughout this work we tried to elucidate the mechanisms of action of the nitrogen compounds, further studies can be performed in order to find other targets for their action. Nevertheless, we are aware that care should be taken before extrapolating the results herein obtained to *in vivo* conditions. In fact, it is known that, after administration, compounds distribute differently through the different body compartments and even undergo some structural modifications, as a consequence of biotransformation. Therefore, other studies (including *in vivo* studies), which involve the use of more complex experimental models, should be conducted, in order to evaluate the role of gastrointestinal absorption, biotransformation and blood-brain barrier permeability, in view of their future application as active principles of drugs, with pharmacological potential.

6.3. References

Areias, F.M. 2006. Novos compostos heterocíclicos de azoto com unidades fenólicas: síntese e actividade biológica. *Ph.D. Thesis*. University of Minho, Braga, Portugal. 259p. Available online at: http://hdl.handle.net/1822/5941.

Dajas, F., Rivera-Megret, F., Blasina, F., Arredondo, F., bin-Carriquiry, J.A., Costa, G., Echeverry, C., Lafon, L., Heizen, H., Ferreira, M. and Morquio, A., 2003. Neuroprotection by flavonoids. *Braz. J. Med. Biol. Res.* **36**: 1613-1620.

Duthie, S. and Dobson, V.L., 1999. Dietary flavonoids protect human colonocyte DNA from oxidative attack. *Eur. J. Nutr.* **38**: 28-34.

ESCODD - European Standard Committee on Oxidative DNA Damage, 2003. Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. *Free Radic. Biol. Med.* **34:** 1089-1099.

Fishel, M.L., Vasko, M.R. and Kelley, M.R., 2007. DNA repair in neurons: So if they don't divide what's to repair? *Mutat. Res.* **614**: 24-36.

Haidara, K., Morel, I., Abaléa, V., Barré, M.G. and Denizeau, F., 2002. Mechanism of *tert*-butylhydroperoxide induced apoptosis in rat hepatocytes: involvement of mitochondria and endoplasmic reticulum. *Biochim. Biophys. Acta* **1542**: 173-185.

Halliwell, B., 2001. Role of free radicals in the neurodegenerative diseases - Therapeutic implications for antioxidant treatment. *Drugs & Aging* **18**: 685-716.

Harris, G.K., Qian, Y., Leonard, S.S., Sbarra, D.C. and Shi, X.L., 2006. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E-2 formation in RAW 264.7 cells. *J. Nutr.* **136**: 1517-1521.

Jung, J.Y., Han, C.R., Jeong, Y.J., Kim, H.J., Lim, H.S., Lee, K.H., Park, H.O., Oh, W.M., Kim, S.H. and Kim, W.J., 2007. Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. *Neurosci. Lett.* **411**: 222-227.

Kohen, R. and Nyska, A., 2002. Invited Review: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol. Pathol.* **30**: 620-650.

L'Ecuyer, T., Horenstein, M.S., Thomas, R. and Heide, R.V., 2001. Anthracycline-induced cardiac injury using a cardiac cell line: Potential for Gene Therapy Studies. *Mol. Gen. Metabol.* **74**: 370-379.

Le Page, F., Klungland, A., Barnes, D.E., Sarasin, A. and Boiteux, S., 2000. Transcription coupled repair of 8-oxoguanine in murine cells: The Ogg1 protein is required for repair in nontranscribed sequences but not in transcribed sequences. *Proc. Natl. Acad. Sci. U. S. A.* **97**: 8397-8402.

Lebeau, J., Furman, C., Bernier, J.L., Duriez, P., Teissier, E. and Cotelle, N., 2000. Antioxidant properties of di-tert-butylhydroxylated flavonoids. *Free Radic. Biol. Med.* **29**: 900-912.

Masella, R., Di Benedetto, R., Vari, R., Filesi, C. and Giovannini, C., 2005. Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes. *J. Nutr. Biochem.* **16**: 577-586.

Nordberg, J. and Arner, E.S., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* **31**: 1287-1312.

Pereira, C., Santos, M.S. and Oliveira, C., 1999. Involvement of oxidative stress on the impairment of energy metabolism induced by Ab peptides on PC12 Cells: protection by antioxidants. *Neurobiol. Dis.* **6**: 209-219.

Piga, R., Saito, Y., Chen, Z., Yoshida, Y. and Niki, E., 2005. Characterization of monochloramine toxicity on PC12 cells and protective effect of tocopherol via antioxidative function. *Arch. Biochem. Biophys.* **436**: 101-109.

Rolseth, V., Rundén-Pran, E., Luna, L., McMurray, C., Bjorás, M. and Ottersen, O.P., 2008. Widespread distribution of DNA glycosylases removing oxidative DNA lesions in human and rodent brains. *DNA Repair* **7**: 1578-1588.

Sies, H., 1997. Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* **82**: 291-295.

Silva, J.P., Areias, F.M., Proença, M.F. and Coutinho, O.P., 2006. Oxidative stress protection by newly synthesized nitrogen compounds with pharmacological potential. *Life Sci.* **78**: 1256-1267.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39**: 44-84.

Yokozawa, T., Chen, C.P., Dong, E., Tanaka, T., Nonaka, G.I. and Nishioka, I., 1998. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2 picrylhydrazyl radical. *Biochem. Pharmacol.* **56**: 213-222.