Original Article

Di(hetero)arylamines in the benzo[*b*]thiophene series as novel potent antioxidants

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ABSTRACT: The damaging consequences of oxidative stress are known to be involved in several pathologies. So, the development of new drugs that can aid cells to cope with excessive levels of free radicals still assumes great relevance. Here, we investigated the antioxidant properties of four novel di(hetero)arylamines (named MJQ1, MJQ3, MJQ4 and MJQ5), sharing a common benzo[*b*]thiophene nucleus (an indole analogue), against oxidative damage induced to H9c2 myoblasts. Cell proliferation, evaluated by the sulforhodamine B assay, was not compromised by the presence of any of these compounds for concentrations below 50 µM (at 24 h) and 1 µM (72 h). Moreover, all of them showed a dose-dependent protective effect against *tert*-butylhydroperoxide (*t*-BHP)-induced cell death for concentrations in the nanomolar range. Their ability to scavenge free radicals seems to account for their protective effects, as they were able to prevent almost completely, at 25 nM, *t*-BHP-induced intracellular ROS formation, assessed by DCF fluorescence. Furthermore, their relatively high partition coefficient values are indicative of their ability to easily permeate lipid membranes and act intracellularly. Additionally, these novel diarylamines led to a reduction, between 60-70%, of the amount of DNA strand breaks induced by *t*-BHP, evaluated by the Comet assay, and lipid peroxidation (TBARS assay) induced by the oxidant pair ascorbate/iron. In all these parameters, which show their ability to prevent the oxidation of the main biomolecules, their protective role was superior to the traditional antioxidant Trolox. Although the mechanisms underlying the action of these diarylamines are currently under investigation, the data obtained so far reveals their high pharmacological potential as antioxidant molecules.

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Keywords: Di(hetero)arylamines, benzo[*b*]thiophene derivatives, antioxidants, cardiac oxidative stress, H9c2 myoblasts

1. Introduction

Reactive oxygen (ROS) and nitrogen (RNS) species can accumulate intracellularly as a result of both exogenous and/or endogenous factors (*1*). In normal physiological conditions, these species are maintained in equilibrium by the cells' antioxidant defence systems and are known to play important roles in the regulation of physiological functions (*2,3*). However, an imbalance in the equilibrium, favouring the oxidants, can result in a situation defined as oxidative stress (*4,5*), which causes damage to all biomolecules, including DNA, lipids and proteins, and is ultimately involved in the regulation of mechanisms leading to cell death (*5*). In addition, it is often implicated in the etiology of several pathologies, such as atherosclerosis (*6*), neurodegenerative diseases (*7,8*) and ischemia-reperfusion injury (*9*).

Cells possess effective enzymatic (*e.g.* superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (*e.g.* glutathione, thioredoxin, coenzyme Q) antioxidant systems to help them cope with oxidative stress (*10*). Also, several compounds present in plants and vegetables (*e.g.* vitamins C and E, polyphenols) have been suggested to have the ability to react with free radicals (*11*), protecting cells from damage. However, there are some drawbacks associated to these natural compounds, as some reports describe pro-oxidant effects for many of them, which may eventually result in cell death too (*12-14*). In this regard, the undergoing development of novel synthetic compounds with antioxidant activity that may help the endogenous defence system, assumes special relevance (*15*).

Secondary amines, in particular diarylamines, are regarded as important molecules that can aid in the antioxidant protection, since their reducing properties

make them suitable as good radical scavengers, able to react either with O-centered or C-centered radicals (*16,17*).

Esteves and collaborators (*16*) evaluated the redox properties and free radical scavenging activity of some diarylamines by cyclic voltammetry and the DPPH radical reduction assay, respectively. They observed that the compounds with a *p*-methoxyphenyl moiety in their structures presented lower oxidation potentials and higher radical scavenging activity. In another study (*18*), a high antioxidant activity was also demonstrated for diarylamines in the benzo[*b*]thiophene series, which proved to be even better antioxidant molecules than the well-known synthetic standards butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). These results were the first indication of the potential of diarylamines in the benzo[*b*]thiophene series to be used as antioxidants in biological systems.

Later on, Queiroz and co-workers (*19*) reported the synthesis and evaluation of the antioxidant properties of 7-aryl or 7-heteroarylamino-2,3-dimethylbenzo[*b*]thiophenes, by assessing their free radical scavenging activity and reducing power, establishing some structure-activity relationships based on the presence and position of different substituents (1 or 2 OMe and a nitrile group) on the phenyl ring, or on the presence of a pyridine ring and on the position of its nitrogen atom relative to the N-H bond. In a more recent study (*20*), cyclic voltammetry was used to evaluate the antioxidant activity of those molecules (7-aryl or 7-heteroarylamino-2,3-dimethylbenzo[*b*]thiophenes), while comparing their oxidation potentials with those of the classical antioxidants BHA and BHT, showing the importance of the presence of the methoxylated arylamine moiety to the antioxidant properties of the molecule compared with the presence of a pyridine ring. By its turn, it was also shown that the presence of electron-donating groups (*e.g.* methoxy) on the arylamine moiety showed lower oxidation potential than compounds with electron-withdrawing groups. The position of the methoxy group on the arylamine moiety also seemed to affect the oxidation potential, being that a methoxy group in the *para* position relative to the NH group, or two methoxy groups, one in the *para* and other in *meta* position, increased the antioxidant activity in comparison with molecules containing a single methoxy group in the *meta* position.

In this study, we evaluated the antioxidant potential of four di(hetero)arylamines in the benzo[*b*]thiophene series (named MJQ1, MJQ3, MJQ4 and MJQ5), which differ in the number of methoxy (OCH_3) and methyl (CH_3) groups present within their structures (Figure 1). Three of them are entirely new synthetic molecules not tested in biological systems. For MJQ1 we have previously reported good antioxidant properties, particularly at mitochondria level (*21*). In the same study we have tested another benzo[*b*]thiophene derivative, aminated in the thiophene ring with a pyridine (MJQ2), which did not show to be as good as antioxidant.

So in the present work we focused on the MJQ1 analogues. We intended to address their protective role against oxidative injury induced by pro-oxidant stimuli on a cardiomyocyte model, the H9c2 cell line, since cardiac cells are recognized to be highly susceptible to this kind of damage (*22*).

2. Materials and Methods

2.1. *Chemistry*

The compounds in study, named MJQ1, MJQ3, MJQ4 and MJQ5 were synthesized by palladium-catalyzed C-N Buchwald-Hartwig coupling of 6-bromotri or tetra methylbenzo[*b*]thiophenes with methoxylated anilines (*23*) in the Center of Chemistry of the University of Minho (Braga, Portugal). They all share a common benzo[*b*]thiophene nucleus and differ mainly in the number of methoxy (OCH_3) and methyl (CH_3) groups present within their structures. The synthesis and chemical characterization of the compounds MJQ1 and MJQ5 have been previously published (*21,23*). For the other two compounds (MJQ3 and MJQ4) the synthesis procedure is described below. The dry powders obtained were reconstituted in DMSO, aliquoted and maintained at –20°C, protected from light. After thawed each aliquot was used only once.

2.2. *General methods of synthesis*

Melting points (°C) were determined in a Stuart SMP3 apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Plus (300 and 75.4 MHz, respectively) or an Avance III Bruker (400 MHz and 100.6 MHz, respectively). Chemical shifts are given in ppm and coupling constants in Hz. The mass spectra were obtained by electronic impact

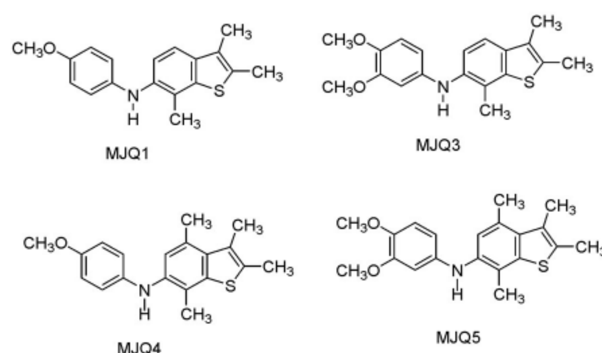

Figure 1. Schematic structure of the di(hetero)arylamines studied MJQ1, MJQ3, MJQ4, and MJQ5. They all possess a tri or tetramethylated benzo[*b*]thiophene moiety and differ mainly in the number of methoxy (OCH_3) and methyl (CH_3) groups present within their structures.

unless stated in the mass spectrometry external service of the University of Vigo (Spain). Elemental analysis was performed on a LECO CHNS 932 elemental analyser. The reactions were monitored by thin layer chromatography (TLC). Column chromatography was performed on Macherey-Nagel silica gel 230-400 mesh. Petroleum ether refers to the boiling range 40-60°C. Ether refers to diethylether. When solvent gradient was used, the increase of polarity was made gradually from petroleum ether to mixtures of ether/petroleum ether increasing 10% of ether until the isolation of the product.

A dry Schlenk tube was charged, under Argon, with dry toluene (3-4 mL), the benzo[*b*]thiophene, $Pd(OAc)_2$ (3 mol%), *rac*-BINAP (4 mol%), Cs_2CO_3 (1.4 equiv.), the methoxyaniline, and the mixture was heated at 100°C for several hours. After cooling, ether was added and the mixture was filtered under vacuum. The filtrate was evaporated under reduced pressure to give a residue which was submitted to column chromatography using solvent gradient from neat petroleum ether to mixtures of diethyl ether/petroleum ether, increasing 10% of ether till the isolation of the product, unless stated otherwise.

2.2.1. *6-(3,4-Dimethoxyanilino)-2,3,7-trimethylbenzo [b]thiophene (**MJQ3**)*

From 6-bromo-2,3,7-trimethylbenzo[*b*]thiophene (150 mg, 0.590 mmol), 3,4-dimethoxyaniline (95.0 mg, 0.649 mmol), heating for 7 h, and using solvent gradient from neat petroleum ether to 20% ether/petroleum ether in the column chromatography, compound **MJQ3** was obtained as a white solid (150 mg, 78%), m.p. 119-121°C. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 2.21 (3H, s, Me), 2.29 (3H, s, Me), 2.41 (3H, s, Me), 2.56 (3H, s, Me), 3.66 (3H, s, OMe), 3.67 (3H, s, OMe), 6.33 (1H, dd, *J* = 8.6 and 2.4 Hz, 6'-H), 6.56 (1H, d, *J* = 2.4 Hz, 2'-H), 6.77 (1H, d, *J* = 8.4 Hz, 5'-H), 7.15 (1H, *J* = 8.4 Hz, Ar-H), 7.27 (1H, s, N-H), 7.35 (1H, d, *J* = 8.4 Hz, Ar-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-*d6*): δ 11.17 (Me), 13.41 (Me), 15.98 (Me), 55.24 (OMe), 56.23 (OMe), 102.37 (CH), 107.51 (CH), 113.60 (CH), 117.60 (CH), 119.10 (CH), 121.28 (C), 127.47 (C), 130.43 (C), 135.19 (C), 138.00 (C), 139.23 (C), 139.76 (C), 142.23 (C), 149.59 (C). MS (EI) m/z (%) 327 (M+, 82), 312 (100). HRMS M+ calct. for $C_{19}H_{21}NO_2S$ 327.1293, found 327.1297.

2.2.2. *6-(4-Methoxyanilino)-2,3,4,7-tetramethylbenzo [b]thiophene (**MJQ4**)*

From 6-bromo-2,3,4,7-tetramethylbenzo[*b*]thiophene (150 mg, 0.730 mmol), 4-methoxyaniline (99 mg, 0.803 mmol), heating for 7 h, and using solvent gradient from neat petroleum ether to 20% ether/petroleum ether in the column chromatography, compound **2a** was obtained as a white solid (102 mg, 45%), m.p. 136-138°C. ^{1}H NMR (300 MHz, DMSO-*d6*): δ 2.20 (3H, s, Me), 2.36 (3H, s, Me), 2.40 (3H, s, Me), 2.56 (3H, s, Me), 3.36 (3H, s, OMe), 6.79 (coalesced ABq, 2', 3', 5' and 6'-H), 6.80 (1H, s, 5-H), 7.18 (1H, s, N-H) ppm. ^{13}C NMR (75.4 MHz, DMSO-*d6*): δ 13.70 (Me), 14.95 (Me), 15.56 (Me), 21.10 (Me), 55.29 (OMe), 114.54 (CH), 118.09 (CH), 118.38 (C), 120.13 (CH), 129.04 (C), 129.53 (C), 130.44 (C), 133.30 (C), 137.62 (C), 138.98 (C), 140.02 (C), 152.81 (C). Anal. Calcd for $C_{19}H_{21}NOS$: C 73.28, H 6.80, N 4.50, S 10.29; found: C 73.27, H 6.89, N 5.53, S 10.14.

2.3. *Other chemicals*

Fetal bovine serum (FBS) was obtained from BioChrom KG (Berlin, Germany); 6-hydroxi-2,5,7,8-tetrametilcromano-2-carboxylic acid 97% (Trolox), Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium, dimethyl sulfoxide (DMSO), EDTA, trypsin, *tert*-butylhydroperoxide and sulforhodamine B were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was obtained from Invitrogen (Eugene, OR, USA).

2.4. *H9c2 cell culture*

H9c2 cell line was originally derived from embryonic rat heart tissue using selective serial passages (*24*). It was originally purchased from America Tissue Type Collection (Manassas, VA, USA). This cell line has been used extensively in the literature as a model for cardiomyoblasts and has also been considered as a proper model to study molecular responses of the cardiomyocyte to oxidative damage (*25,26*). 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. To prevent loss of differentiation potential, cells were not allowed to become confluent. So, they were fed every 2-3 days, and sub-cultured once they reached 70-80% confluence, by treatment with a 0.05% trypsin/EDTA solution. Cells were seeded at a density of 35,000 cells/mL, either in 24-well plates (final volume of 1 mL/well) for sulforhodamine B assays, or 6-well plates (final volume of 2 mL medium/well) for other assays. To detect intracellular ROS, cells were seeded in coverslips placed in the bottom of 6-well plates.

2.5. *Sulforhodamine 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, as previously described (*27*). Briefly, H9c2 cells were seeded in 24-well plates and incubated with the diarylamines for different time points, in the presence or absence of *tert*-butylhydroperoxide (*t*-BHP). Following this treatment, cells were fixed in ice cold methanol, containing 1% acetic acid, for at least 1 h, and then incubated with 0.5% (w/v) sulforhodamine B dissolved in 1% acetic acid for 1 h at 37°C. 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 dissolving the SRB through agitation of the plates, 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.

2.6. *Detection of intracellular reactive oxygen species*

Intracellular ROS formation was assessed by measuring the fluorescence of DCF, the oxidation product of the non-fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), according to a method previously described (*27*), with slight modifications.

Briefly, cells plated in coverslips placed in the bottom of 6-well plates were treated with *t*-BHP and the diarylamines for 3 h and then incubated with 10 µM $DCFH_2$-DA for 30 min, at 37°C, in the dark. Cells were then observed by fluorescence microscopy using a fluorescein filter in a Leica DM 5000B microscope. The intracellular mean fluorescence intensity was quantified using the ImageJ 1.40g software (National Institutes of Health, USA).

2.7. *Single cell gel electrophoresis (Comet assay)*

Protection against oxidative damage to DNA was assessed by the Comet assay, as previously described (*28*). In brief, cells plated at a density of 5 × 10^5 cells/well were treated with 50 µM *t*-BHP and the diarylamines (at 10 and 25 nM) for 3 h to induce the formation of strand breaks. After treatment with the compounds, cells were trypsinised, spread on agarose-coated slides and lysed. The DNA that remained embedded in the agarose was subjected to an electrophoresis in alkaline conditions, for 20 min at 1 V/cm. The electric field 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 with 0.4 M Tris, pH 7.5 and fixed with absolute ethanol. Comets were stained with ethidium bromide and analysed under a fluorescence microscope. Comet quantification was performed by visual scoring. This method, which correlates well with computer-assisted image analysis of the % of DNA in the comets' tail (*28*), is based in the classification of comets into one of five classes of damage (from 0 to 4) in 100 nucleoids, giving a score range between 0 and 400.

2.8. *Measurement of lipid peroxidation – TBARS assay*

The extent of lipid peroxidation was determined by measuring the levels of Thiobarbituric Acid-Reactive Substances (TBARS), as described in a previous publication (*29*). Cells were plated at a density of 1.7 × 10^6 cells/well in 6-well plates and TBARS formation was induced by the oxidant pair 2 mM ascorbate/100 µM iron (II) for 1 h, at 37ºC. The amount of TBARS produced was calculated using the molar absorption coefficient of 1.56 × 10^5 $M{\bullet}cm^{-1}$, normalized for the total protein content and expressed as nmol TBARS/mg protein. The protection of the diarylamines against lipid peroxidation was determined according to the following formula:

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

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

2.9. *Partition coefficients*

The diarylamines degree of hydrophobicity was determined by measuring the partition coefficients (PC) in an *n*-octanol/HEPES system, as previously described (*29*). The diarylamines 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 10 min, at room temperature. The two different phases formed were then separated by centrifugation. The absorbance peaks of each compound, required to assess their concentrations in each solution, were determined at 305 nm for MJQ1, 307 nm for MJQ3 and 308 nm for both MJQ4 and MJQ5. PC values were then calculated using the formula:

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

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

2.10. *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 $p < 0.05$ was considered significant.

3. Results

3.1. *Effects on toxicity and cell proliferation*

The effects of the diarylamines on toxicity and cell proliferation were assessed by the sulforhodamine B assay. The compounds were tested at a range of concentrations between 1 and 50 μM, during a period of 72 h.

In Figure 2, it is possible to observe that none of the compounds is toxic to H9c2 cells at any of the concentrations tested, since the percentage of proliferation does not decrease below the levels measured at time zero (0 h). However, some effects on cell proliferation could be observed, which varied according to the compound tested. In this way, MJQ5 was the one that less affected this parameter, since that only after 48 h, and for the highest concentration (50 μM), cell proliferation was reduced. By its turn at 72 h incubation with this diarylamine, only concentrations of 10 μM and above affected cell proliferation. At that same concentration a decrease in cell proliferation could be observed earlier (at 24 h), for all other compounds. At 48 h, MJQ4 showed some deleterious effects for a 25 μM concentration, while MJQ1 and MJQ3 revealed to affect proliferation in a statistically significant way for lower concentrations, namely 1 and 5 μM, respectively. After 72 h of cell incubation in the presence of MJQ1, MJQ3, and MJQ4, proliferation was also significantly affected for the lower concentrations tested (1 μM for MJQ1 and MJQ4, and 5 μM for MJQ3).

To determine if the diarylamines were able to afford protection against cell death induced by an oxidant stimulus, H9c2 cells were incubated for 6 h in the presence of 50 μM *t*-BHP and different concentrations of the diarylamines. Cell viability was then evaluated by the SRB assay. The thiol-oxidizing agent *t*-BHP is metabolized intracellularly, leading to the formation of *tert*-butoxyl radicals, being commonly used as an inducer of oxidative stress in several cell models (*30,31*).

As shown in Figure 3, *t*-BHP induces a decrease of 55.7% in cell viability, which is prevented by all the diarylamines, in a dose-dependent manner, for concentrations at the nanomolar range. This protection was statistically significant at 10 nM and above for MJQ3, MJQ4, and MJQ5. For MJQ1 the protective effect was only significant for concentrations equal or superior to 25 nM. At this concentration (25 nM) all the compounds showed about 100% protection. It should be noted that the concentrations at which the compounds

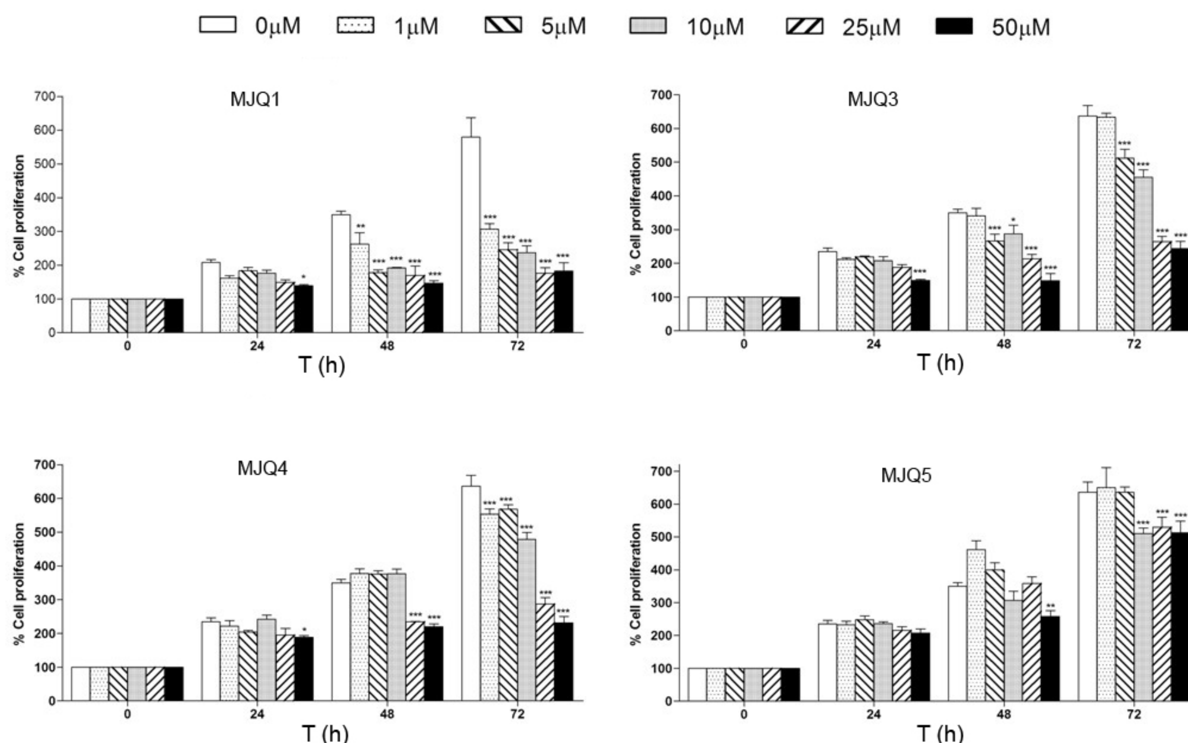

Figure 2. Cellular proliferation assessed with the sulforhodamine B assay. H9c2 cells proliferation was followed up to 72 h, in the presence of different compounds concentrations, as indicated. The percentage of cellular proliferation was calculated relatively to t = 0 h. Proliferation in control cells (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 shown and the respective controls for each time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relatively to control cells (white bars) at respective time period.

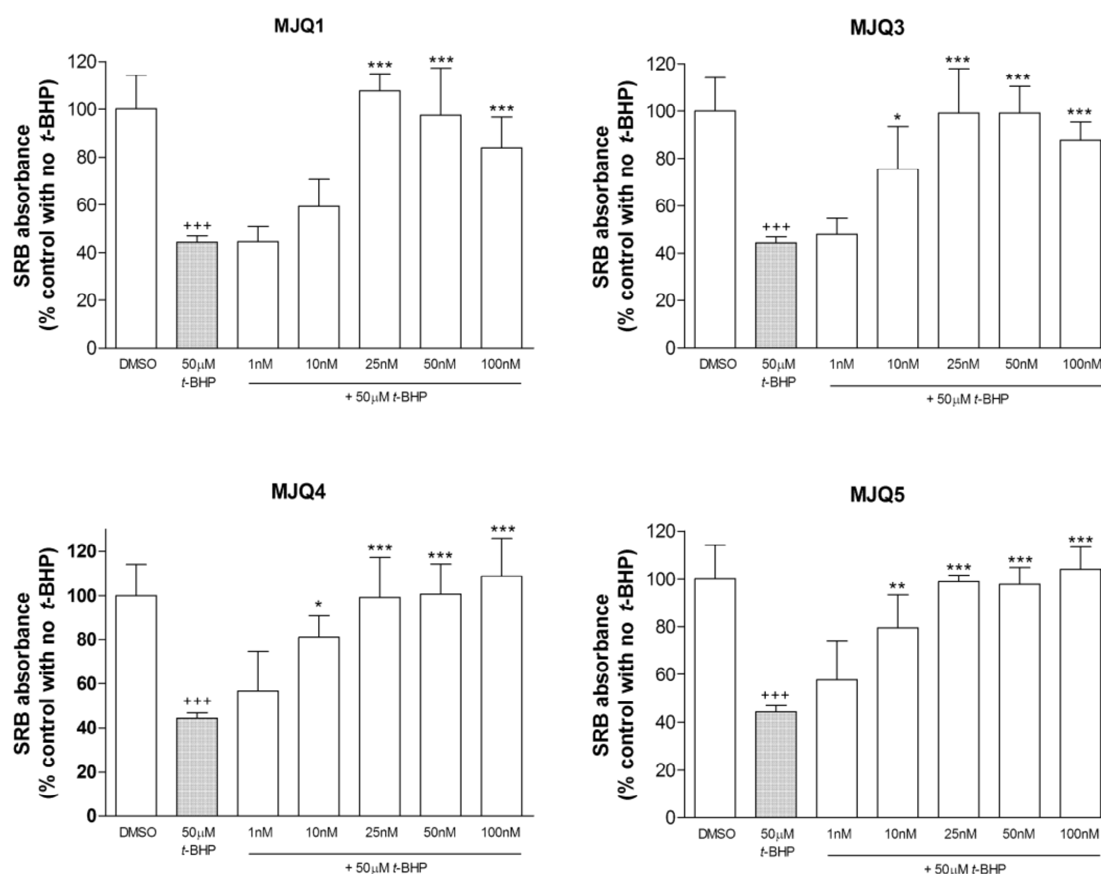

Figure 3. Effects of the diarylamines on the reduction of cell viability evaluated by the sulforhodamine B assay. H9c2 cells were incubated for 6 h in the presence of the compounds and 50 µM *t*-BHP. Results are presented in terms of percentage of cell viability, determined relatively to the control 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. $^{+++}p < 0.001$, compared to control cells (DMSO); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared to 50 µM *t*-BHP.

showed to protect against cell death were far below the ones that affected cell proliferation.

These results show the elevated potential of these novel diarylamines to protect cells against oxidative stress-induced cell death.

3.2. *Scavenging of intracellular reactive oxygen species*

The effect of the compounds on the intracellular formation of reactive oxygen species was determined by fluorescence microscopy, through the detection of the oxidized form (DCF) of dichlorofluorescein probe. Oxidative stress was induced by incubating cells with 50 µM *t*-BHP for 3 h, and the diarylamines (at 10 and 25 nM) added simultaneously with the pro-oxidant.

Incubation with the pro-oxidant alone evidenced an increase in the cell mean fluorescence (Figure 4) corresponding to ROS formation. However, in the presence of all the compounds tested, the amount of those reactive species was greatly decreased, to values approximate to the control. Both concentrations assayed (10 and 25 nM) showed the same levels of protection. In addition, the novel compounds proved to have a higher protective effect than the classical antioxidant Trolox, regardless of the concentration used.

In this way, these results indicate that the protective role of the new diarylamines against *t*-BHP-induced cell death may be somehow related with their ROS scavenging activity.

3.3. *Protection against oxidative DNA damage*

Once formed, reactive oxygen species may interact with biomolecules, resulting in damaging consequences to all of them (*32*). In particular, the attack of ROS to DNA may lead to several oxidative modifications, including strand breaks formation. This appearance of strand breaks was evaluated by the Comet assay, after treating cells in the same conditions used to assess the compounds' protection against intracellular ROS formation.

As depicted in Figure 5, cells treated with the pro-oxidant stimulus showed an increase in the levels of strand breaks formation. However, a significant reduction of about 60-70% in the extent of DNA damage was observed in the presence of

all the diarylamines, when added to cells at 25 nM concentration. This protective effect was also noticed for a lower concentration (10 nM), although in this case the reduction in the amount of damage was slightly lower. Nevertheless, at both concentrations, the protection of diarylamines against this kind of oxidative damage was higher than the one observed in cells treated with Trolox. Moreover, it should be noted that the compounds, by themselves, do not induce the formation of strand breaks, as observed in cells treated with the compounds alone.

Therefore, these results indicate the elevated potential of the novel molecules in study to protect cardiomyocytes against oxidative DNA damage.

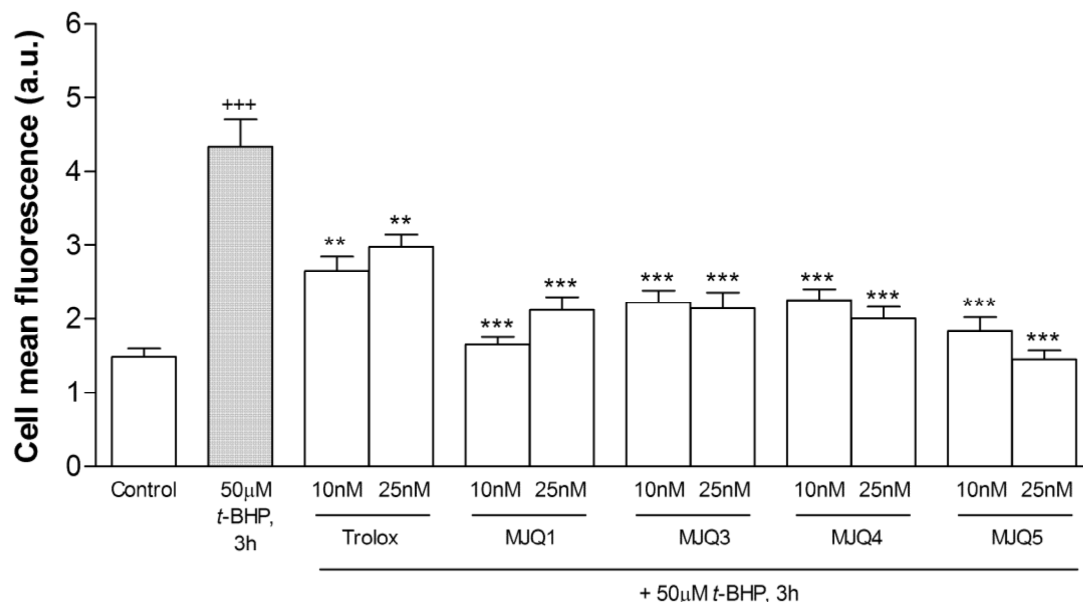

Figure 4. Effects of the compounds on *t*-BHP-induced intracellular ROS formation. H9c2 cells were incubated in the presence of *t*-BHP and the diarylamines for 3 h. Increase in intracellular oxidative stress was detected by oxidation of the fluorescent probe dichlorofluorescein (DCF) by fluorescence microscopy. 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. $^{+++}p < 0.001$, compared with control; $^{**}p < 0.01$, $^{***}p < 0.001$, relatively to *t*-BHP.

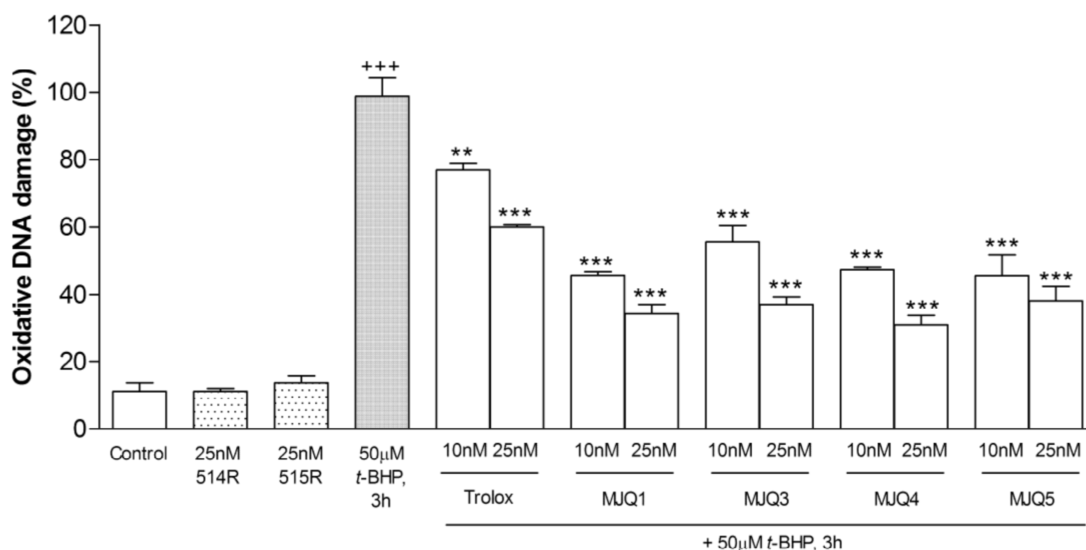

Figure 5. Diarylamines' protection against *t*-BHP-induced formation of DNA strand breaks, evaluated by the Comet assay. Oxidative DNA damage was induced by incubating H9c2 cells for 3 h in the presence of 50 μM *t*-BHP. The compounds were added to the cells simultaneously with the pro-oxidant stimulus. Each bar represents the mean ± S.E.M. for at least three independent experiments. $^{+++}p < 0.001$, compared to control cells; $^{**}p < 0.01$, $^{***}p < 0.001$, compared to 50 μM *t*-BHP.

3.4. *Effects of the compounds on lipid peroxidation*

Membrane lipid peroxidation can also occur as a consequence of oxidative stress. In fact, polyunsaturated fatty acids, because of their multiple double bonds, are extremely sensitive to oxidation by free radicals (*33*).

The oxidant pair ascorbate/iron(II), which has been previously shown to induce high levels of lipid peroxidation in other cell models (*34,35*) was chosen to induce this kind of damage in this cardiomyocyte cell model. Ascorbate acts as a pro-oxidant agent, by reducing Fe^{3+} to Fe^{2+}, which then leads to the formation of hydroxyl radicals *via* the Fenton reaction, thereby causing the oxidation of membrane lipids (*3,36*).

Therefore, after incubating cells with the oxidant pair for 1 h, we observed an increase in lipid peroxidation, indicated by 4-fold increase in the levels of TBARS (Figure 6). These high levels were significantly reduced by simultaneous treatment with the diarylamines at 25 nM, which is indicative of their ability to prevent the oxidation of membrane lipids, once again for a low concentration. In particular, MJQ3 presented a slightly higher percentage of protection (86.95 ± 8.23%) when compared with the other three diarylamines (Figure 6, **Insert**). The high protection observed for MJQ1 is in agreement with the one previously observed in a neuronal cell model and in isolated mitochondria by using a similar deleterious agent (*21*). Again the protective effects observed revealed to be quite superior to the one observed for Trolox.

3.5. *Determination of the compounds' liposolubility*

The partition coefficient (PC) is a measure of the relative lipophilic/hydrophobic behaviour of a chemical and has been commonly associated to a drug's biological activity (*37*). Therefore, the degree of hydrophobicity of the diarylamines was measured by determining this parameter in an *n*-octanol/HEPES system.

As shown in Table 1, all the diarylamines show high partition coefficient values, which is indicative of their high ability to cross lipid membranes and act intracellularly. Among the tested diarylamines, MJQ3 showed the highest affinity for the octanol phase (highest PC values), followed by MJQ4 and MJQ5, with similar values, and finally by MJQ1. In general, the values here obtained were higher than the ones presented by other compounds tested in our lab (*34*) and were similar (and, in the case of MJQ3, even higher) to some natural compounds tested by others in the same octanol/HEPES system (*38*), thus confirming a superior

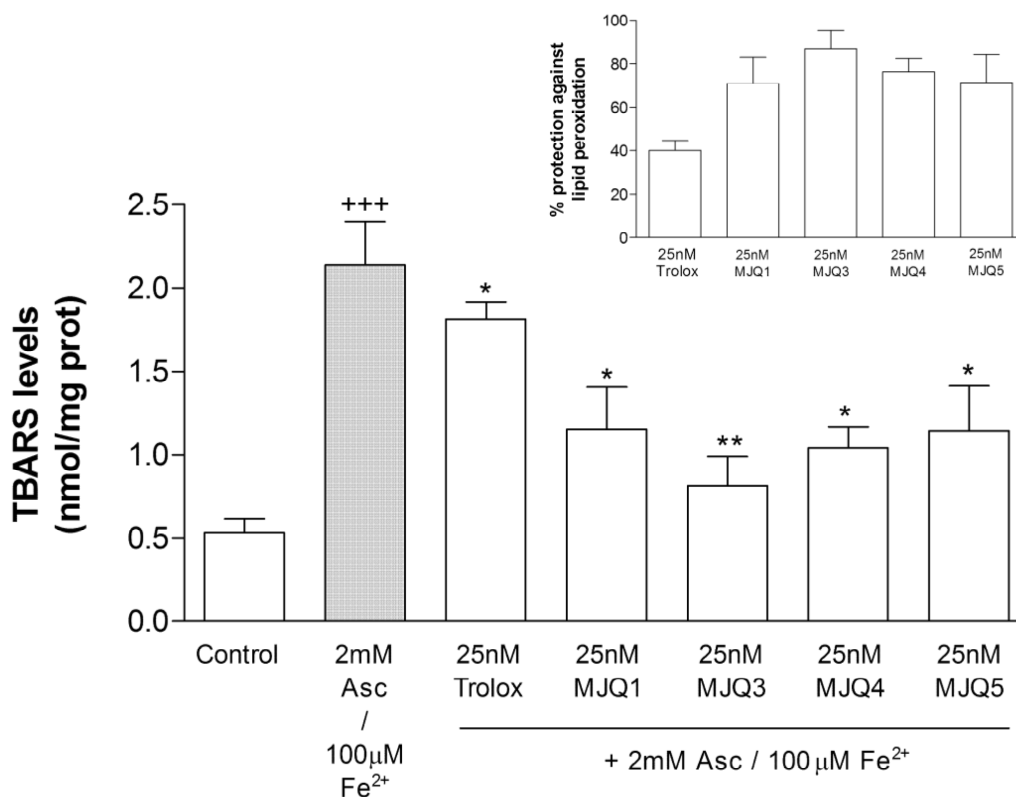

Figure 6. Relative drug protection against lipid peroxidation. Cells were incubated for 1 h with the oxidant pair 2 mM ascorbate/100 µM Fe^{2+}. The diarylamines were added to the cells at the same time as the oxidant pair. Each column represents the mean ± S.E.M., considering the results obtained for at least three different experiments. $^{+++}p < 0.001$, compared to control cells; $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the oxidant stimulus. **Insert**: Percentage of protection relatively to the controls as described in "Material and Methods".

Table 1. Partition coefficients (PC) of the diarylamines

Compound	PC values
MJQ1	1.87 ± 0.06
MJQ3	2.95 ± 0.40
MJQ4	2.14 ± 0.32
MJQ5	2.10 ± 0.23

PC values were measured in an *n*-octanol/HEPES system as described in "Materials and Methods". A higher PC value means higher lipossoluble profile. Results represent the mean ± S.E.M. for at least three independent experiments.

ability of diarylamines to cross the lipid bilayer and to act inside cells.

4. Discussion

A redox imbalance in the cell can cause oxidative damage to all types of biomolecules and even lead to cell death as an ultimate consequence. In this way, oxidative stress is commonly associated to the etiology of several pathological conditions (*2*).

This work comprises a small part of a broader project aiming the development and/or improvement of new synthetic molecules with the ability to scavenge free radicals, acting as antioxidants, and thus preventing oxidative stress. Although the four diarylamines herein tested share the same benzo[*b*]thiophene nucleus, they exhibit some structural differences. In particular, they differ in the number of methoxy (one in MJQ1 and MJQ4, and two in MJQ3 and MJQ5, in the same positions) and methyl groups (three in MJQ1 and MJQ3, and four in MJQ4 and MJQ5, in the same positions). It should be noted that besides being able to scavenge a free radical by donating one $H^{\cdot}$, an antioxidant should also be able of stabilizing the unpaired electron that is formed in its own molecule as a consequence of the radical scavenging activity. With the introduction of these groups, namely the methoxy ones, it was expected a greater stabilization of the radical formed after scavenging of the free radical.

In this study, we evaluated the antioxidant potential of these four diarylamines, by addressing their protective role against oxidative injury on a cardiomyocyte model, the H9c2 cell line, since cardiac cells have for long been recognized to be highly susceptible to this kind of damage.

As a first approach, we observed that none of the diarylamines was toxic to the cells. However, for high concentrations (in the micromolar range), they interfered with the process of cell proliferation to some extent, especially after a 48 h incubation period. Nevertheless, it should be taken into account that they present protection at concentrations far below the ones that affected cell proliferation, and for shorter incubation time periods. In fact, they were able to revert cell death induced by *t*-BHP at concentrations in the nanomolar range, in a dose-dependent manner.

The thiol-oxidizing agent *t*-BHP is known to be metabolized intracellularly, where it generates *tert*-butoxyl radicals (*31*). In this way, *t*-BHP-induced cell death is associated to an increase in the levels of oxidative stress, as it has also been reported in H9c2 cells (*27*). Therefore, we determined the protective role of the diarylamines against the intracellular increase of reactive oxygen species, induced by that agent, by analysing the changes in DCF fluorescence. We observed that all the diarylamines led to a reduction in the levels of intracellular ROS formation at very low concentrations (10 and 25 nM), an effect that seems to correlate well with the protection observed against *t*-BHP-induced cell death. Therefore, these results confirm the diarylamines' elevated antioxidant activity and suggest that their ROS scavenging ability may indeed result in a decrease in cell death induced by high levels of oxidative stress.

As previously stated, the presence of excessive levels of reactive oxygen species inside the cells is known to also cause damage to all main biomolecules. In this way, we determined if the diarylamines were able to prevent ROS-induced oxidative modifications to both DNA and membrane lipids. Indeed, the amount of DNA strand breaks induced by the pro-oxidant *t*-BHP was significantly reduced in the presence of the compounds. Since cardiac cells are post-replicative, oxidative damage to these cells' DNA could result in the deterioration of cardiac function (*39*). Therefore, the protection observed for the diarylamines on oxidative DNA damage in this myoblast cell line is of great relevance.

In addition, the incubation of the cells with the diarylamines led to a reduction in the levels of lipid peroxidation induced by the oxidant pair ascorbate/iron(II). This oxidant pair, which generates free radicals *via* the Fenton reaction, was chosen since it has been commonly used in other cell models, like PC12 (*34*) and retinal (*35*) cells, and because it induced higher levels of TBARS levels, in comparison with *t*-BHP (data not shown). It should be noted that, although all the diarylamines show similar protective effects at this level, MJQ3 percentage of protection was slightly higher than the ones observed for the other related compounds. This may be explained, at least partly, by its higher partition coefficient value, which confers a more liposoluble profile to this compound, in comparison with the other ones, thus allowing it to better cross lipid membranes and act on this kind of damage.

In fact, the partition coefficients obtained for the diarylamines are indicative of their high liposolubility, which enables them to permeate cells, contributing to their intracellular action. In terms of liposolubility, these diarylamines resembled or even performed better than some common natural antioxidant compounds (*40*). However, the correlation between the diarylamines structures and their liposolubility profiles is not

straightforward. As expected, the presence of two non-polar methoxy group increases the liposolubility, as is observed by comparing the diarylamines containing three methyl groups in their structures (MJQ3 > MJQ1). The presence of four methyl groups was also expected to increase the liposolubility of the compounds, which is observed through comparison of MJQ4 and MJQ5 with MJQ1. However, the expected increase in liposolubility observed by the presence of more methoxy groups is not observed when we put MJQ4 side by side with MJQ5, indicating that the methoxy non-polar effect disappears when four methyl groups are present in the diarylamines' structures.

Overall, no significant differences in terms of the antioxidant protection profile could be observed when comparing all the diarylamines tested. This suggests that the modifications introduced to the molecules, namely the presence of one or two methoxy and three or four methyl groups, may not in fact be the main responsible for the scavenging activity of the diarylamines, though may alter their liposolubility. Nevertheless, the importance of those functional groups in these molecules seems to be relevant, as they aid in the stabilization of the radical formed upon scavenging of a free H radical.

It should also be taken into consideration that in all parameters, namely intracellular ROS formation, oxidative DNA damage and lipid peroxidation, the diarylamines evoked protection profiles higher than the ones observed with the classical antioxidant and hydrosoluble vitamin E analogue, Trolox. This adds further value to the compounds in study, as they show an increased ability to prevent oxidative injury when compared to some existing molecules that are commonly used for its antioxidant activity.

In conclusion, we present evidence for the elevated antioxidant potential of novel molecules that demonstrated a high protective role against intracellular ROS formation and oxidative injury to biomolecules like lipids and DNA, resulting in an overall protection against oxidative stress-induced cell death. These effects were attained at a nanomolar range and even at a so low concentration they were superior to the classical antioxidant Trolox. Nevertheless, this work should be regarded as a first approach in the evaluation of the effects of the diarylamines as antioxidants in cells, and further studies are being conducted in order to address other potential intracellular action targets for these molecules. The results described allow us to introduce modifications to the structures of these molecules, in order to ameliorate them and thus increase their antioxidant activity and possibly direct them to specific molecular targets. In this regard, a recently proposed quantitative structure-activity relationship (QSAR) model (*41*) can be used to predict the antioxidant activity, and thus guide the synthesis, of derivatives of compounds used in this work.

Altogether, the data herein obtained encourage us to continue to study the potential of these promising diarylamines as molecules with a possible pharmacological application against pathological situations in which oxidative stress-mediated injury occurs.

Acknowledgements

We wish to thank Dr. Paulo Oliveira (CNC, University of Coimbra, Portugal) for gently providing the H9c2 cell line. This work was supported by FCT research project PTDC/QUI/68382/2006 and R. Calhelha's PhD grant (SFRH/BD/29274/2006).

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(Received March 9, 2010; Accepted May 30, 2010)