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Antioxidant effect of 1,3,4-thiadiazolium mesoionic derivatives on isolated mitochondria

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ABSTRACT

Mesoionic compounds have shown antitumor and citotoxic activity against different tumor cells lines, which has been attributed to their physical and chemical characteristics. Among these compounds, the 1,3,4-thiadiazolium-2-phenylamine derivatives have been highlighted due to their important anti-melanoma activity. In this work, the effects of three derivatives that belong this class, MI-J, MI-4F and MI-2,4diF, on the oxidative stress parameters were evaluated using rat liver mitochondria. All the derivatives prevented natural and calcium induced oxidation of pyridine nucleotides at lower concentrations (6.5 and 32.5 nmol/mg protein). The calcium uptake was inhibited by all the derivatives at higher concentrations (65 and 130 nmol/mg protein), whereas the cation efflux was inhibited only by the MI-J (52%) and MI-4F (50%), possibly by inhibiting the formation of the permeability transition pore (PTP) by 100% and 50%, respectively, as observed in the same experimental conditions. MI-2,4diF did not inhibit the mitochondrial permeability transition or calcium efflux. The enzymatic activity of glutathione reductase, glutathione peroxidase and catalase was not affected by any derivative, but superoxide dismutase was inhibited by all the derivatives. MI-J inhibited enzyme activity significantly (85%) at the highest concentration (130 nmol/mg protein); on the other hand, their activity was less affected by fluorine derivatives (MI-4F-20% and MI-2,4diF-32%). These results suggest that these derivatives exert antioxidant effects on isolated mitochondria.

1. Introduction

1,3,4-thiadiazolium-2-phenylamine derivatives (Fig. 1) are a class of mesoionic compounds that possess important biological activities, including antitumor activity (Cardoso et al., 2004; Da Silva et al., 2002; Gozzi et al., 2015; Grynberg et al., 1997; Rodrigues et al., 2007; Senff-Ribeiro et al., 2003; Senff-Ribeiro et al., 2004a; Senff-Ribeiro et al., 2004b). MI-D (4-phenyl-5-(4-nitrocinnamoyl)-1,3,4-thiadiazolium-2-phenylamine – Fig. 1) is an important derivative of this class and the one studied most extensively. In an earlier paper, we showed that MI-D possesses anti-inflammatory activity similar to nonsteroidal anti-inflammatories (Cardoso et al., 2004) and antitumor and cytotoxic activity against murine and human melanoma, respectively (Senff-Ribeiro et al., 2003; Senff-Ribeiro et al., 2004a; Senff-Ribeiro et al., 2004b). Its mean lethal dose (LD-50) was determined at 181 mg/kg in mice, which is 22-fold and 181-fold greater than the effective dose for analgesic, antipyretic and anti-inflammatory activities, respectively, and 7-fold higher than the effective dose for antimelanoma activity (Romão et al., 2009; Cardoso et al., 2004). With regard to the antimelanoma activity of other derivatives of the same class, which differ from each other only in terms of the cinnamoyl ring substituent (MI-J, X=OH; MI-4F, X=F; MI-2,4diF, X=Y= F – Fig. 1), significant activity was also demonstrated. In a comparison, MI-D was found to be the most effective inhibitor of tumor growth *in vivo*, decreasing the viability and proliferation of B16-F10 melanoma cells in *in vitro* assays. MI-4F was more effective than MI-

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*–tetraacetic acid; FCCP, p-trifluoromethoxycarbonylcyanide phenylhydrazone; GSH, glutathione; GSSG, glutathione disulfide; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MI-J, (4-phenyl-5-(4-hydroxycinnamoyl)-1,3,4–thiadiazolium-2-phenylamine chlorides); MI-4F, (4-phenyl-5-(4-chlorocinnamoyl)-1,3,4–thiadiazolium-2-phenylamine chlorides); MI-2,4diF, (4-phenyl-5-(2,4-chlorocinnamoyl)-1,3,4–thiadiazolium-2-phenylamine chlorides); MPT, mitochondrial permeability transition; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; PTP, permeability transition pores; ROS, reactive oxygen species; Tris, tris(hydroxymethyl)aminomethane

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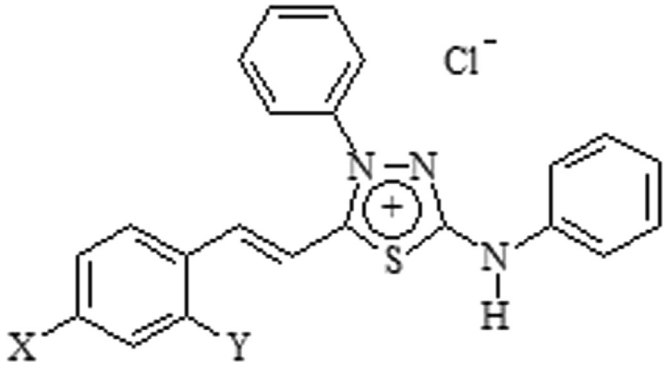

Fig. 1. : Chemical structure of the derivatives of 4-phenyl-5-(2-Y-4-X-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides: MI-J (X=OH; Y=H), MI-4F (X=F; Y=H), MI-2,4diF (X=Y=F) and MI-D ($X=NO_2$; Y=H).

2,4diF in *in vitro* assays, but had little effect on tumor growth *in vivo*, while MI-J showed no effect (Senff-Ribeiro et al., 2004a). Recently we showed that the derivatives MI-J, MI-4F, MI-2,4diF and MI-D were selectively cytotoxic to HepG2 cells in comparison to non-tumoral hepatocytes. In addition, these derivatives were only weakly, or not at all, transported by the main multidrug transporters, namely P-glycoprotein, ABCG2 and MRP1 (Gozzi et al., 2015). To elucidate the mechanism of antitumor activity, and considering the involvement of mitochondria with cell death mechanisms, we showed that 1,3,4-thiadiazolium-2-phenylamine derivatives inhibit the respiratory chain complexes, collapse the transmembrane potential and stimulate ATPase activity in intact rat liver mitochondria (Cadena et al., 1998; Pires et al., 2010). These effects were ascribed to the diminished fluidity and elasticity of mitochondrial membrane (Cadena et al., 2002; Pires et al., 2011). We also demonstrated that these compounds are able to inhibit lipid peroxidation induced by Fe^{3+}-ADP/2-oxoglutarate and AAPH, and that this effect is related to its $O_2^{\bullet -}$ scavenging activity (Méndez-Sánchez et al., 2009; Pires et al., 2011) and important uncoupling effect, which could bypass their inhibitory effect on the respiratory chain and hence reduce the latter's production of superoxide anions. In addition, these derivatives showed distinct scavenging abilities and the intensity of their inhibitory effect on lipid peroxidation was also different (MI-2,4diF=MI-4F > MI-D > MI-J), which was ascribed to the hydrophobicity constants of these compounds (Patrick, 1995), reinforcing the idea that the effects of the derivatives on intact mitochondria are due to their interaction with mitochondrial membranes.

In view of the significant biological activities of the mesoionic 1,3,4- thiadiazolium derivatives and their potential antioxidant role, we now investigate their effects on the modulation of certain parameters of oxidative stress in isolated mitochondria. This approach was based on the involvement of oxidative stress in mechanisms of cell death mediated by these organelles (Sinha et al., 2013). The effects of the mesoionic derivatives on calcium transport, antioxidant enzyme activity, the oxidative state of pyridine nucleotides, and the induction of mitochondrial permeability transition were also evaluated.

2. Materials and methods

2.1. Chemicals

Sucrose, D-mannitol, potassium phosphate, succinic acid, rotenone, BSA, HEPES, Tris, NBT, PMS, EGTA, EDTA, GSH, GSSG, NADH, NADPH, cyclosporin A and arsenazo III were obtained from Sigma. DMSO and KCl was purchased from Merck. All the other commercially available chemicals used in this study were of the highest purity.

The derivatives MI-J (4-phenyl-5-(4-hydroxycinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride), MI-4F (4-phenyl-5-(4-chlorocinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride) and MI-2,4diF (4-phenyl-5-(2,4-chlorocinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride) (Fig. 1) were synthesized at the Department of Chemistry of the Federal Rural University of Rio de Janeiro, Brazil and their structures were confirmed by ^1H NMR, ^{13}C NMR and mass spectrometry (Dos Santos and Echevarria, 2001). For their use in this study, the derivatives were dissolved in DMSO and then further diluted with the assay medium. Controls with DMSO (1% v/v) were carried out in each assay.

2.2. Animals

Male Wistar rats (180–200 g) were supplied by the Central Animal House of the Federal University of Paraná (PR, Brazil). The animals were housed at 22 ± 1 °C and had free access to standard laboratory food (Purina®) and tap water. All the animals were starved for 12 h before being killed by decapitation. The experiments were conducted following the recommendation of the Brazilian Law 6638 of 05 Nov 1979 for the scientific management of animals, and the procedures were approved by the institution's Animal Ethics Committee.

2.3. Isolation of rat liver mitochondria

Mitochondria were isolated from rat liver by differential centrifugation (Voss et al., 1961), using a suspension medium consisting of D-mannitol 250 mmol/L, HEPES-KOH 10 mmol/L pH 7.2, EGTA 1 mmol/L, and BSA 0.1% (m/v). Only mitochondrial preparations with a respiratory control above 4.0 were used. Before beginning the assays, the derivatives were incubated for 2 min with the mitochondrial preparations. Duplicate controls with DMSO were included to validate each assay. At the concentrations used in these experiments, DMSO had no effect on the mitochondrial properties.

2.4. Evaluation of the redox state of pyridine nucleotides in mitochondria

The redox state of mitochondrial pyridine nucleotides was monitored fluorometrically using 366 and 450 nm as excitation and emission wavelengths, respectively. The reaction medium containing sucrose 125 mmol/L, KCl 65 mmol/L and HEPES-KOH 10 mmol/L, pH 7.4, was supplied with rotenone 2.5 µmol/L, sodium succinate 5 mmol/L and mitochondrial protein 1 mg/mL[1]. The reaction was accompanied by a decrease in NADPH and NADH fluorescence after 10 min at 30 °C (Pigoso et al., 1998).

2.5. Estimation of calcium transport from mitochondria

Calcium influx and efflux from mitochondria was estimated by monitoring changes in the absorbance spectrum of arsenazo III at the 675–685 nm wavelength pair (Scarpa, 1979). The medium containing D-mannitol 250 mmol/L, BSA 0.1% (m/v) and HEPES-KOH 10 mmol/L, pH 7.2, was treated with Chelex-100 resin (0.1%) before use. Mitochondria (1 mg/mL) were incubated in this medium, which was supplied with potassium phosphate 0.3 mmol/L, arsenazo III 20 µmol/L, rotenone 5 µmol/L and $CaCl_2$ 45 µmol/L at 30 °C. The influx was started with 5 mmol/L sodium succinate and efflux was induced by FCCP 1 µmol/L.