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The antioxidant effect of the mesoionic compound SYD-1 in mitochondria

Gustavo Jabor Gozzi [a], Amanda do Rocio Andrade Pires [a], Glaucia Regina Martinez [a], Maria Eliane Merlin Rocha [a], Guilhermina Rodrigues Noleto [a], Aurea Echevarria [b], André Vinicius Canuto [b], Sílvia Maria Suter Correia Cadena [a,*]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, Paraná, Brazil
[b] Departamento de Química, Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Brazil

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ABSTRACT

The sydnone SYD-1 (3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate] possesses important antitumor activity against Sarcoma 180 and Ehrlich tumors. We previously showed that SYD-1 depresses mitochondrial phosphorylation efficiency, which could be involved in its antitumoral activity. Considering the important role of mitochondria in the generation of reactive oxygen species (ROS) and the involvement of ROS in cell death mechanisms, we evaluated the effects of SYD-1 on oxidative stress parameters in rat liver mitochondria. SYD-1 (0.5 and 0.75 μmol mg^{-1} protein) inhibited the lipoperoxidation induced by Fe^{3+}/ADP-oxoglutarate by approximately 75% and promoted total inhibition at the highest concentration tested (1.0 μmol mg^{-1} protein). However, SYD-1 did not affect lipoperoxidation started by peroxyl radicals generated by α-α′-azodiisobutyramidine dihydrochloride. The mesoionic compound (0.25–1.0 μmol mg^{-1} protein) demonstrated an ability to scavenge superoxide radicals, decreasing their levels by 9–19%. The activities of catalase and superoxide dismutase did not change in the presence of SYD-1 (0.25–1.0 μmol mg^{-1} protein). SYD-1 inhibited mitochondrial swelling dependent on the formation/opening of the permeability transition pore induced by Ca^{2+}/phosphate by approximately 30% (1.0 μmol mg^{-1} protein). When Ca^{2+}/H_2O_2 were used as inducers, SYD-1 inhibited swelling only by approximately 12% at the same concentration. NADPH oxidation was also inhibited by SYD-1 (1.0 μmol mg^{-1} of protein) by approximately 48%. These results show that SYD-1 is able to prevent oxidative stress in isolated mitochondria and suggest that the antitumoral activity of SYD-1 is not mediated by the increasing generation of ROS.

1. Introduction

The potential biological activities of mesoionic compounds, which are associated with their chemical properties, have motivated the synthesis of several mesoionic compounds within the last few decades [1–4]. These compounds possess a five member heterocyclic ring that cannot be represented by a covalent or polar structure. The negative charge present in an atom or an exocyclic group with which the ring associates is balanced by the positively charged ring, resulting in an overall neutral molecule. These properties allow mesoionic compounds to cross biological membranes and to interact with biomolecules such as DNA and proteins [4]. Several biological activities have been attributed to different classes of mesoionic compounds (sydnones, sydnonymines, and mesoionic 1,3,4-thiadiazoles); these activities include anti-inflammatory, analgesic, antibacterial [3,5–7], antiplatelet, fibrinolytic and thrombolytic activities, as well as broncholytic and antitumor

Abbreviations: AAPH, α-α′-azodiisobutyramidine dihydrochloride; BHT, butylhydroxytoluene; BSA, bovine serum albumin; CAT, catalase; CsA, cyclosporine A; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis(β-aminoethyl-ether)-*N,N,N′,N′*-tetraacetic acid; FCCP, *p*-trifluoro-methoxycarbonylcyanide phenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MI-D, (4-phenyl-5-(4-nitrocinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride); Mn-SOD, manganese superoxide dismutase; 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; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; PTP, permeability transition pore; ROS, reactive oxygen species; SYD-1, 3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate]; TBARS, thiobarbituric acid reactive substances; TRIS, tris(hydroxymethyl)-aminomethane.

* Corresponding author. Address: Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Coronel Franscisco H. dos Santos, C. Postal 19046, Curitiba 81531-990, Paraná, Brazil. Tel.: +55 41 33611535; fax: +55 41 3266 2042.
E-mail address: silvia.cadena@ufpr.br (S.M.S.C. Cadena).

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effects [2,8–9]. Among these compounds, the sydnone SYD-1 (3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate) (Fig. 1) has shown important cytotoxic and antitumor effects *in vivo* [10–11]. However, the molecular mechanisms involved in these effects are not known. *In vitro* studies suggest that alterations to the permeability of mitochondrial membranes and the consequent alteration of functions linked to energy provision may be involved in the mechanisms of cell death induction, particularly apoptosis [12]. We have shown that SYD-1 was able to promote both an important inhibition of the respiratory chain as well as the uncoupling of oxidative phosphorylation [13].

Several studies have shown that electron transport chain inhibitors can induce ROS overproduction and promote apoptosis (intrinsic pathway) in tumor cells [14–16]. The key event in this process is the permeabilization of the mitochondrial membrane and the release of pro-apoptotic factors into the cytoplasm. These pro-apoptotic factors can activate caspases responsible for the process of cell death. The disruption of the mitochondrial membrane is usually caused by BAX activation or calcium-mediated permeability transition pore formation. These events are normally accompanied by the dissipation of the mitochondrial membrane potential [17]. It has been demonstrated that lipid peroxidation, a process that can be initiated by oxidative stress, promotes mitochondrial membrane permeabilization and induces apoptosis through the modulation of PTP constituents [18].

To contribute to the understanding of the molecular pathways involved in the biological action of SYD-1, we investigated in this work the effects of SYD-1 on redox parameters that may be related to the mitochondrial dysfunction, cytotoxicity and antitumoral activity previously attributed to this mesoionic compound.

2. Materials and methods

2.1. Chemicals

D-mannitol, HEPES, EGTA, rotenone, BSA, glutamic acid, ADP, NADH, NBT, PMS, BHT and succinic acid were obtained from Sigma. Monobasic potassium phosphate and trichloroacetic acid were obtained from Synth. AAPH was purchased from Fluka. All other chemicals used were of the highest commercially available purity. SYD-1 was synthesized by the Department of Chemistry of the Federal Rural University of Rio de Janeiro, Brazil and its structure was confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry [10]. For use in assays, the compound was dissolved in DMSO and then further diluted with the assay medium. The mesoionic compound was incubated for two min with mitochondrial preparations before assays began. To validate each assay, duplicate controls with DMSO were included at the concentrations used in the experiments. DMSO had no effect on the parameters analyzed.

2.2. Animals

Male Wistar rats (180–200 g) were obtained from the Central Animal House of Federal University of Paraná (PR, Brazil). They were housed at 22 ± 1 °C under a 12 h light – 12 h dark cycle (lights on at 08:00 h) with free access to standard laboratory food (Purina®) and tap water. The animals were starved for 12 h and then killed by decapitation. Experiments were conducted following the recommendations of Brazilian Law 11.794, 08/10/2008 for the scientific management of animals and procedures and were approved by the institution's Animal Ethics Committee.

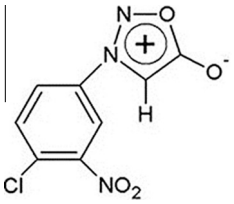

Fig. 1. Chemical structure of 3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate] (SYD-1).

2.3. Isolation of rat liver mitochondria

Mitochondria were isolated from rat liver by differential centrifugation [19] using an extraction medium consisting of 250 mM D-mannitol, 10 mM HEPES–KOH (pH 7.2), 1 mM EGTA, and 0.1% BSA. Specifically for mitochondrial permeability transitions and the oxidation of pyridine nucleotides induced by calcium the last centrifugation was performed in the absence of EGTA. Disrupted mitochondria were obtained by freeze–thawing and were used to determine Mn-SOD activity.

2.4. Oxygen uptake

Oxygen uptake of intact mitochondria was evaluated at 28 °C in a 1.3 mL closed, thermostatically controlled water-jacketed chamber under magnetic stirring. Oxygen consumption was measured polarographically using a Clark-type electrode connected to a Gilson oxygraph [20–21] and a standard medium containing 125 mM D-mannitol, 65 mM KCl, 10 mM HEPES–KOH (pH 7.2) and 0.1% BSA. The medium was supplemented with 1.0 mM Pi, 0.1 mM ADP, 5 mM sodium glutamate, 0.5 mM sodium malate and 1.5 mg mL^{-1} of mitochondrial protein. State 3 and 4 respiration rates were measured after the addition of substrates in the presence (state 3) and after exhaustion (state 4) of ADP. The respiratory control coefficient was calculated as the ratio of state 3 respiration to state 4 respiration [22]. Only mitochondrial preparations with respiratory controls above 4 were used (data not shown).

2.5. Determination of lipoperoxidation

Two systems were used as inducers of lipoperoxidation in intact mitochondria. One system consisted of AAPH, an inducer of free radicals in an aqueous phase at a concentration of 30 mM [23], and the other system consisted of 2 mM ADP, 0.2 mM $FeCl_3$ and 5 mM 2-oxoglutarate [24], which is able to induce the production of free radicals from the respiratory chain. Lipoperoxidation was performed as described by Buege and Aust [25] in medium containing mitochondrial protein (1 mg mL^{-1}), 10 mM HEPES (pH 7.2), 250 mM mannitol and a system inducer of free radicals. Lipid peroxidation was estimated by the absorbance of TBARS (ε_{535} - = 1.56×10^5 M^{-1} cm^{-1}) and expressed as a percentage of control.3

2.6. Superoxide radical assay

The scavenging of superoxide radicals was assessed by the method described by Nishimiki et al. [26], with some modifications. The reaction mixture consisted of 10 mM Tris–HCl (pH 8.0), 70 μM NADH, 21 μM NBT and 9 μM PMS. The reaction was followed at 560 nm for 1 min and the scavenging of superoxide radicals was calculated using the following equation [27]:

$$\text{Scavenging effect} = \left(1 - \frac{\text{Abs sample}}{\text{Abs control}}\right)$$

2.7. Determination of mitochondrial permeability transition

The mitochondrial permeability transition was followed by analyzing the decrease in absorbance at 540 nm due to mitochondrial