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Impairment of oxidative phosphorylation increases the toxicity of SYD-1 on hepatocarcinoma cells (HepG2)

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ABSTRACT

Toxicity of the SYD-1 mesoionic compound (3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate) was evaluated on human liver cancer cells (HepG2) grown in either high glucose (HG) or galactose (GAL) medium, and also on suspended cells kept in HG medium. SYD-1 was able to decrease the viability of cultured HepG2 cells in a dose-dependent manner, as assessed by MTT, LDH release and dye with crystal violet assays, but no effect was observed on suspended cells after 1–40 min of treatment. Respiration analysis was performed after 2 min (suspended cells) or 24 h (cultured cells) of treatment: no change was observed in suspended cells, whereas SYD-1 inhibited as well basal, leak and uncoupled states of the respiration in cultured cells with HG medium. These inhibitions were consistent with the decrease in pyruvate level and increase in lactate level. Even more extended results were obtained with HepG2 cells grown in GAL medium where, additionally, the ATP amount was reduced. Furthermore, SYD-1 appears not to be transported by the main ABC multidrug transporters. These results show that SYD-1 is able to change the metabolism of HepG2 cells, and suggest that its cytotoxicity is related to impairment of mitochondrial metabolism. Therefore, we may propose that SYD-1 is a potential candidate for hepatocarcinoma treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent cancer in the world and is the third most common cause of cancer-related deaths. It has a poor long-term survival rate with 600,000 deaths recorded in 2015 [1,2]. The traditional therapies such as resection, transplantation or transarterial interventions have a limited efficacy, and the drugs available for treatment may also affect non-tumor cells, promoting many serious side effects [3,4]. Then, it is essential to establish more efficient therapies with fewer side effects. One potential candidate to this aim is SYD-1 (Fig. 1). It is a mesoionic compound belonging to the sydnone class, for which several biological activities have been described, such as anti-inflammatory, analgesic, antibacterial and free radical scavenging [5]. These activities are associated with the betaine-like character of mesoionic compounds, with a partial positive charge in the heterocyclic ring balanced by a negative charge of an atom or group exocyclic, which allows them to interact with biomolecules such as proteins and DNA [6]. SYD-1 is noteworthy for its important anti-tumor activity against Ehrlich carcinoma and Sarcoma 180, and its *in vitro* toxicity on several tumor cell lines [7–9], suggesting that it may also be effective against HCC cells. This possibility became further more interesting when we observed that SYD-1, at low concentrations, was not cytotoxic for non-tumor hepatocytes [10]. In this work, besides the evaluation of this hypothesis we also

Abbreviations: FBS, Fetal bovine serum; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide; TRIS, tris(hydroxymethyl)-aminomethane; DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; HBSS, Hanks balanced salt solution; PBS, phosphate buffered saline; SYD-1, 3-[4-cloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate.

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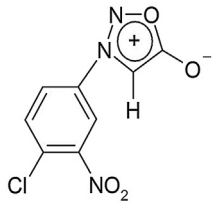

Fig. 1. Chemical structure of 3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate-SYD-1.

investigated if the impairment of oxidative phosphorylation promoted by SYD-1, as shown in our previous studies [11,12] is relevant for its cytotoxic effect. For this purpose, we promoted a metabolic shift by replacing the glucose of the culture medium of HepG2 cells by galactose plus glutamine at higher concentration, making these cells more dependent on oxidative phosphorylation than glycolysis for energy provision [13–17].

Resistance to the treatment also constitutes a great problem in chemotherapy [18,19]. Tumor cells may become resistant to drugs through different mechanisms including the overexpression of ATP-binding cassette transporters (*P*-glycoprotein (Pgp)) [20], multidrug resistance protein 1 (MRP1) [21] and breast cancer resistant protein (ABCG2) [22]. The hard feature of these transporters is the efflux of several types of drugs with unrelated structures and mechanisms of action which became a main obstacle to effectiveness of chemotherapy against HCC [23]. In this study we then also investigated the effects of SYD-1 on the multidrug resistance proteins Pgp, ABCG2 and MRP1. Our results evidenced that the effects of SYD-1 involve mitochondria as a primary target and showed a potential selectivity of this compound for these cells in comparison to our previous results with non-tumor hepatocytes.

2. Materials and methods

2.1. Chemicals

D-Mannitol, HEPES, DMSO, Rotenone, FCCP, Oligomycin, NADH, NAD^+, L-lactate dehydrogenase, MTT, Trypsin, Galactose, Glutamine and Dulbecco's modified Eagle's medium (DMEM), were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest commercially available purity. SYD-1 was synthesized by the Department of Chemistry at the Federal Rural University of Rio de Janeiro, Brazil, and its structure was confirmed by ^1H NMR, ^{13}C NMR and mass spectrometry. For use in assays, the compound was dissolved firstly in DMSO and then into the assay medium. Controls with DMSO were carried out in each assay, and the solvent had no effect on the evaluated parameters.

2.2. Cells culture and treatment

The HepG2 human hepatoma cell line, known to express significant amounts of the three main ABC multidrug transporters ABCB1/Pgp, ABCC1/MRP1 and ABCG2 [24,25], was purchased from Rio de Janeiro Cell Bank (Brazil) and maintained in Dulbecco's modified Eagle's medium (DMEM), containing either 25 mM glucose and 4 mM glutamine (HG medium), or 10 mM galactose and 6 mM glutamine (GAL medium). Both of them were supplemented with 10% (v/v) FBS, and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) and kept at 37 °C under 5% CO_2. To experimental proceedings, the HepG2 cells were firstly thawed in HG medium and kept in 5% CO_2 at 37 °C. The shift to GAL medium was done gradually, and the cells were kept in this new medium for fifteen days before starting the assays. After this time, the cells grown both in HG or GAL-containing medium were seeded in plates at confluence according necessary to each individual experiment and treated with different concentrations of SYD-1 for different times. For the assays of oxygen consumption and of ATP lactate and pyruvate levels, HepG2 cells were cultured in a 60 cm^2-well plate with DMEM HG or GAL medium, with or without SYD-1 (25 or 50 μM) for 24 h. The conditions for other assays are specified in the respective item.

For transport experiments by multidrug transporters, the human embryonic kidney (HEK293) cell line was stably transfected with either *ABCG2* (HEK293*ABCG2*) [26] or *MRP1* (HEK293*ABCC1*), and their respective parental HEK293 (wild-type) or HEK293*pcDNA5* (empty-vector) cells, were maintained at 37 °C (5% CO_2) in high-glucose DMEM medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. The mouse embryonic fibroblasts, of either wild-type (NIH3T3) or overexpressing Pgp (NIH3T3*ABCB1*) [27], were maintained under the same conditions. The cell culture media were drug supplemented with either 0.75 mg/mL G418 (HEK293*ABCG2*), 200 μg/mL hygromycin B (HEK293*pcDNA5* and HEK293*ABCC1*) or 60 ng/mL colchicine (NIH3T3*ABCB1*). The three transfected cell lines specifically overexpressed the corresponding multidrug ABC transporter, namely ABCB1/Pgp [28], ABCC1/MRP1 [29] and ABCG2 [30], respectively.

2.3. Cellular viability assays

The viability of HepG2 cells was evaluated by three methods: tetrazolium dye reduction (MTT) [31], LDH release and dying with violet crystal [32]. HepG2 cells (1×10^4) were seeded in a 96-well plate with DMEM HG or GAL in the presence or absence of SYD-1 (at 0, 15, 25, 35, and 50 μM) for 24 h. After, the medium was collected and centrifuged at 1500 rpm for 5 min, and the LDH activity in the supernatant was determined using LDH assay kit (Labtest®, Lagoa Santa, Brazil) according to the manufacturer's recommended protocol. The results were expressed as percentage of LDH released. To adherent cells was added a MTT solution (0.5 mg/mL) following incubation at 37 °C for 3 h, or violet crystal (0.2% (m/v) in 2% of ethanol (v/v)) for 3 min. The MTT-formazan crystals were dissolved with DMSO and the absorbance was measured at 540 nm using a microplate reader. HepG2 dyed with violet crystal were washed many times with PBS, and sodium citrate (0.05 M in ethanol 50%) was added. Finally, the absorbance was measured at 550 nm using a microplate reader. Cell viability was expressed as percentage of the control. For the experiments with multidrug resistant cells, the MTT reduction was utilized to determinate the IG_{50}. All HEK293 cells were seeded at a density of 1×10^4 cells/well into 96-well culture plates, and incubated for 24 h at 37 °C in 5% CO_2. NIH–3T3 and NIH–3T3*ABCB1* cells were seeded at a density of 3.5×10^3 and 5.0×10^3 cells/well, respectively, and maintained under the same conditions before treatment. The cells were treated with SYD-1 for 72 h; then, 20 μL of MTT solution (5 mg/mL) were added to each well and incubated for 4 h at 37 °C. The culture medium was discarded, and 100 μL of a DMSO:ethanol (1:1) solution was added into each well and mixed by gently shaking for 10 min. Absorbance was measured in a microplate reader at 570 nm, from which the value measured at 690 nm was subtracted [31].

2.4. Oxygen uptake

The respiration of the intact HepG2 cells was measured by high-resolution respirometry with an Oxygraph-2k (OROBOROS INSTRUMENTS, Innsbruck, Austria) at 37 °C under gentle agitation. The oxygen consumption was rated in different states of respiration