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Effects of a new antiprotozoal drug, *N,N′*-diphenyl-4-methoxy-benzamidine, on energy-linked functions of rat liver mitochondria

Lyvia Lintzmaier Petiz[a], Amanda do Rocio Andrade Pires[a], Aurea Echevarria[b], Cláudio Eduardo Rodrigues-Santos[b], Maria Eliane Merlin Rocha[a], Alexandra Acco[c], Silvia 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*
[c] *Departamento de Farmacologia, Universidade Federal do Paraná, Curitiba, Paraná, Brazil*

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ABSTRACT

Amidines are chemically characterized by the presence of two nitrogen atoms that bind to the same carbon atom in its structure. Several biological activities have been ascribed to these compounds. Pentamidine, an aromatic diamidine, is effective in the treatment against *Pneumocystis carinii* and leishmaniasis, but it can also have severe side effects. New amidine derivatives have been synthesized, among them *N,N′*-diphenyl-4-methoxy-benzamidine (methoxyamidine), which is effective against *Leishmania amazonensis* (LD_{50} = 20 μM) and *Trypanosoma cruzi* (LD_{50} = 59 nM). In the present study, methoxyamidine toxicity was evaluated in isolated rat liver mitochondria at the same range of concentrations that exert antiprotozoal activity. In these organelles, actively oxidizing glutamate + malate inhibited state 3 respiration (25 nmol mg^{-1} of protein) by ~15%. The sites of inhibition in the respiratory chain were complex I and the segment between ubiquinone and complex III. Methoxyamidine also stimulated state 4 respiration by ~32% and ~43% at 50 and 65 nmol mg^{-1} of protein, respectively. Its uncoupling effect was confirmed by a dose-dependent increase in oxygen consumption in state 4 respiration that was induced by oligomycin, reaching up to ~69% (65 nmol mg^{-1} of protein) and an increase in ATPase activity in intact mitochondria by ~27% and ~83% at 50 and 65 nmol mg^{-1} protein, respectively. Swelling that was supported by the oxidation of glutamate + malate in the presence of sodium acetate was reduced by methoxyamidine by ~16% and 32% at 50 and 65 nmol mg^{-1} protein, respectively. Mitochondrial swelling in the absence of substrate and in the presence of K^+ and valinomycin was inhibited by ~20% at the same concentrations, suggesting that methoxyamidine affects mitochondrial membrane permeability and fluidity. Our data show that methoxyamidine has slight effects on the energy-linked functions of isolated mitochondria at concentrations that correspond to the LD_{50} against *Leishmania amazonensis* and *Trypanosoma cruzi*. These findings may prompt further studies that evaluate methoxyamidine toxicity *in vivo*.

1. Introduction

Amidines are chemically characterized by the presence of two nitrogen atoms that bind to the same carbon in a structure that permits a range of substitutions that ascribe several biological activities to them [1], including antiplatelet [2], antidegenerative [3], antitumoral [4], antimicrobial [5], antiinflammatory [6], neuroprotective [7], antitrypanosomal [8], and antileishmanial [9–12] effects. Pentamidine is an aromatic diamidine that is widely used for the treatment of leishmaniasis. The first report of the beneficial effects of pentamidine dates back to 1938 concerning the treatment of African trypanosomiasis. Today it is applied for the treatment of antimony-resistant leishmaniasis, including *Trypanosoma brucei* and *Pneumocystis jiroveci* (*carinii*) [8]. Although effective, pentamidine can have severe side effects [13], such as hypotension, pancreatitis, and leukopenia, which are dose- and time-dependent and may be irreversible [14]. In patients with acquired

Abbreviations: $\Delta\psi_m$, mitochondrial transmembrane potential; A, absorbance; ADP, adenosine diphosphate; AIDS, acquired immunodeficiency syndrome; ANOVA, analysis of variance; ATP, adenosine triphosphate; AUF, arbitrary units of fluorescence; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; HEPES, 2-(4-[2-hydroxyethyl]piperazin-1-yl)ethanesulfonic acid; LD_{50}, median lethal dose; MOPS, 3-morpholinopropane-1-sulfonic acid; NMR, nuclear magnetic resonance; RCR, respiratory control ratio; TRIS, tris(hydroxymethyl)-aminomethane

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

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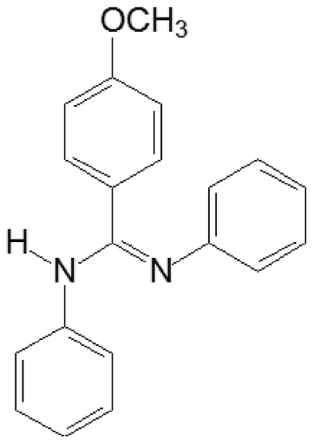

Fig. 1. Chemical structure of *N*,*N*′-diphenyl-4-methoxybenzamidine (methoxyamidine).

immunodeficiency syndrome (AIDS) who often contract *Pneumocystis jiroveci* pneumonia because of immunodepression, pentamidine administration at low (0.5 g kg^{-1}), medium (1.0–1.4 g kg^{-1}), and high (3.38–6.6 g kg^{-1}) doses resulted in accumulation of the drug in the kidneys, the spleen, the lungs, and especially the liver where major accumulation was observed [15].

Considering the side effects of pentamidine, novel amidines are continually synthesized and tested for their antiprotozoal activity. *N*,*N*′-diphenyl-4-methoxybenzamidine (methoxyamidine) is a new triarylamidine (Fig. 1) [16] that has potential therapeutic applications. This compound was reported to have an LD_{50} of 20 μM and 59 nM against *Leishmania amazonensis* and *Trypanosoma cruzi,* respectively [9]. Methoxyamidine has also been shown to decrease parasitic infection *in vitro* without adversely affecting host cells [10]. It also reduced more than twofold nitric oxide ($NO^{\bullet}$) release by *L. amazonensis* and *T. cruzi* compared with pentamidine [11]. Methoxyamidine also prevented parasite internalization by host cells (macrophages) when the parasites were pretreated with this compound, and this beneficial effect was not observed for pentamidine; moreover, methoxyamidine did not alter $NO^{\bullet}$ production by macrophages [12]. These results suggest that methoxyamidine is a promising molecule for *L. amazonensis* and *T. cruzi* treatment.

The liver is the main organ of accumulation of pentamidine and is the target of toxicity for most xenobiotics, mainly because of its anatomic location and metabolic detoxification pathways, including the P450 enzymatic family that metabolizes drugs within the endoplasmic reticulum [17,18]. Drug-induced hepatotoxicity can lead to cell death through necrosis or apoptosis. Necrosis is induced mainly by inflammation that is triggered by the release of chemotactic factors from nearby cells. Apoptosis is an adenosine triphosphate (ATP)-dependent process. Apoptosis that is triggered by intrinsic pathways leads to the activation of proapoptotic mitochondrial proteins, such as Bax, Bid, and Bim, and the release of cytochrome *c* into the cytoplasm [19]. Mitochondria are usually a target of xenobiotic-induced hepatotoxicity because of their structural and functional characteristics. As the main source of ATP production, cell life or cell death depends on the correct functioning of mitochondria. Some drugs affect mitochondrial functions, leading to bioenergetic collapse [20]. Indeed, clinical tests of new drugs are frequently interrupted because of severe hepatotoxic effects that are linked to mitochondrial damage [21]. For example, some drugs can lead to oxidative stress through glutathione depletion, leading to the dysfunction of enzymatic complexes from the respiratory chain, which can impair ATP production and mitochondrial permeability transition pore formation [22]. Several symptoms are related to drug-induced intoxication, such as hypotension, fatigue, nausea, and hallucinations, which may be related to mitochondrial injury [23]. Mitochondrial impairment has been associated with hepatic diseases, such as microvesicular steatosis, non-alcoholic steatohepatitis, and cytolytic hepatitis [24]. Thus, the toxic effects of new drugs on mitochondrial function linked to energy provision need to be evaluated.

Considering the promising effects of methoxyamidine against *L. amazonensis* and *T. cruzi* [9–12], the present study investigated its effects on the energy-linked functions of isolated rat liver mitochondria as an indicator of its potential toxicity in liver cells.

2. Material and methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), D-mannitol, 2-(4-[2-hydroxyethyl]piperazin-1-yl)ethanesulfonic acid (HEPES), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), rotenone, glutamic acid, malic acid, ADP, ATP, NADH, sodium succinate, cytochrome *c* (IV type, bovine heart), phosphoenolpyruvate, pyruvate kinase (type I), lactate dehydrogenase (type I), carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), magnesium sulfate, valinomycin, oligomycin, antimycin-A, sodium cyanide, safranin, 3-morpholinopropane-1-sulfonic acid (MOPS), and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). *N*,*N*′-diphenyl-4-methoxybenzamidine was synthesized in the Department of Chemistry, Federal Rural University of Rio de Janeiro, Brazil, and its structure was confirmed by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and gas chromatography coupled to mass spectrometry [16]. DMSO was used as the methoxyamidine vehicle, with a maximum volume of 0.5% when in solution. Solvent controls with DMSO were performed in each assay. The range of methoxyamidine doses (5, 25, 50, and 65 nmol mg^{-1} of mitochondrial protein) was based on the LD_{50} of 20 μM for *L. amazonensis* [9]. Methoxyamidine was incubated for 2 min with the mitochondrial preparations before the assays began.

2.2. Animals

Male Wistar rats (180–220 g) were provided from the Central Animal House of the Federal University of Paraná, Brazil. The animals remained in their home cages in a room with controlled temperature (22 °C ± 1 °C) and a 12 h/12 h light/dark cycle with food and water available *ad libitum*. Before the experiments, the animals were fasted for 12 h and then euthanized by decapitation. This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Paraná (permit no. 548).

2.3. Isolation of rat liver mitochondria

Mitochondria were isolated by differential centrifugation according to Voss et al. [25] using extraction media that contained 250 mM D-mannitol, 10 mM HEPES buffer (pH 7.2), 1 mM EGTA, and 0.1% (w/v) BSA. For the mitochondrial swelling assays, the extraction media contained 330 mM sucrose, 1 mM Tris buffer (pH 7.5), and 1 mM EDTA [26]. Mitochondrial suspension subjected to freeze-thaw treatment were used to determine enzymatic activity related to the respiratory chain and ATPase.

2.4. Oxygen consumption

Mitochondrial oxygen consumption was monitored by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Measurements were made in two chambers at 28 °C under constant agitation. The reaction medium contained 125 mM D-mannitol, 10 mM HEPES-KOH (pH 7.2), 65 mM KCl, and 0.1% (w/v) BSA supplemented with 5 mM sodium glutamate, 2.5 mM sodium malate,