Mutation Research 806 (2016) 34–39

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

The antileishmanial drug miltefosine (Impavido®) causes oxidation of DNA bases, apoptosis, and necrosis in mammalian cells

Patrícia Valéria Castelo Branco[a,*], Rossy-Eric Pereira Soares[a], Luís Cláudio Lima de Jesus[a], Vanessa Ribeiro Moreira[a], Hugo José Alves[a], Marta Regina de Castro Belfort[a], Vera Lucia Maciel Silva[b], Silma Regina Ferreira Pereira[a]

[a] Laboratory of Genetics and Molecular Biology, Department of Biology, Federal University of Maranhão, Cidade Universitária do Bacanga, São Luís, Maranhão, Brazil
[b] University of State of Maranhão, São Luís, Maranhão, Brazil

ARTICLE INFO

Article history:
Received 3 May 2016
Accepted 9 June 2016
Available online 24 June 2016

Keywords:
Genotoxicity
Comet assay
Micronucleus test

ABSTRACT

Miltefosine was developed to treat skin cancer; further studies showed that the drug also has activity against *Leishmania*. Miltefosine is the first oral agent for treating leishmaniasis. However, its mechanism of action is not completely understood. We have evaluated the induction of DNA damage by miltefosine. Cytotoxicity and genotoxicity (comet assay) tests were performed on human leukocytes exposed to the drug *in vitro*. Apoptosis and necrosis were also evaluated. *In vivo* tests were conducted in Swiss male mice (*Mus musculus*) treated orally with miltefosine. Oxidation of DNA bases in peripheral blood cells was measured using the comet assay followed by digestion with formamidopyrimidine glycosylase (FPG), which removes oxidized guanine bases. The micronucleus test was performed on bone marrow erythrocytes. Miltefosine caused DNA damage, apoptosis, and necrosis *in vitro*. Mice treated with miltefosine showed an increase in the DNA damage score, which was further increased following FPG digestion. The micronucleus test was also positive.

1. Introduction

Miltefosine (hexadecylphosphocholine) was developed as an anticancer drug. The antileishmanial activity of this agent was discovered through *in vitro* and *in vivo* tests [1]. Miltefosine has been in use since 2002 as the first and only orally administered drug to treat leishmaniasis, in India, South America, and Germany [2]. In 2011, miltefosine was included as an antileishmanial drug in the World Health Organization list of essential medicines [3].

Miltefosine is a simple, very stable, relatively safe, and highly effective drug. Cure rates in India are 97% for *Leishmania donovani*, 91% for *L. panamensis* in Colombia, and 53% for *L. braziliensis* and *L. mexicana* in Guatemala [4]. In Brazil, the response rate for *L. braziliensis* is 75% [5] and 71.4% for *L. guyanensis* [6].

Miltefosine belongs to the class of zwitterionic surfactants; it is structurally similar to biological membrane phospholipids [7]. It interacts with protozoan cell membranes, then quickly reaches subcellular membranes, where it can affect metabolism [8,9]. Hence, the cell membrane is considered the main site of action; mitelfosine is inserted in the phospholipid membrane by miscibility and through interaction with membrane sterols [10]. The drug is transported into the cell through a protein complex in the parasite's plasma membrane [3].

The use of miltefosine for the treatment of leishmaniasis is considered very beneficial due to its lower toxicity and side effects compared to those of pentavalent antimonials and also due to its easier administration [11]. Effective oral medication against leishmaniasis is critical because this disease is prevalent in poor regions, such as India, Bangladesh, Nepal, and Brazil; oral administration would increase adherence to the treatment [12].

Miltefosine is well absorbed orally and is rapidly distributed throughout the body. It accumulates in the liver, spleen, kidneys, lungs, and adrenal glands; it is slowly metabolized by liver phospholipases, and urinary excretion is minimal [13,14]. Prolonged treatment (28 d) is required and the drug has a long half-life (approximately 150 h) [15].

Pentavalent antimonial agents are the most commonly used antileishmanial drugs worldwide, despite studies that have

* Corresponding author at: Av dos Portugueses, 1966, Cidade Universitária do Bacanga. São Luis - MA. CEP 65080-805, Brazil.
E-mail address: ifepv@yahoo.com.br (P.V. Castelo Branco).

http://dx.doi.org/10.1016/j.mrgentox.2016.06.007
1383-5718/

reported their high toxicity [16,17], genotoxicity, and mutagenicity [18].

The Food and Drug Administration (FDA) described possible mutagenic effects of miltefosine, based on seven mutagenicity tests. Only one of the tests gave positive results [19]. The objective of the present study was to evaluate the interaction of miltefosine with the genetic material.

2. Materials and methods

Miltefosine capsules (Impavido®–50 mg) were kindly provided by Prof. Dr. Carlos Costa from the Leishmaniasis Laboratory (Laboratório de Leishmanioses – [LabLeish]) of the Federal University of Piauí (UFPI), Brazil. For the assays, capsules were dissolved in distilled water. All concentrations were previously assayed for cellular viability based on the World Health Organization recommended dose of 2.5 mg/kg/day.

2.1. In vitro assays

All *in vitro* assays were carried out on peripheral blood leukocytes obtained from three male and three female volunteers. Inclusion criteria were: 19–30 years of age, healthy, non-smoker, medication-free, and chemotherapy- or radiation therapy-free for the last six months (Ethics Committee Opinion n° 1.284.493-UFMA). Peripheral blood (20 mL) was collected from each subject in heparinized tubes; leukocytes were obtained using Ficol Paque™ Plus. Cells were uniformly diluted in RPMI culture medium, 5 mL, exposed to the drug immediately (time 0), and harvested after 3 and 24 h (for the comet assay and cell viability) and after 48 h for the cell death assays. For the negative control, cells were grown in RPMI 1640 medium alone. For the positive control, cells were treated with H_2O_2, 10 vol (30 mg/mL), 10 μL, for 10 min.

2.1.1. Cell viability

Cell viability was determined in a pilot test (100, 50, 25, and 10 μg/mL) by adding trypan blue (0.4%, 10 μL) to cell suspension, 10 μL. One hundred cells were counted for each treatment and the cultures had to have >70% viable cells to be used [20]. We performed cytotoxicity tests at both exposure times (3 and 24 h). The final concentrations established were C1 (0.25 μg/mL), C2 (0.5 μg/mL), and C3 (1.0 μg/mL).

2.1.2. Comet assay

Leukocytes were grown for 3 and 24 h in PB-MAX™ medium (Gibco® Life Technologies™). Then, low-melting-point agarose (0.5%, 200 μL) was added to cell suspension, 100 μL. This mixture was dropped on slides pre-treated with normal melting point agarose (1.5%) and cooled for 5 min. After solidification, the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% dimethyl sulfoxide, 1% Triton X-100, pH 10.0) and refrigerated overnight at 4 °C. Subsequently, the slides were incubated in alkaline solution (10 M NaOH, 0.2 M EDTA, and distilled water, pH 13.0) for 20 min at 4 °C. Electrophoresis was performed for 25 min/25 V (0.72 V/cm)/300 mA. Slides were neutralized (0.4 M Tris/HCl, pH 7.5), dried at room temperature (22–25 °C) and fixed in 100% ethanol for 4 min. Then, slides were submitted to 20 μg/mL ethidium bromide [21,22] and analyzed under a fluorescence microscope (Olympus BX61).

One hundred nucleoids were analyzed by considering size and amount of DNA present in the comet tail. The damage was classified into five levels: Class 0, no damage (<5%); Class 1, low damage (5–20%); Class 2, moderate damage (20–40%); Class 3, high damage (40–94%); Class 4, total damage (>95%) [23]. The damage score was obtained by multiplying the number of nucleoids in each class by the respective class value, according the equation:

$$\text{Score} = [(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/\text{total number of cells}$$

2.1.3. Cytotoxicity assay

After 48 h culture, an aliquot (50 μL) of cell suspension was stained with 2.5 μL of a fluorescent dye mixture (5 μg/mL propidium iodide, 15 μg/mL fluorescein diacetate, 2 μg/mL Hoechst 3334 and 15 μL PBS, pH 8.0) and then incubated at 37 °C for 5 min [24]. Six repetitions were performed for each treatment; 500 cells from each animal were analyzed using a fluorescence microscope (triple-band filter), totaling 3000 cells per treatment group. Cells were classified as normal (spherical nuclei stained in blue and cytoplasm stained in green), necrotic (spherical nuclei stained in red), or apoptotic (blue-stained nucleus with apoptotic bodies and green cytoplasm) [25].

2.2. In vivo assays

2.2.1. Animals

The Animal Ethics Committee of the Federal University of Maranhão (Protocol no. 23115-009229/2014-21) approved all experimental procedures. Male Swiss *Mus musculus* mice (n = 25) weighing 30 g were obtained from the Animal Breeding Unit (Federal University of Maranhão, São Luís, MA, Brazil). The animals were kept in a well cross-ventilated room at 21 ± 2 °C with relative humidity 44–56% and light and dark cycles of 12 h. They had access ad lib to sterilized food and water.

2.2.2. Doses and treatment

To perform *in vivo* tests, miltefosine (Impavido®) doses were established based on the recommended dose for treating leishmaniasis, according to the WHO, which is 2.5 mg/kg over 28 d, for cumulative dose = 70 mg/kg. Thus, each group of animals received an acute treatment at one of three doses (35; 70; and 140 mg/kg). All treatments were administered orally in volumes of 0.1 mL/10 g of body weight. The negative control group received distilled water and the positive control group received cyclophosphamide (50 mg/kg) intraperitoneally. The animals were euthanized 24 h after receiving the drug.

2.2.3. Comet assay

The standard alkaline comet assay was performed using peripheral blood cells. After 24 h treatment, a small incision was made on the extremity of the animal's tail to collect 5 μL peripheral blood, which was mixed with 100 μL low-melting-point agarose (0.5%). Then, this mixture was transferred to slides with normal-melting-point agarose (1.5%). The subsequent steps were as previously described for the comet assay *in vitro*.

The comet assay was followed by FPG (formamidopyrimidine-DNA glycosylase) digestion to test whether miltefosine induces DNA damage by base oxidation. The FPG FLARE™ kit (Trevigen, Gaithersburg, MD) was used following the protocol described by Gajski et al. [26]. After the lysis step, the slides were immersed in 1 × FLARE TM (Trevigen) for 30 min. Then, each slide was treated with 150 μL FPG solution, covered with a coverslip, and incubated at 37 °C for 45 min in a moist chamber. The coverslips were then removed and the electrophoresis, neutralization, fixation, staining, and analysis steps were performed as previously described for the *in vitro* comet assay.

2.2.4. Micronucleus test

The micronucleus test was performed in bone marrow cells collected from the same animals used for the comet assay. Briefly,