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A N   I N T E R N A T I O N A L   J O U R N A L

In Vitro Antileishmanial Properties of Neutron-Irradiated Meglumine Antimoniate

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ABSTRACT

Pentavalent antimony, as meglumine antimoniate (Glucantime®) or sodium stibogluconate (Pentostam®), is the main treatment for leishmaniasis, a complex of diseases caused by the protozoan Leishmania, and an endemic and neglected threat in Brazil. Despite over half a century of clinical use, their mechanism of action, toxicity and pharmacokinetic data remain unknown. The analytical methods for determination of antimony in biological systems remain complex and have low sensitivity. Radiotracer studies have a potential in pharmaceutical development. The aim of this study was to obtain a radiotracer for antimony, with suitable physical and biological properties. Meglumine antimoniate was neutron irradiated inside the IEA-R1 nuclear reactor, producing two radioisotopes ^{122}Sb and ^{124}Sb, with high radionuclidic purity and good specific activity. This compound showed the same antileishmanial activity as the native compound. The use of the radiotracers, easily created by neutron irradiation, could be an interesting tool to solve important questions in antimonial pharmacology.

Key words: Meglumine antimoniate, leishmaniasis, *Leishmania (L.) chagasi*, radiotracer

INTRODUCTION

Leishmaniasis is a complex of diseases caused by the protozoan parasite of the genus *Leishmania*, transmitted by the phlebotomine sand fly vector. Human leishmaniasis is distributed worldwide, but occurs mainly in the tropics and subtropics, with a prevalence of 12 million cases, causing diseases ranging from skin lesions in cutaneous leishmaniasis (CL) to a progressive and frequently fatal hepatosplenomegaly in visceral leishmaniasis (VL) (Croft and Coombs, 2003). Co-infection with HIV makes VL a priority for the World Health Organization (Guerin et al., 2002).

Pentavalent antimonials, such as meglumine antimoniate (Glucantime®) or sodium stibogluconate (Pentostam®), are the main drugs recommended in the treatment of all forms of leishmaniasis (Murray, 2001). Other alternative drugs used in the treatment are pentamidine and amphotericin B, but their use has been limited by high toxicity and cost (Singh and Sivakumar, 2004). Despite several gaps in the knowledge of action, toxicity and pharmacokinetic parameters, pentavalent antimonials have been used for over 60 years (Roberts et al., 1998) and the definition of its pharmacokinetic profile may suggest a better therapeutic protocol for doses, administration

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interval and duration of the antimonial therapy, reducing resistance, relapse and severe side effects.

The analytical methods for determination of the amount of antimony in biological systems remain complex and have low sensitivity (Rath et al., 2003). Radiotracer studies performed on animals have the potential to play a major role in pharmaceutical development, pharmacological studies and basic biochemistry research (Meikle and Eberl, 2001). Therefore, the aim of this study was to obtain a radiotracer, by neutron irradiation of Glucantime®, resulting in radioactive antimony salts, with biocompatibility and the same antileishmanial activity as the native compound.

MATERIALS AND METHODS

Production and analysis of antimony radiotracer

For standardization, samples of 0.5-0.8 mL of meglumine antimoniate (Glucantime®; Aventis, S.P., Brazil, 81 mg Sb^V/mL) were sealed in quartz ampoules and irradiated at a thermal neutron flux of $1x10^{13}$n/cm^2.s, for 20, 15 and 7 minutes, inside the IEA-R1 nuclear reactor (IPEN-CNEN/SP). Radionuclidic purity was determined by γ-spectrometry, using an HPGe detector (Canberra Company) coupled to the Geniepec program. Radioactive concentration was also measured with the same system after efficiency calibration with standard ^{60}Co, ^{137}Cs and ^{152}Eu sources. UV-visible spectrometry Ultrospec 3000 (Pharmacia Biotech) was used for the chemical determination, of samples of the neutron-irradiated meglumine antimoniate (IMA) and non-neutron-irradiated meglumine antimoniate (NMA) by scanning the spectrum from 200 to 700 nm,.

Determination of the 50% Effective Concentration (EC_{50})

The antileishmanial activity against promastigotes was determined as described elsewhere (Tempone et al., 2005). Cold irradiated and non-irradiated meglumine antimoniate were diluted in M199 medium supplemented with 10% FBS, at different concentrations and incubated with $1x10^6$ L. (L.) chagasi promastigotes (M6445 strain)/well in 96-well micro plates. The plates were incubated for 24 h at 24 °C and the viability of promastigotes was verified by the MTT assay. The sigmoid dose-response analysis was accomplished using Graph Pad Prism 3.0 software.

Cytotoxicity assay.

RAW 264.7 (ATCC TIB-71) cells were seeded in 96-well micro plates at $4x10^4$/well. Drugs were serial diluted in RPMI – PR-1640 with 10% FBS medium at different concentrations. The plates were incubated for 48 h at 37 °C in a 5% CO_2 incubator. The viability of the macrophages was determined by the MTT assay. The sigmoid dose-response analysis was performed using Graph Pad Prism 3.0 software.

Antileishmanial activity against intracellular amastigote.

Macrophages were collected from the peritoneal cavity of female BALB/c mice by washing with RPMI – PR-1640 medium with 10% FBS. They were then seeded at $4x10^5$/well into 24-well plates containing glass cover slips for a period of 2 h at 37 ºC in a humidified 5% CO_2 95% air incubator for attachment. Non-adherent cells were removed by one-step washing with medium and the subsequent incubation was performed for 24 h undert the same conditions. L. (L.) chagasi amastigote (M6445 strain) were obtained from the spleen of the golden hamster by differential centrifugation and added at a ratio of 1:10 (macrophage/amastigote) and incubated for 18 h. Macrophages were treated with cold irradiated and non-neutron-irradiated meglumine antimoniate during 96 h. Samples without drugs were used for control (100% infected). At the end of the assay, macrophages were fixed with methanol and stained with Giemsa. The parasite burden was verified as the number of infected macrophages in a total of 400 cells. The data were analyzed by a sigmoid dose-response curve using Graph Pad Prism 3.0 software.

RESULTS AND DISCUSSION

The neutron irradiation of meglumine antimoniate in a nuclear reactor produced two radioisotopes of antimony: ^{122}Sb and ^{124}Sb. High radionuclidic purity was verified, where ^{122}Sb ($t_{1/2}$ = 2.7 days) gamma peaks were observed in 563.99 keV (69.3%) and 692.94 keV (3.78%) and ^{124}Sb ($t_{1/2}$ = 60.2 days) in 602.66 keV (97.8%), 645.77 keV

(7.38%), 668.87 keV, 709.26 keV (1.35%), 713.73 keV (2.40%), 722.75 keV (10.76%), 790.46 keV (0.74%), 968.31 keV (1.8%), 1045.15 keV (1.84%), 1368.43 keV (2.62%) e 1691.51 keV (47.34%). Activity at the end of irradiation was 33.2 MBq (0.897 mCi) of ^{122}Sb and 0.77 MBq (0.021 mCi) of ^{124}Sb, corresponding to a specific activity of 22.12 MBq, ^{122}Sb/mL of meglumine antimoniate (0.598 mCi/mL), and 0.52 MBq ^{124}Sb/mL of meglumine antimoniate (0.014 mCi/mL). After analysis of the IMA, the samples neutron-irradiated for a longer period (20 and 15 minutes) showed an intense color change, with spectrophotometric modifications (data not shown) and with physical-chemical alterations, as compared to NMA. Samples neutron-irradiated for seven minutes presented very little absorbance at 300 nm, with a slight color change, probably due to the meglumine polymer formation. The same effect was verified when the product was exposed to unsuitable conditions of temperature, lighting, and bad storage conditions (Romero et al., 1996).

During the *in vitro* assessment of biological activity of the meglumine antimoniate, neutron irradiated for seven minutes, the same effectiveness against amastigotes, tested *in vitro* with *L. (L.) chagasi* infected macrophage (Fig. 1),

as with the native product, was observed, despite some enhancement of activity against promastigotes or living cells (Table 1).

It has been suggested that Sb^V is a prodrug and is intracellularly reduced to trivalent form (Sb^{III}), the active form of the drug, at or near the site of action. The antileishmanial activity of Sb^V has been found to be dependent on its reduction to Sb^{III} inside the parasite (Shaked-Mishan et al., 2001). The greater susceptibility of Sb^V to intracellular amastigotes than promastigotes suggests that the reductive activation of the drug occurs within the intracellular amastigotes (Yan et al., 2003).

The meglumine antimoniate, neutron irradiated for seven minutes, showed high radionuclidic purity, good specific activity, maintenance of the biological activity and suitable physiological characteristics, allowing its use in biodistribution studies, It furnished evidence to advance the pharmacokinetic study for antileishmanial therapy. The use of neutron irradiation for developing antimony radiotracers could be an interesting tool for solving important questions in antimonial pharmacology.
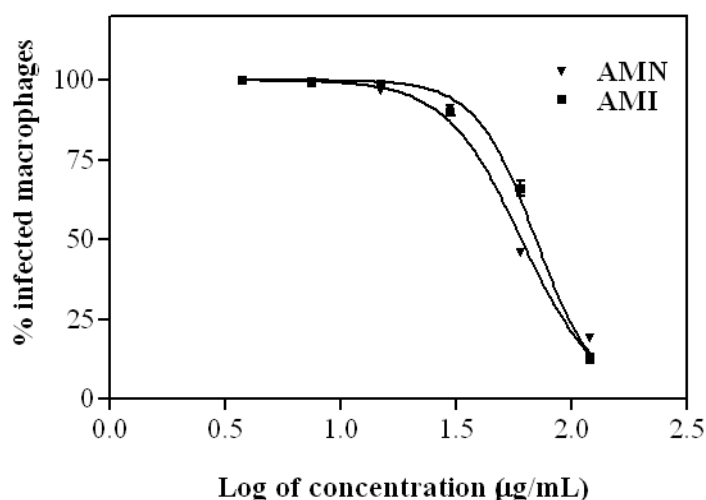

Figure 1 - Determination of EC_{50} of IMA and NMA against *L. chagasi*-infected macrophages. Macrophages were treated for 96 h at 37°C with drugs, and the number of infected macrophages in Giemsa-stained glass cover slips was determined by light microscopy. Dose-response curves were obtained with GraphPad Prism 3.0 software. Data are mean and standard deviation. IMA = neutron-irradiated meglumine antimoniate (1×10^{13}n/cm^2.s, 7 minutes); NMA = non-neutron-irradiated meglumine antimoniate

Table 1 - Effectivity of the neutron-irradiated meglumine antimoniate in *L. (L.) chagasi promastigotes,* macrophages infected with *L. (L.) chagasi* amastigotes and mammalian cells. IMA= neutron-irradiated meglumine antimoniate (thermal neutron flux of $1x10^{13}n/cm^2.s$, for 7 minutes); NMA= non-neutron-irradiated meglumine antimoniate. EC 50% (IC95%), (n=2).

DRUG CELL	IMA	NMA
L. (L.) chagasi promastigotes	20.94 mg Sb/mL (16.73 – 26.2)	41.73 mg Sb/mL (32.67 – 53.30)
L. (L.) chagasi amastigotes	70.62 µg Sb/mL (64.22 – 77.67)	61.14 µg Sb/mL (52.68 - 70.96)
Mammalian cells	1.43 mg Sb/mL (0.99 – 2.07)	3.56 mg Sb/mL (2.37 – 5.35)

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RESUMO

Os antimoniais pentavalentes, como o antimoniato de meglumina (Glucantime®) ou estibogluconato de sódio (Pentostam®), são o principal tratamento para a leishmaniose, um complexo de doenças causadas pelo protozoário parasita *Leishmania*, uma doença endêmica e negligenciada no Brasil. Apesar do seu uso clínico por mais de meio século, seu mecanismo de ação, toxicidade e dados de farmacocinética permanecem desconhecidos. Os métodos analíticos para determinação de antimônio em sistemas biológicos são complexos e apresentam baixa sensibilidade. Estudos utilizando radiotraçadores têm papel potencial no desenvolvimento farmacológico. O objetivo deste estudo foi desenvolver um radiotraçador de antimônio, com propriedades físicas e biológicas adequadas. O antimoniato de meglumina foi irradiado por nêutrons no reator nuclear IEA-R1, produzindo dois radioisótopos: ^{122}Sb e ^{124}Sb, com alta pureza radionuclídica e boa atividade específica. Este composto mostrou atividade antileishmania similar ao fármaco não irradiado. O uso de radiotraçadores, facilmente produzidos por irradiação por nêutrons pode ser um importante instrumento para elucidar questões sobre a farmacologia dos antimoniais.

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