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Research Paper

Antiparasitic activity of menadione (vitamin K_3) against *Schistosoma mansoni* in BABL/c mice

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ABSTRACT

Schistosomiasis is one of the neglected tropical diseases affecting nearly quarter of a billion people in economically challenged tropical and subtropical countries of the world. Praziquantel (PZQ) is the only drug currently available to treat this parasitic disease in spite being ineffective against juvenile worms and concerns about developing resistance to treat reinfections. Our earlier *in vitro* viability studies demonstrated significant antiparasitic activity of menadione (MEN) (vitamin K_3) against *Schistosoma mansoni* adult worms. To gain insight into plausible mechanism of antischistosomal activity of MEN, its effect on superoxide anion levels in adult worms were studied *in vitro* which showed significant increases in both female and male worms. Further confirmation of the deleterious morphological changes in their teguments and organelles were obtained by ultrastructural analysis. Genotoxic and cytotoxic studies in male Swiss mice indicated that MEN was well tolerated at the oral dose of 500 mg/kg using the criteria of MNPCE frequency and PCE/RBC ratio in the bone marrow of infected animals. The *in vivo* antiparasitic activity of MEN was conducted in female BALB/c mice infected with *S. mansoni* and significant reductions ($P < 0.001$) in total worm burden were observed at single oral doses of 40 and 400 mg/kg (48.57 and 61.90%, respectively). Additionally, MEN significantly reduced ($P < 0.001$) the number of eggs in the liver of infected mice by 53.57 and 58.76%, respectively. Similarly, histological analysis of the livers showed a significant reduction ($P < 0.001$) in the diameter of the granulomas. Since MEN is already in use globally as an over-the-counter drug for a variety of common ailments and a dietary supplement with a safety record in par with similar products when used in recommended doses, the above antiparasitic results which compare reasonably well with PZQ, make a compelling case for considering MEN to treat *S. mansoni* infection in humans.

1. Introduction

Schistosomiasis is a chronic disease caused by worms of the genus *Schistosoma* that affects more than 200 million people worldwide (Colley et al., 2014; Rollinson et al., 2013). *S. mansoni* is the major etiological agent of human schistosomiasis which is currently endemic in Africa, the Middle East, the Caribbean and South America (Clerinx and Soentjens, 2015; WHO, 2014). To-date the development of a vaccine for this tropical disease remains an arduous task due to the existence of multiple species of schistosomes which are capable of building resistance and the fact that they can survive in human host for more than 30 years (Ismail et al., 1999). Currently, the control of schistosomiasis relies on a single drug, praziquantel (PZQ), a broad spectrum anthelmintic, administered in one oral dose at a low cost for infection from all *Schistosoma* species with high margin of safety (Caffrey, 2015). However, due to the extensive and excessive use of PZQ, concerns about this drug developing resistance have recently emerged which limit its use in treating reinfections that occur frequently (Qi and Cui, 2013; El Ridi and Tallima, 2013; Wang et al., 2012; Doenhoff et al., 2008). Additionally, PZQ is less effective against juvenile worms during the early stages of infection (Hines-Kay et al., 2012; Pica-Mattoccia and

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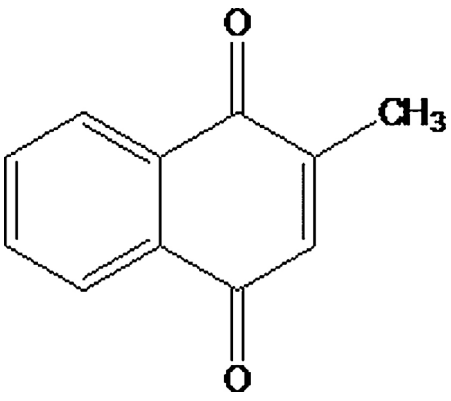

Fig. 1. Chemical structure of Menadione (2-methyl-1,4-naphthoquinone) (MEN).

Cioli, 2004). Thus, there is urgent need for affordable, new drugs to supplement or replace PZQ in managing this communicable disease now spreading rapidly in both developing and developed tropical countries of the world.

Menadione (2-methyl-1,4-naphthoquinone) (MEN) (Fig. 1) is a synthetic provitamin (vitamin K_3) which is metabolized in liver to vitamin K (Dialameh et al., 1971), an essential vitamin for the formation of prothrombin, a glycoprotein responsible for blood clotting and Gla-containing proteins that function as clotting factors and bone calcification regulators (Hassan, 2013; Ferland, 2012). It is also a catabolic product of orally ingested vitamins K, K_1 and K_2 groups in the intestine (Hirota et al., 2013; Thijssen et al., 2006). MEN is widely used around the world as an over-the-counter (OTC) drug for treatment of diarrhea, colitis, abdominal cramps, hemorrhage, hypoprothrombinemia, hay fever and joint pains, and as a nutrient in vitamin K deficiency and in pet foods (European Food Safety Authority, 2014; Ferland, 2012; IARC, 2000). Such use of MEN is banned in the U.S. due to concerns about its utilization in excessive quantities might lead to hemolytic anemia, and neonatal brain and liver damages (Ferland, 2012; IARC, 2000). However, numerous homeopathic drug formulations, cosmetic products and nutritional supplements containing MEN are readily available in most U.S. drug and dietary supplement stores. It is noteworthy that MEN is affirmed by Federal Drug Administration (FDA) as GRAS (generally recognized as safe) and has approved its use in the U.S. as a feed ingredient for poultry and swine, and as an antifungal agent in agriculture (FDA, 2015; IARC, 2000). While toxicity of MEN is a legitimate concern, it is noteworthy that its safety is proven over the years to be in par with other common OTC drugs and supplements when used in recommended doses.

During our recent investigations of plant-derived and synthetic simple naphthoquinones (NAPQs) for antiparasitic activity (Magalhães et al., 2014; Layzama-Davila et al., 2012; Kapadia et al., 2001), we demonstrated that MEN was the most active synthetic 1,4-NAPQ among a series of related eight compounds tested *in vitro* against *S. mansoni* with minimum lethal concentration (MLC) of 12.5 μM and lethal **c**oncentration (LC_{50}) of 5.8 μM when incubated for 72 h (Magalhães et al., 2014). Thus, these *in vitro* results identified MEN to be the best candidate among the NAPQs tested for further *in vivo* studies in animal models of schistosomiasis.

The present study reports additional *in vitro* investigations and *in vivo* antiparasitic activity of MEN in female BALB/c mice infected with *S. mansoni* using PZQ as the positive control. Also, genotoxicity and cytotoxicity of MEN were assessed in male Swiss mice. Additionally, to gain insight into potential mechanism of antischistosomal activity of MEN, ultrastructural alterations by transmission electron microscopy (TEM) and effect on superoxide anion production in MEN-treated female and male *S. mansoni* parasites were investigated.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals were purchased from Aldrich Chemical Company, Milwaukee, WI, USA: dimethylsulfoxide (DMSO), ethanol, formaldehyde, glutaraldehyde, lead citrate, menadione (MEN) (2-methy1,4-naphthoquinone), methanol, methylthiazolyldiphenyl-tetrazolium bromide (MTT), methyl methanesulfonate (MMS); *p*-nitrotetrazolium blue (NBT), osmium tetroxide, praziquantel (PZQ), potassium hydroxide (KOH), uranyl acetate and xylol. RPMI-1640 culture medium, penicillin/streptomycin and bovine fetal serum (FBS) were purchased from Gibco Life Technologies, Gaithersburg, MD, USA. Araldite 6005 resin was purchased from EMS, Hatfield, PA, USA. Paraffin was purchased from Merck, Darmstadt, Germany.

2.2. Animals

Female BALB/c mice, 6 weeks old and weighing 20–25 g (for *S. mansoni* maintenance and *in vivo* antiparasitic studies) and male Swiss mice, 6 to 8-week-old and weighing ~30 g (for cytotoxic and genotoxic studies) were supplied by the Animal House of the University of São Paulo, Brazil. All animals were acclimated for a period of one week before the beginning of experiments. Mice were housed in plastic bins with wire tops and wood chip bedding (5 mice per bin) at the university animal research facility under controlled conditions of temperature (22 ± 2 °C) and humidity (50 ± 10%), and a 12-h light–dark cycle. They were fed standard rat chow (Labina, São Paulo, Brazil) with access to water *ad libitum*. All experiments were authorized by the Ethical Committee for Animal Care of the University of Franca (Approval number: 048/15 or 001/14) and were handled with good animal practice as defined by the University of Franca which is in accordance with the Brazilian legislation (CEUA, 11.794/2008).

2.3. Parasite maintenance

The life cycle of *S. mansoni* (Luiz Evangelista strain) was maintained by serial passage through *Biomphalaria glabrata* snails (invertebrate host) and female BALB/c mice (vertebrate host) at the University of Franca animal facility. Cercariae were obtained from infected snails exposed to light for 1 h after 38 days of infection according to the standard procedures of our laboratory (Magalhães et al., 2014). For the *in vitro* study, adult worm pairs were harvested under aseptic conditions after 49 ± 2 days from mice previously infected with 200 cercariae by perfusion of their livers and mesenteric veins (Smithers and Terry, 1965).

2.4. In vitro *schistosomicidal assays*

2.4.1. Parasite viability assay

To analyze the parasite viability when exposed to test compound, one adult worm pair of *S. mansoni* was placed in each well of a 24-well culture plate containing RPMI 1640 medium, pH 7.4 with 20 mM HEPES buffer and supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml) and 10% FBS. Plates were incubated at 37 °C in a humid atmosphere containing 5% CO_2 for 24 h for adaptation. Then, the adult worm pairs were incubated for 6, 12 or 24 h with different concentrations of MEN (6.25–100 μM) or PZQ (0.39–12.5 μM). During incubation period, the worms were monitored every 6 h using an inverted microscope (Zeiss AG, Germany). The phenotypic changes were scored using a viability scale of 0–3 (3 = normally active; 2 = slower activity when compared with control medium; 1 = minimal activity, occasional movement of head and body; 0 = death of all worms) (Manneck et al., 2010). The