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Repositioning of chlorambucil as a potential anti-schistosomal agent

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ABSTRACT

As parasites and cancer cells share many lifestyle and behavioral resemblances, repositioning of anti-cancerous agents as anti-parasitic is quite trendy, especially those sharing the same therapeutic targets. Therefore, the current study investigated the *in vitro* efficacy of ascending concentrations of chlorambucil (0.5–20 μg/ml) against adult *Schistosoma mansoni* worms, over 72 h. Additionally, its *in vivo* effects against the different developmental stages of the worm were assessed, after an oral dose of 2.5 mg/kg/day for five successive days, through evaluating the worm load reduction and worms' morphological alterations and oogram changes. In addition to tissue egg count, a histopathological study of the liver was conducted. *In vitro*, chlorambucil demonstrated noticeable anti-schistosomal effects in the form of progressive reductions of the worms' viability in a dose dependent manner. Complete worm death was achieved at 72 h incubation with 5 μg/ml drug concentration. *In vivo*, chlorambucil induced a significant reduction in the total worm load against all developmental stages. Its highest impact was evident against the juvenile stage, where it induced 75.8% total worm load reduction, and 89.2% and 86.7% intestinal and hepatic egg counts reduction, respectively, along with ogram alterations. Besides, it induced significant shortening of both male and female worms and promoted an amelioration of hepatic histopathology. Results show that chlorambucil possesses favorable *in vitro* and *in vivo* anti-schistosomal activity. The highest *in vivo* efficacy was against the juvenile stage of *S. mansoni*, significantly superior to praziquantel, with extended potency to the adult stage. Further studies are recommended for chlorambucil target verification and to enhance its therapeutic efficacy.

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1. Introduction

Schistosomiasis is a chronic water-borne helminthic disease, which is endemic in many tropical and subtropical countries. The current global estimate indicates that 258 million people, mostly children, are affected, with 90% of them living in Africa. It was reported that in 2014, 61.6 million patients have been treated for schistosomiasis. The estimated annual loss is nevertheless very high, with more than 200 thousand deaths in sub-Saharan Africa (WHO, 2016).

The preventive chemotherapy of schistosomiasis is based on regularly targeted treatment with praziquantel (PZQ), which is effective against all schistosome species causing the disease. This drug needs to be regularly applied in mass treatment programs to achieve sustainable control over schistosomiasis, as it lacks activity against young developing stages of the parasite (Doenhoff et al., 2008). As a result of the intensive and widespread use of PZQ, reduced rates of cure and treatment failure have been reported (Gryseels et al., 2001). Moreover, reduced susceptibility of *S. mansoni* isolates to PZQ has been reported in many areas (Ismail et al., 1999). The appearance of resistant strains is a first step towards widespread drug resistance, therefore; it is urgent to seek potential alternative anti-schistosomal agents (Wang et al., 2012).

The development of new drugs from lead compounds faces many obstacles, the most important among those are the long time and high costs consumed during this process. Drug repositioning provides an alternative way for drug discovery that can identify new therapeutic uses for existing drugs. The availability of clinical safety, tolerance and efficacy data of these drugs prominently reduce time and failure risk compared to traditional drug discovery (Thomson Reuters, 2012).

The lifestyle similarities between parasites and cancer have attracted parasitologists to use the same therapeutic target approaches used in cancer. Both cancer cell and parasites are

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self-directed and independent of the regular signaling mechanisms, although they get their own benefit of signals and resources (Oliveira, 2014). Additionally, they share several benchmarks including; high metabolic activities, dependence on lactate fermentation as an energy source within the human host, uninhibited cell division and a degree of hiddenness to the host immune responses. Schistosomes are considered as cancerous growths as they have extreme metabolic activities and a high rate of division, especially female worms for the production of eggs that is beyond the control of the host (Pierce et al., 2012). Hence, a potentially profitable starting point for new drugs discovery to fight parasites is to examine available compounds already developed against cancer for anti-parasitic properties. As a result an anti-cancerous drug with a good tolerability profile is an excellent candidate for anti-schistosomal drug repurposing (Klinkert and Heussler, 2006).

Promising results were obtained with anti-cancerous drugs like miltefosine that proved to have anti-schistosomal activity, showing comparable advantage over PZQ in being effective against the different developmental stages of *S. mansoni* (Eissa et al., 2011, 2015). Nilutamide, an anti-androgen, displayed a potent anti-schistosomal activity. However, it exhibited a low activity against the juvenile stage (Keiser et al., 2010).

Chlorambucil is an-orally active anti-neoplastic alkylating agent that cross-links DNA during all phases of the cell cycle, hence resulting in disruption of DNA function, cell cycle arrest and apoptosis (Calabresi and Schein, 1993). It is used for the therapy of chronic lymphocytic leukemia, low-grade non-Hodgkin's lymphoma and Hodgkin's disease (British Columbia (BC) Cancer Agency, 2013). Its toxicity limited at lower doses and it is relatively well tolerated by patients (Nicolle et al., 2004). It is quickly metabolized in the liver to active metabolite phenylacetic acid mustard. It has a high plasma protein binding (British Columbia (BC) Cancer Agency, 2013). Chlorambucil efficiently inhibits mammalian thioredoxine reductase (TrxR) (Witte et al., 2005), which shares the same active sites of schistosomal thioredoxine glutathione reductase (TGR), one of the candidate chemotherapeutic targets in the redox metabolism of schistosome worms (Song et al., 2012). The limited antioxidant capability of schistosomes, as they lack catalase and have a low activity of glutathione peroxidase, make them more prone to oxidative burst and provide a point of weakness in the parasite biology for anti-schistosomal drug development (Mkoji et al., 1988; Sayed et al., 2006). In contrast to vertebrates, *S. mansoni* are dependent on a single multifunctional oxidoreductase, TGR, which replaces both TrxR and glutathione reductase (GR) in the parasite. This suggests that the worm's redox system is subject to a bottleneck dependence on TGR, an essential enzyme for schistosome's survival, as the antioxidant pathway primarily depends on it to provide reduced thioredoxine (Trx) and glutathione disulfide (GSSG). Moreover, the schistosomal TGR differs from that of mammalian TrxR and GR in the biochemical structure by having a glutaredoxin domain at the amino terminal (Alger and Williams, 2002). Due to its ability to inhibit TrxR, its relatively low toxicity, the possibility of its oral administration and its low cost, this study has investigated the potential *in vitro* and *in vivo* anti-schistosomal activity of chlorambucil against different developmental stages of *S. mansoni*.

2. Materials and methods

2.1. Drugs

- 1.1.1PZQ (Distocide ™- 600 mg tablets, EIPICO, Egypt) was used as therapeutic control. For the *in vitro* assay, it was dissolved in 0.5% dimethyl sulfoxide (DMSO, Sigma-Aldrich). For *in vivo* assay, freshly prepared solution in 60% ethanol, with a concentration of 10 mg/ml, was given orally at a dose of 200 mg/kg/day for five successive days (Botros et al., 2004).
- 1.1.2Chlorambucil (Leukeran ™- 2 mg tablets, GlaxoSmithKline, Germany) stored in the refrigerator at 4 °C (British Columbia (BC) Cancer Agency, 2013). For the *in vitro* assay, it was dissolved in 0.5% DMSO immediately before being used to obtain stock solution of 400 μg/ml (Moraes, 2012). For the *in vivo* assay, ten tablets were dissolved in 100 ml 96% ethanol; with a concentration of 200 μg/ml and were divided into aliquots and kept frozen at −20 °C until use. Shortly before use, aliquots were thawed and further down diluted to reach ethanol concentration 60% (Tomenendalova et al., 2008a). The used solution had a drug concentration of 120 μg/ml.

2.2. Experimental animals

Male Swiss strain albino mice, 4–6 week old, weighing 20–25 g each, were used. They were allowed free access to drinking water and food and were used for parasite maintenance, pilot study and experimental design. All work with laboratory animals was conducted in accordance with the Egyptian National Animal Welfare Standards and was approved by the Ethics Committee of Alexandria Faculty of Medicine (Protocol approval number: 0102426).

2.3. Snail, parasite and animal infection

Swiss strain albino mice, previously infected seven weeks earlier with the Egyptian strain of *S. mansoni*, and susceptible laboratory-bred non-infected *Biomphalaria alexandrina* snails, were both purchased from the Schistosome Biologic Supply Center, Theodor Bilharz Research Institute, Giza, Egypt. The snails were infected by *S. mansoni* eggs; obtained seven weeks post infection (p.i.) from livers of the infected mice (El Naga et al., 2010). Four weeks later, cercariae were gathered from infected snails and were used for animal infection at a dose of 100 ± 10 cercariae/mouse by the paddling tail immersion method described by Smithers and Terry (1965).

2.4. In vitro anti-schistosomal assay

Ten mice, infected seven weeks earlier with 100 ± 10 *S. mansoni* cercaria, were perfused for adult worm recovery according to Smithers and Terry (1965). Recovered worms were washed three times with a culture medium, composed of RPMI 1640 medium with 25 mM HEPES (pH 7.5) supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Three pairs of adult worms (single male and female) were transferred to 35 mm diameter polystyrene dishes with 2 ml of the same medium supplemented with 10% bovine foetal serum (Moraes, 2012). The chlorambucil stock solution was diluted in phosphate buffered saline to obtain final concentrations of 0.5, 1, 5, 10 and 20 μg/ml in culture plates with a final volume of 2 ml (Bentley and Blackmore, 1992). Three replicates were set up, in each set the worms were divided into three groups as follows: Group I, a medium containing 0.5% DMSO was used as negative control; Group II, a medium containing 10 μg/ml praziquantel was used as a positive control; Group III, chlorambucil-treated group where the worms were incubated with the five different drug concentrations (0.5, 1, 5, 10 and 20 μg/ml). Worms were incubated at 37 °C in a humid atmosphere containing 5% CO2 and were monitored every 24 h for three days using an inverted microscope. Worms were examined for their viability using a viability scale as described by Manneck et al. (2011). Briefly, drug activity was defined by grades; grade 3, normal motor activity with no morphological changes; grade 2, slow motor activity with primary morphological changes such as shortening or extra-lenghthening; grade 1, minimal motor

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