**In Vitro** Activity of Novel Metronidazole Derivatives on Larval Stages of *Echinococcus granulosus*

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**Abstract**

The effects of metronidazole (MTZ) and novel synthesized MTZ derivatives on **in vitro** cultured *Echinococcus granulosus* protoscoleces (PSCs), 30 day old segmentation stage and hydatid cysts (HC) developing secondarily in BALB/c mice were compared to those caused upon treatment with comparable doses of albendazole (ABZ) and mebendazole (MBZ) drugs. The highest protoscolicidal action resulted from the use of a non-schiff based MTZ derivative (MTZ-w: 4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyloxy] benzylddehyde). Incubation of PSCs with MTZ-w concentrations of 25, 12.5 and 6.25 µg/ml resulted in significantly higher mortality rates than those caused by ABZ or MBZ at all periods post incubation. Total mortality of PSCs always occurred one day earlier using MTZ-w. Moreover, incubation of PSCs with MTZ-w at 6.25 µg/ml concentration resulted in greater mortality of PSCs than that caused by ABZ at 25ug/ml concentration. Three other MTZ derivatives showed similar **in vitro** effects on PSCs to those caused by ABZ or MTZ. Light microscopy revealed that changes in PSCs exposed to MTZ derivatives and ABZ reflected their relative actions in targeting scolex hooks, suckers and tegument. MTZ-w and ABZ caused rupture of hooks, deformation in suckers and disintegration in tegument of both PSCs and **in vitro** cultured segmentation stage. Less detrimental changes occurred upon the exposure to other MTZ derivatives. Exposure of HC to MTZ-w and ABZ caused regression in their size, damage in germinal membrane, fragmentation of underlying tissue, and scaling of laminated membrane. MTZ-w warrants further assessment as a potential chemotherapy drug against cystic echinococcosis in both animals and humans.

**Keywords**: *Echinococcus granulosus*, Protoscolex, Hydatid cysts, Albendazole, Mebendazole, Metronidazole, Metronidazole derivatives.

**1. Introduction**

Cystic echinococcosis (CE) or unilocular hydatidosis is a cosmopolitan cyclozoonotic helminthic disease of livestock and humans with great public health and economic effects in various continents. While it is currently spreading into new developing countries and increasing in prevalence, CE is still classified with the emerging or re-emerging neglected diseases (Moro and Schantz, 2009; McManus, 2010; Da Silva, 2010).

The disease is caused by the ingestion of embryonated eggs of the tiny dog tapeworm *Echinococcus granulosus* (Eucestoda, Platyhelminthes) whose adult stage inhabits the small intestine of dogs, or any of the canid family as the main definitive host. In livestock and humans, unilocular hydatid cysts (HC) develop in various visceral organs – mainly liver and lungs. Each HC contains an outer a cellular laminated layer (LL) and inner cellular germinal layer (GL) that undergoes asexual reproduction resulting in huge number of protoscoleces (PSC) in a fluid filled environment. Symptoms are often caused when cysts make mechanical pressure on the surrounding tissues and by cyst rupture and aggregated secondary infection. Moreover, spillage of cyst fluid containing PSC leads to secondary hydatidosis (Eckert and Deplazes, 2004; McManus, 2010).

Current treatment of CE depends on one or a combination of the following strategies: surgery, puncture of cyst- aspiration-injection of protoscolicidal chemicals...
and re-aspiration (PAIR), and chemotherapy (Eckert and Deplazes, 2004; Kern, 2006). However, none of these strategies is a conclusive treatment of human CE. Chemotherapeutic treatment of CE depends mainly on the use of benzimidazole compounds particularly albendazole (ABZ) and alternatively mebendazole, praziquantel and nitazoxanide (Hemphill and Muller, 2009). However, the non-optimal efficacy of these drugs, long periods of treatment needed, and the suffering caused to patients from serious side effects warrant careful search for alternative therapeutic approaches (Moros and Schantz, 2009; Hemphill and Muller, 2009; Vuitton, 2009). Chemotherapeutic applications based on the discovery of novel drugs for treatment of CE are thus needed (Vuitton, 2009; Ceballos et al., 2009; Gavidia et al., 2009). Such drugs should have selective and rapid scolicidal effects for both PSCs and HC stages with minimal local and systemic adverse effects on the host. It has been postulated that drugs which have found to be effective against other eukaryotic protozoal and helminthic parasites and/or cancer cell lines are primary candidate choices for testing against CE (Hemphill and Muller, 2009). Metronidazole (MTZ) and many of its newly synthesized derivatives match these properties, and also have been found to inhibit certain cancer cell lines and the growth of cultured Giardia intestinalis and Entamoeba histolytica (Abu Shaireh et al., 1992; Saadeh et al., 2010; 2011).

This study was designed to investigate the effects of MTZ and many of its newly synthesized Schiff-based and non-Schiff based derivatives against freshly prepared PSC and in vitro cultured stages of E. granulosus. Moreover, the ultrastructural effects of the most effective scolicidal compounds on secondary HC developing in mice were explored.

2. Materials and Methods

2.1. Parasites

PSCs were isolated from the livers of infected indigenous sheep slaughtered at abattoirs in Jordan as described previously (Hijjawi et al., 1992). All steps were done under sterile conditions using a vertical laminar flow hood (Flow lab, Irvine, Scotland, UK). Infected sheep offal was washed using soap, and well defined cysts were separated individually and washed three times with 1% iodine in 95% ethyl alcohol. The hydatid fluid (HF) containing PSCs was aspirated using 20 ml sterile syringe fitted with a 19g needle. PSCs were collected asexptically from the HF of fertile cyst or by scraping the GL of fertile cysts. The viability of fertile cysts was measured as a relative number of live PSC to total number of them. At least three samples were counted to determine PSC viability with a minimum of 100 PSC/sample. Discrimination between live and dead PSC was made using methylene blue dye as a vital stain (Gold, 1997, Liu et al., 2013). Only HC with at least 80% viability and free from bacterial contamination were used. Live PSC were separated from dead ones that were digested out using 0.25% trypsin suspension solution prepared in phosphate buffer saline (PBS) in a 1:10 ratio. Tryptsin treatment was made in water bath at 37°C with gentle shaking (60 cycles per minutes) for 30 min. In vitro culturing of freshly prepared PSCs and subsequent developing stages was carried out as described by Hijjawi et al. (1997). All experiments were carried out in 24 well culture plates. RPMI 1640 containing 20% (v/v) fetal calf serum (Invitrogen, Grand Island, New York, USA), 0.45% (w/v) yeast extract, 0.4% (w/v) glucose, penicillin/streptomycin suspension containing 400 IU penicillin and 400 μg/ml streptomycin (Flow Lab, Irvine, Scotland) and amphotericin B suspension containing 400 μg (Hyclone Labs, Thermo Scientific, Logan, Utah, USA) was used as the standard culture medium (SCM).

To prepare the first segmentation stage of E. granulosus, PSCs were cultured in RPMI-1640 SCM for 30 days in 160 ml culture flasks. The culture medium was changed weekly. These 30 day old cultured stages reached the first segmentation stage (S5 stage using Smyth’s designation) (Smyth, 1967).

Secondary hydatidosis was developed in five BALB/c female mice which were injected subcutaneously with 1000 freshly isolated PSCs prepared in 1ml PBS (pH 7.2) when mice were two weeks old (Kakru et al., 2008). After four months, mice were killed by cervical dislocation and developing HCs were dissected out from subcutaneous tissue and maintained in RPMI-1640 medium. Clumped cysts were separated individually and washed three times in PBS (pH 7.2) containing 400 IU/ml penicillin and 400 μg/ml streptomycin before being exposed to standard drugs and chemical compounds (see below).

2.2. Drugs and Chemical Compounds

Drugs and chemical compounds (Figure 1) that were tested for their efficacy against cultured PSCs and metacestode stages include Albendazole (ABZ) [Methyl 5-propylthio-2-benzimidazolecarbamate] (Satish Joshi, Kikma Pharmaceuticals, Mumbai, India) which was used as a positive control drug of choice for the treatment of CE, Mebendazole (MBZ) [5-benzoyl-1H-benzimidazole-2-yl] (Satish Joshi, Kikma Pharmaceuticals, Mumbai, India) which was used as another positive control commercial drug, Metronidazole (MTZ) [1-(2-Hydroxy-1-ethyl)-2-methyl-5-nitroimidazole] (Acrós Organics, New Jersey, USA) and the following novel MTZ derivatives that were prepared, purified, and characterized previously (Abu Shaireh et al., 2009; Saadeh et al., 2010; 2011).

(MTZ-a): (4-Fluoro-benzylidine)-[2-(2-methyl-5-nitroimidazol-1-yl)-ethyl]-amine,
(MTZ-b): (4-Chloro-benzylidine)-[2-(2-methyl-5-nitroimidazol-1-yl)-ethyl]-amine,
(MTZ-c): (4-Methoxy-benzylidine)-[2-(2-methyl-5-nitroimidazol-1-yl)-ethyl]-amine,
(MTZ-d): (4-Nitro-benzylidine)-[2-(2-methyl-5-nitroimidazol-1-yl)-ethyl]-amine,
(MTZ-e): 2-[2-(2-Methyl-5-nitroimidazol-1-yl)-ethyl]-amino-[methyl]-phenol,
(MTZ-f): 4-Chloro-2-[2-(2-methyl-5-nitroimidazol-1-yl)-ethyl]-amino-[methyl]-phenol,
(MTZ-g): 2-Chloro-benzylidine)-[2-(2-methyl-5-nitroimidazol-1-yl)-ethyl]-amine,
(MTZ-h): [2-(2-Methyl-5-nitroimidazol-1-yl)-ethyl]-thiophen-2-yl methylene-amine,
(MTZ-w): 4-[2-(2-methyl-5-nitroimidazol-1-yl) ethyloxy] benzyldehyde.
In vitro effects of MTZ derivatives on fresh PSCs

Among all MTZ derivatives tested, the highest protoscolicidal action resulted from the use of MTZ-w compound in which the mortality rates were consistently higher than comparable ABZ or MBZ drug concentrations (25, 12.5 and 6.25 µg/ml) throughout the periods of post-incubation with these compounds (Figures 2-4).
Figure 2. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 25 μg/ml ABZ, MBZ, MTZ or its derivatives. "@: standard deviation was not placed because the values represent only one or two observations".

Figure 3. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 12.5 μg/ml ABZ, MBZ, MTZ or its derivatives. "@: standard deviation was not placed because the values represent only one or two observations".
Figure 4. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 6.25 μg/ml ABZ, MBZ, MTZ or its derivatives. "@: standard deviation was not placed because the values represent only one or two observations".

Moreover, total mortality always occurred one day earlier upon the use of MTZ-w compared to that when PSCs were exposed to ABZ. Mortality of cultured PSC in the presence of ABZ increased slowly during the first 8 days, while in the presence of MTZ-w it increased steadily in form of straight line during the same period. Thus, the death of 50% of cultured PSCs due to MTZ-w occurred at least one day prior to their exposures to comparable concentrations of ABZ or MBZ, respectively (Figures 2-4). During the early periods, incubation with 25 μg/ml MTZ-w resulted in 3-5 fold mortality rates that caused by the standard positive control drug ABZ as depicted in Figure 5.

For the three drug concentrations used, MTZ showed poor protoscolicidal effect that was significantly less than that caused by MTZ-w or ABZ. However, incubation of cultured PSCs with MTZ-a, MTZ-b and MTZ-d caused significantly less mortality values than those caused by MTZ-w but were closer to those caused by the standard positive control drug ABZ (Figures 2-4). In contrast, incubation with the three different concentrations of MTZ-c and MTZ-f was less effective in killing PSCs and MTZ-e, MTZ-g and MTZ-h were the least effective compared to other MTZ derivatives and standard drugs used.

Figure 5 shows that the protoscolicidal effect of MTZ-w followed a concentration gradient and the most effective was at concentration of 25 μg/ml and the lowest at a concentration of 0.78 μg/ml. Evidently, incubation with an MTZ-w concentration as low as 6.25 μg/ml was more effective in killing PSCs than that caused by ABZ at a concentration of 25 μg/ml.

Light microscopy of *in vitro* cultured stages incubated with various drugs and MTZ-derivatives for 14 days reflected the relative detrimental changes caused by these compounds. The greatest morphological changes which included disruption of scolex hooks, deformation of suckers, and disintegration of the tegument was seen in case of PSCs exposed to MTZ-w or ABZ (Figure 6). Less drastic changes in form of dentated suckers, disrupted hooks and tegument were observed when MTZ-a, MTZ-b and MTZ-d were used. The use of MTZ-c caused tegumental and scolex changes which were intermediate between those caused by the above mentioned compounds in one hand and those caused by MTZ-e, MTZ-g and MTZ-h which were the least effective (Figure 6). Incubation of cultured PSCs to MTZ-a, MTZ-f and ABZ, appeared to shift PSCs differentiation into a globose shape. The degree of degenerative changes that included disruption of hooks, rupture of tegument and peri-tegumental accumulation of disrupted tissue increased with time following incubation with various compounds.
3.2. Effect of MTZ and its derivatives on 30 day old cultured stages in vitro

The effects of ABZ and MTZ drugs as well as MTZ derivatives (all at 25 µg/ml concentration) on 30 day old cultured stage (S5 developmental stage) were followed for an additional 14 days. The metacestode stages in cultures treated with ABZ, MTZ-w, MTZ-a, MTZ-b, MTZ-d and MTZ-f revealed dentated suckers and disruption of both hooks and tegument. Less detrimental effects were observed using MTZ and other derivatives.

3.3. In vitro effects of MTZ and its derivatives on secondary developing HC in mice

Figure 7 displays typical ultrastructural effects of ABZ drug and MTZ-w on metacestodes cysts that were incubated in RPMI-1640 containing 25 µg/ml of each compound. During culturing and incubation with these compounds, HC regressed in size. Under SEM, the wall of HC incubated with RPMI-1640 appeared intact with smoothly lined LL and GL with intact tegument. In contrast, ABZ treated HC showed damaged GL, fragmentation of underlining tissue and scaling of LL with oval depressions that appear to lead to the involution and regression in HC size. HC incubated in MTZ-w revealed greater dentated damage in GL and more patchy LL with many deep depressions than those seen in cysts treated with ABZ.

4. Discussion

The present study documented for the first time the effects of several Schiff based and non-Schiff based MTZ derivatives on cultured E. granulosus PSCs and subsequent in vitro cultured stages. Indeed, one of the non-Schiff based MTZ derivative, MTZ-w, revealed remarkable activity and showed more protoscolicidal activity than ABZ, the drug of choice in CE treatment, even at one fourth the concentration of the latter drug. The mortality of PSCs and metacestode stages exposed to MTZ-w was about twice than ABZ at the same concentration and exceeded three times that of ABZ during early periods of exposure. Moreover, the damaging effects on hydatid cyst LL and GL incubated with MTZ-w was more than that on those incubated with ABZ at the
same concentration. In terms of molarity, exposure of PSCs and other in vitro cultured metacestodes to 25 μg/ml concentration of MTZ-w or ABZ is equivalent to 110 and 94 μM solutions, respectively. Taking molarity into consideration does not change the comparative parasitidal effects of these two compounds in vitro. MTZ-w remains significantly more effective than ABZ. Even the exposure of cultured metacestodes to as low as 27.5 μM solution of MTZ-w was significantly more lethal than that caused by exposure to 90 μM solution of ABZ.

Some other Schiff based MTZ derivatives, particularly MTZ-a, MTZ-b and MTZ-d showed protoscolicidal effects and mortality values close to those caused by the standard positive control drug, ABZ. These, in addition to the most potent MTZ-w, are thus important candidates for assessment as alternatives for ABZ both in vitro and in vivo. In contrast, MTZ itself does not seem to be a suitable drug against CE as it showed a much less protoscolicidal than ABZ. ABZ and MTZ must have different modes of action from that of MTZ-w which showed significant activity against PSCs and other cultured stages. MTZ-w, is an imidazole benzeyldehyde analogue, having imidazole ring as in ABZ [Methyl 5-propylthio-2-benzimidazole carbamate]. However, MTZ-w (4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethoxy]-benzeyldehyde) has 2-methyl-5-nitro with para-aldehyde benzene ring. Whether there is a synergistic effect of MTZ-w nucleus and the benzeyldehyde group that renders it more effective on cultured PSCs than ABZ remains to be investigated. The activity of ABZ includes disruption of glucose uptake by inhibition of β-tubulin of the endoplasmic reticulum and mitochondria of parasite GL (Polat et al., 2009). As with MTZ (Halloran et al., 2010; Lofmark, et al., 2010), the activities of Schiff bases is comparable to MTZ which suggests a similar mechanism of action. The differences in protoscolicidal activity between the several MTZ derivatives may reflect differences in stability and transport properties.

The in vivo dose of ABZ for chemotherapy against CE in human and livestock is 50 mg/kg body weight. Lower dosages of ABZ were given after the surgical treatment for maintenance purposes (Moreno et al., 2001; Adas et al., 2009; Creul et al., 2012). In the present study, the doses that were chosen to test the effects of ABZ and the various other drugs and MTZ derivatives in vitro cultures of PSCs and subsequent stages were 25 μg/ml or lower. It should be pointed that lower dosages that prove effective against CE are more beneficial than higher dosages. In addition to decrease in cost, fewer side effects are expected with the use of lower dosages. Although the in vivo effect of MTZ derivatives, particularly MTZ-w requires further intensive assessment, comparisons of their effects on in vitro cultured metacestode stages with those caused by standard drugs are important initial steps towards searching for effective and safe drug alternatives. Thus, MTZ-w and other derivatives that showed sufficient in vitro parasitidal activity at lower dosages should be followed further for potential use as chemotherapeutic drugs. Moreover, the fast action shown by MTZ-w and some other derivatives is of great importance. Fast action lowers the number and volume of drugs for treatment (Taylor et al., 1990; Todorov et al., 1992).

Cultures of PSCs reaching 30 days old stage are useful to assess the effect of the compounds on developing parasite stages. The effect of MTZ derivatives on this stage was studied after 14-days of incubation with single dose of 25 μg/ml and MTZ-w showed the greatest detrimental effect. However, daily follow up is needed to compare the effect of these compounds and the timing needed to reach total (100%) parasitidal effect. This should be carried out on various pre-segmentation and post-segmentation stages as well as adult worms. If proved effective, drug development against the developing and adult parasite stages in the dog definitive host are valuable. It should be pointed out that the experimental setup of in vitro culturing of PSCs and metacestode stages was done in a microenvironment where oxygen was in excess. MTZ and possibly its derivatives normally function under anaerobic or low oxygen tension conditions. As Echinococcus granulosus metacestode stages possess both aerobic and anaerobic respiratory systems (Cue et al., 2013), there is urgent need to explore further the effect of MTZ derivatives, particularly MTZ-w on in vitro cultured stages that are maintained under low oxygen microenvironment.

The target of MTZ-w and other effective derivatives on PSCs appear to be the tegument with subsequent effects on suckers and hooks. Tegument disruption, sucker collapse, and hook rupture were all noted using the most effective MTZ-w compound in addition to ABZ. The loss of rigidity and the size reduction of treated HCs with ABZ or MTZ-w may be due to changes in osmolarity inside and outside HC layers as a result of drug internalization through the cyst wall. Scanning electron microscopy results showed additional evidence about disruptive action of MTZ-w on HC. Here, we provide strong evidence of its potential as an anti-helminthic compound using the E. granulosus model. These findings build on the uniquely wide spectrum of this compound as antiprotozoal and antimicrobial activity (Gavidia et al., 2009; Abu Shaireh et al., 2009; Saadeh et al., 2010). There is an urgent need to examine the chemotherapeutic potential of this compound in vivo using the mouse secondary hydatidosis model. This is a prerequisite for further studies on its toxicity, side effects, and bioavailability.

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