JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 187 - 194

Jordan Journal of Biological Sciences

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

Wafa'a T. Nasr[1], Haythem A. Saadeh[2,3], Mohammad S. Mubarak[2] and Sami K. Abdel-Hafez[1,*]

[1]*Department of Biological Sciences, Yarmouk University, Irbid, Jordan;*

[2]*Department of Chemistry, The University of Jordan, Amman, Jordan;* [3]*Department of Chemistry, United Arab Emirates University, Al Ain, Abu Dhabi, United Arab Emirates*

Received: April 22, 2014 *Revised: May 16, 2014* *Accepted: May 22, 2014*

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 albandazole (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) ethyoxy] benzeyldehyde). 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 underlining tissue, and scaling of laminated membrane. MTZ-w warrants further assessment as a potential chemotherapeutic 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

* Corresponding author. e-mail: skhafez@yu.edu.jo;skhafez@yahoo.com.

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(Moro 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 been 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.,* 2009; 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 protoscolicidal 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 painted three times with a solution of 1% iodine in 95% ethanol. The hydatid fluid (HF) containing PSCs was aspirated using 20 ml sterile syringe fitted with a 19g needle. PSCs were collected aseptically from the HF of fertile cyst or by scrapping 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 trypsin suspension solution prepared in phosphate buffer saline (PBS) in a 1:10 ratio. Trypsin 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-benzimidazol-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] (Acros 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-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-b:((4-Methyl-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-c): (4-Methoxy-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-d):(4-Nitro-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine
(MTZ-e):2-{[2-(2-Methyl-5-nitro-imidazol-1-yl)-ethylimino]-methyl}-phenol,
(MTZ-f):4-Chloro-2-{[2-(2-methyl-5-nitro-imidazol-1-yl)-ethylimi-no]-methyl} phenol,
(MTZ-g):(2-Chloro-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-h):[2-(2-Methyl-5-nitro-imidazol-1-yl)-ethyl]-thiophen-2-yl methylene-amine,
(MTZ-w):4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyoxy] benzeyldehyde.

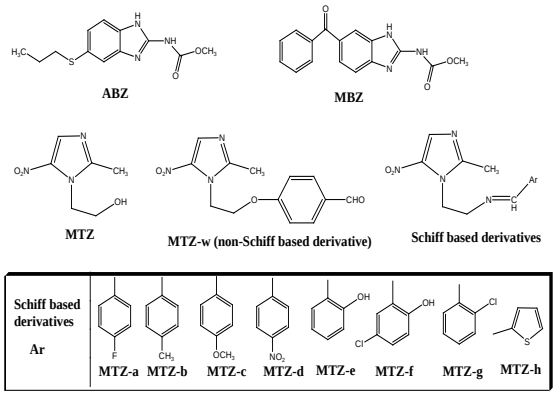

Figure 1. Schematic representation of the chemical structure of: ABZ: Albendazole; MBZ; Mebendazole; MTZ: Metronidazole MTZ derivative compounds (MTZ-w, a non-Schiff based derivative and MTZ-a - MTZ-h Schiff based derivatives).

2.3. Experimental Assays

2.3.1. Exposure of in vitro cultured PSCs and meta cestode stages to standard drugs and MTZ derivatives

For each drug or MTZ derivative, a stock of 200 µg/ml was dissolved in 50% DMSO solution that was initially prepared in sterile double distilled H2O. The stock compounds were sterilized using 0.20 µm filters (Whatman, NJ, USA). Each well received 2 ml of SCM containing 25, 12.5 or 6.25 µg/ml of each compound that was freshly prepared from the original standard drugs or chemical compound stocks. Some 2000 PSCs were added to each culture well and incubated for 13 days at 37 °C with 95% humidity and 5% CO2 in air. The pH of the cultures was adjusted to pH 7.4 at the beginning of the experiment. The PSC viability was tested daily by counting viable and dead PSC in a minimum of 50 PSC taken from each well and various drug and derivatives treatments were carried out in triplicate wells for each concentration used. Viability of PSCs was determined using methylene blue vital stain. The stain penetrates dead PSCs which appeared intensely stained with the dye. ABZ was used as positive control in addition to three negative controls: SCM, 50% DMSO solution and a combination of SCM and 50% DMSO solution in a 1:1 ratio.

2.3.2. Exposure of in Vitro Cultured First Segmentation Metacestode Stage to Standard Drugs and MTZ Derivatives

The first segmentation stages (S5 metacestodes) that were prepared upon *in vitro* culturing of PSCs for 30 days were exposed to the same drug concentrations following a similar protocol to that described above for freshly prepared PSCs. The morphological, anatomical and parasiticidal effects on the developing stages was followed for 14 days.

2.3.3. Exposure of secondarily developed HC to MTZ-w and ABZ

Five secondarily developing HCs that were isolated from BALB/c mice as described above were incubated with SCM containing 25 µg/ml of MTZ-w for 21 days. Other HCs were incubated with a comparable concentration of ABZ that was used as a standard positive control drug, while others were incubated with a solution made of SCM and 50% DMSO in a 1:1 ratio and used as negative control.

2.4. Microscopic Examination

2.4.1. Light microscopy

Morphological and tegumentary changes in cultured PSCs subjected to various drug and MTZ derivatives were studied under light microscopy using aceto-carmine staining protocol (Meyer and Olsen, 1980). Briefly, PSCs or cultured larval stages were fixed in 10% formalin solution for at least 48 hs. The specimens were washed three times with distilled water before being dehydrated through an ascending ethanol series (35%, 50% and 70%) for 30 min each. Then, 70% ethanol was replaced by Semichon'saceto carmine stain for 30 min. Samples were washed with 70% ethanol for few seconds. Excessive staining was avoided by placing specimens in 70% ethanol containing 2-4 drops of HCL for few minutes until they differentiated well. Subsequently, specimens were washed quickly with 70% ethanol for few seconds before they were transferred to 70% ethanol with 2 drops of NaHCO3 solution and kept there for 30-60 min. Further dehydration was made through further ascending ethanol series (85%, 95% and 100%) for 15 min each. After further dehydration with 100% ethanol, samples were transported to glass vials containing xylene and kept there for at least 15 min before being mounted on a glass slide supported with one drop of Canada balsam, covered and dried.

A minimum of 25 stained specimens were examined to determine the microscopic effects of compounds on treated PSCs and *in vitro* cultured metacestode stages.

2.4.2. Scanning electron microscopy

Metacestodes that were exposed to standard positive control drugs or MTZ derivatives were picked from culture medium and washed three times with sodium cacodylate buffer (SCB) (pH 7.2) for 5 min each. Then, they were fixed in 2.5% glutaraldehyde in 0.1M SCB for four h. After washing three times with 0.1 M SCB for 5 min each, specimens were post fixed in 1% osmium tetroxide (OsO4) prepared in the same buffer for two hours. The cysts were washed further three times with SCB for 5 min each. Next, they were dehydrated through an ascending ethanol series (30, 50, 70, 90, and 100%) for 20 min each. Finally, the specimens were dried using Balzers critical point drier 0301 (Wanner *et al.*, 2005) and sputter coated with gold on stubs. The specimens were studied and photographed using Zeiss scanning electron microscope at 20 KV on a rotator.

3. Results

3.1. 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).

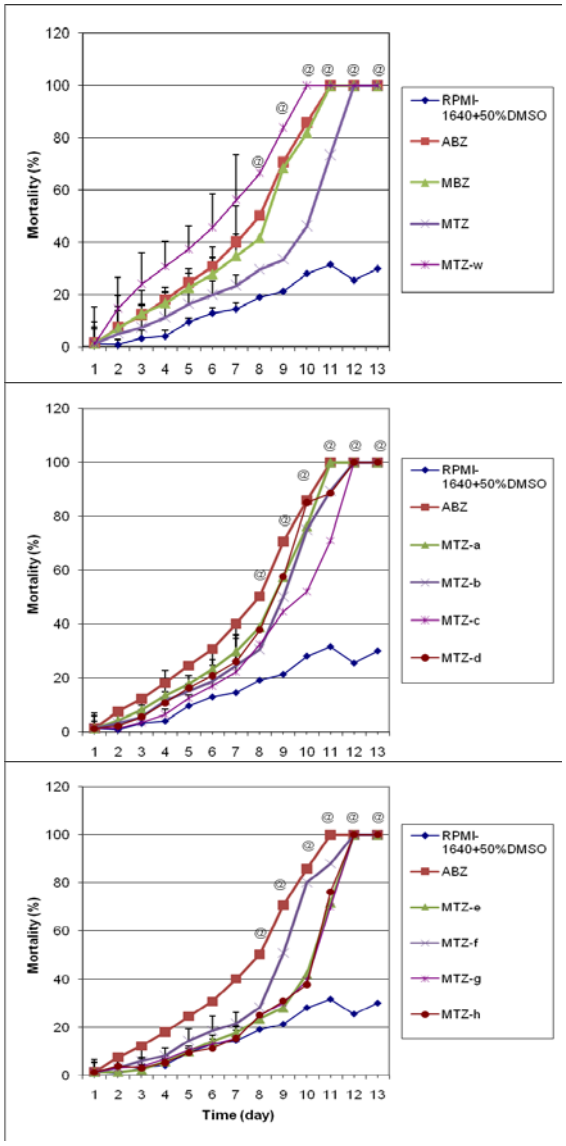

Figure 2. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 25 µg/ml ABZ, MBZ, MTZ or its derivatives. " @: standard deviation was not placed because the values represent only one or two observations".

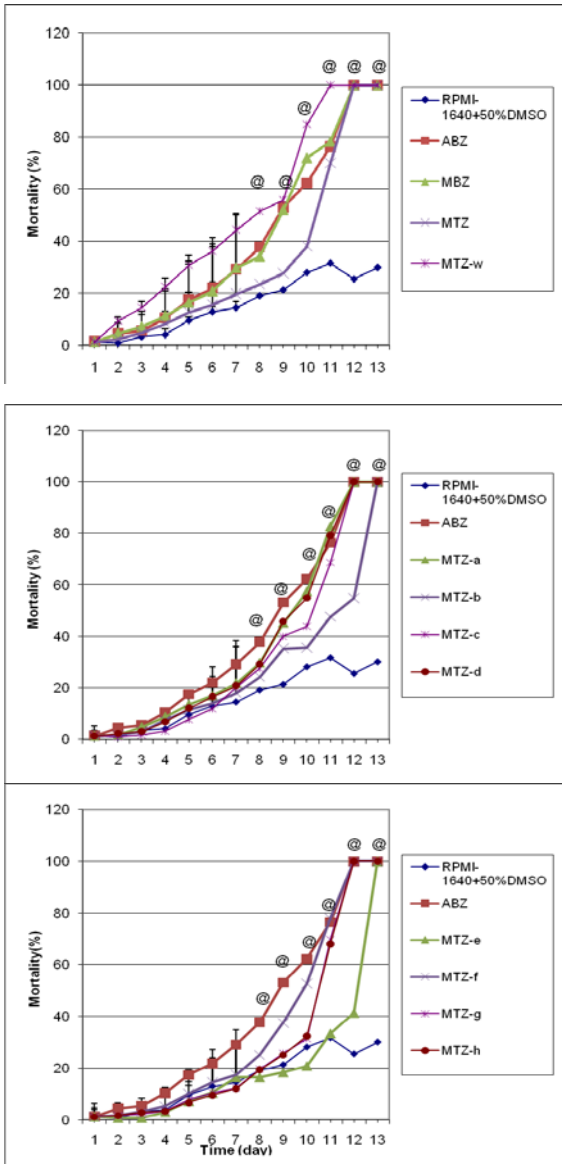

Figure 3. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 12.5 µg/ml ABZ, MBZ, MTZ or its derivatives. " @: standard deviation was not placed because the values represent only one or two observations".

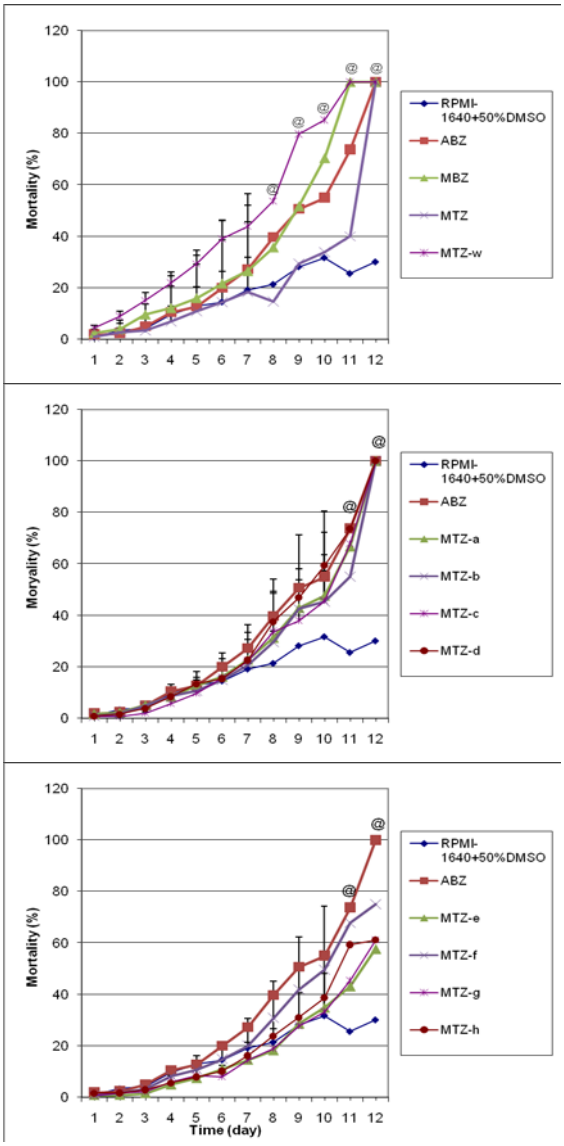

Figure 4. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 6.25 µg/ml ABZ, MBZ, MTZ or its derivatives. " @: standard deviation was not placed because the values represent only one or two observations".

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.

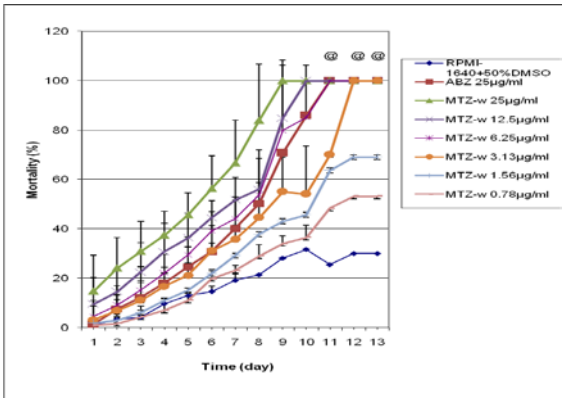

Figure 5. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with different concentration of MTZ-w compared with ABZ (+ control). "@: standard deviation was not placed because the values represent only one or two observations ".

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 25µg/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.

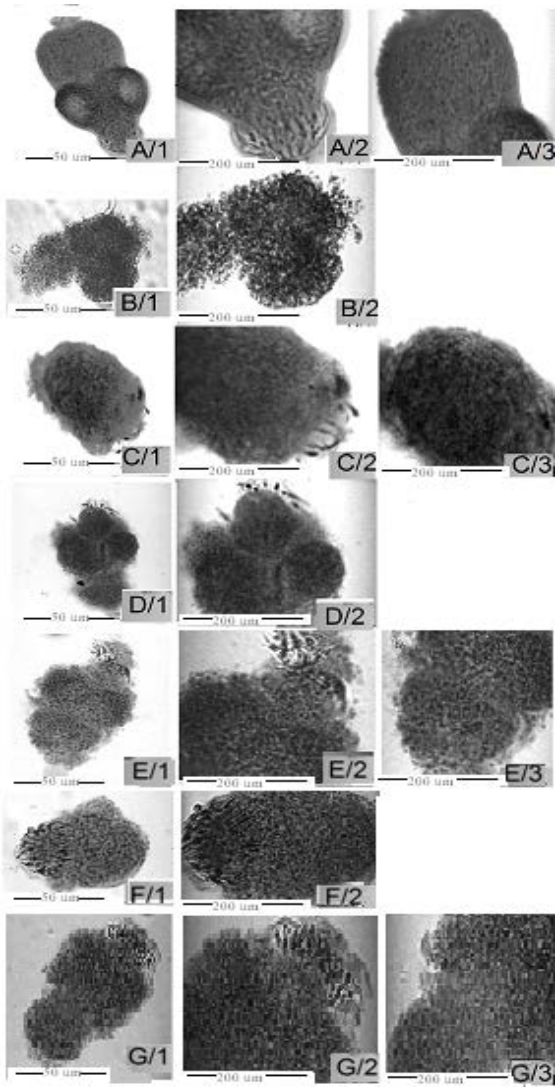

Figure 6. Light microscopy of 14 day old cultures of evaginated PSCs in RPMI-1640 incubated with 25µg/ml ABZ,MTZ or its derivatives in 25µg/ml: (A) RPMI-1640 represent negative control (A/1), (A/2) normal suckers and hooks, (A/3) body tegument; (B) ABZ: (B/1) ,(B/2) dentated suckers, disrupted hooks and disrupt body tegument; (C) MTZ (C/1) , (C/2) dentated suckers and disrupted hooks, (C/3) disrupted body tegument; (D) MTZ-w (D/1) , (D/2) dentated suckers, disrupted hooks and disrupted body tegument; (E) MTZ-a, MTZ-b or MTZ-d: (E/1) , (E/2) dentated suckers and disrupted hooks, (E/3) disrupted body tegument; (F) MTZ-c or MTZ-f: (F/1) , (F/2) dentated suckers and disrupted hooks, (F/3) disrupted body tegument; (G) MTZ-e, MTZ-g or MTZ-h: (G/1), (G/2) dentated suckers and normal hooks (G/3) disrupted body tegument.

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.

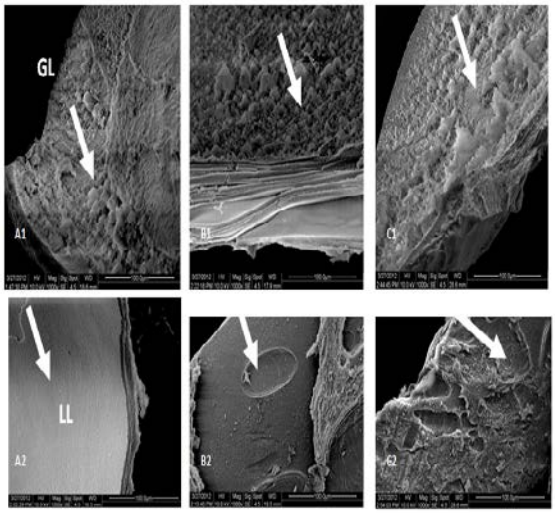

Figure 7. Scanning electron microscopy of laminated (LL) and germinal layer (GL) of secondary hydatid cysts developing in BALB /C mice 14 weeks post subcutaneous inoculation with PSCs: Cysts were incubated for 21 days in following media and compounds: RPMI-1640 as negative control showing intact GL (A1) and LL (A2) with remnants of host tissues towards the periphery of LL; RPMI- 1640 containing 25 µg/ml ABZ as positive control showing scaling of LL (B1), oval depressions and damaged GL with fragmented cellular elements (B2); RPMI- 1640 containing 25µg/ml MTZ-w showing carpet like appearance of GL with dentated endings (C1), and fragmentation of LL with many deep depressions and patchy appearance (C2).

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 25µg/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 parasiticidal 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) ethyoxy] benzeyldehyde) has 2-methyl-5-nitro with *para*-aldehyde benzene ring. Whether there is a synergistic effect of MTZ-w nucleus and the benzyldehyde 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 ß-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 *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* parasiticidal activity at lower dosages should be followed further for potential use as chemotherapeutic drugs. Moreover, the fast action showed 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 25µg/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%) parasiticidal 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 set up 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 i*n vivo* using the mouse secondary hydatidosis model. This is a prerequisite for further studies on its toxicity, side effects, and bioavailability.

Acknowledgements

We thank Dr. Maen Abdel-Hafez and Dar Al-Hikmah Pharmaceutical Company, Amman, Jordan for providing albandazole and mebendazole compounds used in this study. Thanks to Mr. Tareq Ramadneh for technical help. This work was supported by a grant from the Research Council, Deanship of Scientific Research and Graduate Studies, Yarmouk University, Irbid, Jordan.

References

Abu Shaireh E, Saadeh H, Mosleh I, Al-Arif M and Mubarak M. 2009. Metronidazole derivatives as antiparasitic agents. *European Patent Applications*; **EP 2 085 394 A2**.

Adas G, Karatepe O, Altiok M, Battal M, Bender O, Ozcan D, *et al*. 2009. Diagnostic problems with parasitic and non-parasitic splenic cysts. *BMC Surg*., **9**:1186-1192.

Ceballos L, Elissondo M, Bruni S, Denegri G, Alvarez L, and Lanusse C. 2009. Flubendazole in cystic echinococcosis therapy:pharmaco-parasitological evaluation in mice. *Parasitol Int*., **58**:354-358.

Creul C, Codreanu1 R, Mastalier B, Popa1 I, Cordo M, Beuran D, *et al*. 2012. Albendazole associated to surgery or minimally invasive procedures for hydatid disease– how much and how long? *Chirurgia*, **107**:15-21.

Cui SJ, Xu LL, Zhang T, Xu M, Yao J, Fang CY*et al*. 2013. Proteomic characterization of larval and adult developmental stages in *Echinococcus granulosus* reveals novel insight into host-parasite interactions. *J Proteomics*, **84**:158-75.

Da Silva A. 2010. Human echinococcosis: a neglected disease. *Gastroenterol Res Pract*., Article ID 583297.

Eckert J and Deplazes P. 2004. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clin Microbiol Rev*., **17**:107–135.

Gavidia C, Gonzalez A, Lopera L, Jayashi C, Angelats R, Barron E, Ninaquispe B, Villarreal L, Garcia L, Verastegui M and Gilman R. 2009. Evaluation of nitazoxanide and oxfendazole efficacy against cystic echinococcosis in naturally infected sheep. *Am J Trop Med Hyg*., **80**:367–372.

Gold, D. 1997. Assessment of the viability of *Schistosoma mansoni* schistosomula by comparative uptake of various vital dyes. *Parasitol Res.*, **83:** 163-169.

Halloran E, Hogan A and Mealy K. 2010. Metronidazole-induced pancreatitis. *HPB Surg.*, Article ID 523468.

Hemphill A and Muller J. 2009. Alveolar and cystic echinococcosis: towards novel chemotherapeutical treatment options. *J Helminthol*., **83**: 99–111.

Hijjawi N, Abdel-Hafez S and Al-Yaman F. 1992. *In vitro* culture of the strobilar stage of *E. granulosus*of sheep and donkey origin from Jordan. *Parasitol Res.*, **78**: 607-616.

Liu, CS, Zhang, HB, Yen, JH, Jiang, B and Han XM. 2013. *Echinococcus granulosus*: suitable *in vitro* protoscolices culture density. *Biomed Environ Sci*. **26:** 912-915.

Kakru D, Sofi B and Assadullah S. 2008. Novel route of injection in experimental model of hydatid disease. *Indian J PatholMicrobiol*., **51**: 373-375.

Kern P. 2006. Medical treatment of echinococcosis under guidance of good clinical practice (GCP/ ICH). *Parasitol Int*., **55** (Suppl 2): 273-282.

Lofmark S, Edlund C and Nord C. 2010. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infec Dis*., **50**:16–23.

McManus, D. 2010. Echinococcosis with particular reference to Southeast Asia. *Adv Parasitol*., **72**:267-303.

Meyer M and Olsen O. 1980. **Essentials of Parasitology**. Wm C Brown Company, Dubuque, Iowa, p. 236.

Moreno M, Paris M, Casado N and Caabiaro F. 2001. Praziquentel and albendazole in the combined treatment of experimental hydatid disease. *Parasitol Res.*, **87**: 235-238.

Moro P and Schantz P. 2009. Echinococcosis: a review. *Int J Infec Dis*., **13**:125-133.

Polat E, Aslan M, Cakan H, Saribas S, Ipek T and Kocazeybek B. 2009. The effects of albendazole and povidone iodine for antimicrobial agents and chemotherapy of hydatid cysts protoscoleces, *in-vitro* and *in–vivo*. *Afr J Microbiol Res*., **3**:743-746.

Saadeh H, Abu Shaireh E, Mosleh I, Al-Bakri A and Mubarak M. 2011. Synthesis, characterization and biological activity of Schiff bases derived from metronidazole. *Med Chem Res*., **21**: 2969-2974.

Saadeh H, Mosleh I, Al-Bakri A and Mubarak M. 2010. Synthesis, characterization and antimicrobial activity of new 1,2,4- triazole-3- thiol metronidazole derivatives. *Monatsh Chem*., **141**:471-478.

Smyth J. 1967. Studies on tapeworm physiology. XI. *In vitro* cultivation of .*Echinococcus granulosus* from protoscolex to the strobilar stage. *Parasitology*, **57**:111-133.

Taylor D, Morris D and Richards K. 1990. Perioperative prophylactic chemotherapy of *Echinococcus granulosus*: determination of minimum effective length of albendazole therapy *in vitro* protoscoleces culture. *HPB Surg*., **2**:159-164.

Todorov T, Mechkov G, Vutova K, Georgiev P, Lazarova 1, Tonchev Z, *et al*.1992. Chemotherapy of human cystic echinococcosis: comparative efficacy of mebendazole and albendazole.*Ann Trop Med Parasitol*., **86**:59-66.

Vuitton, D. 2009. Benzimidazoles for the treatment of cystic and alveolar echinococcosis: what is the consensus? *Expert Rev Anti Infect Ther*., **7**:145-149.

Wanner G, Schroeder-Reiter E and Formanek H. 2005. 3D analysis of chromosome architecture: advantages and limitations with SEM. *Cytogenet Genome Res*., **109**:70–78.