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Effects of albendazole, artesunate, praziquantel and miltefosine, on *Opisthorchis viverrini* cercariae and mature metacercariae

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ABSTRACT

Objective: To explore larvicidal effects of anthelmintic drugs on *Opisthorchis viverrini* (*O. viverrini*) for alternative approach to interrupting its cycle for developing a field-based control program.

Methods: The larvicidal activities of albendazole (Al), artesunate (Ar), praziquantel (Pzq) and miltefosine (Mf) on *O. viverrini* cercariae and mature metacercariae were investigated. Lethal concentrations (LC_{50} and LC_{95}) of these drugs were determined. Mature metacercariae previously exposed to various concentrations of the drugs were administered to hamsters. Worms were harvested 30 d post infection and worm recovery rates calculated. Al, Ar, Pzq and Mf produced morphological degeneration and induced shedding tails of cercariae after 24 h exposure.

Results: The LC_{50} and LC_{95} of Al, Ar, Pzq and Mf on cercariae were 0.720 and 1.139, 0.350 and 0.861, 0.017 and 0.693, and 0.530 and 1.134 ppm, respectively. LC_{50} and LC_{95} of Ar on mature metacercariae were 303.643 and 446.237 ppm and of Mf were 289.711 and 631.781 ppm, respectively but no lethal effect in Pzq- and Al-treated groups (up to 1 ppt). No worms were found in hamsters administered Pzq-treated metacercariae. The adult worms from Al-treated metacercariae were significantly bigger in size compared to the control group ($P < 0.05$). Fecundity and body width were greater in adults from Mf-treated metacercariae compared to the control group ($P < 0.05$).

Conclusions: The larvicidal effects of these drugs were high efficacy to *O. viverrini* cercariae but lesser efficacy to metacercariae. It should be further studied with the eventual aim of developing a field-based control program.

1. Introduction

Opisthorchiasis, caused by infection with *Opisthorchis viverrini* (*O. viverrini*), is a major risk factor for cholangiocarcinoma (CCA), an important public health problem in northeastern Thailand [1]. Humans acquire infection by eating raw or undercooked cyprinid fish containing the infective stage, mature metacercariae [2]. The parasite life cycle requires the snail *Bithynia siamensis goniomphalos* (*B. siamensis goniomphalos*) as first intermediate host in the northeast region of Thailand and many species of cyprinid fishes as second intermediate host [3].

In the snail host, germinal cells in sporocysts and rediae proliferate asexually, ultimately producing many free-swimming cercariae. Numerous cercariae of *O. viverrini* are shed daily from naturally infected snails, with an average of 1728 cercariae/snail, at the daily peak shedding time of 8.00–10.00 AM [4]. After finding a cyprinid fish host, cercariae penetrate and encyst to become metacercariae, mainly in the head portion and muscles [5]. This can lead to a high prevalence of *O. viverrini* metacercariae [3]. For treatment of opisthorchiasis

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cases, praziquantel has high efficacy [6]. Other drugs widely used for many species of trematodes, including *O. viverrini*, are albendazole and artesunate [7,8]. Efficacy of miltefosine against trypanosomes, schistosomes and *Entamoeba histolytica* has been studied [9,10]. Miltefosine has larvicidal effects on eggs, miracidia and cercariae of *Schistosoma mansoni* (*S. mansoni*) and *Schistosoma haematobium*, and lethal effect to *Biomphalaria alexandrina*, the snail hosts [11]. To date, there has been no study on the effects of any of these drugs against larval stages of *O. viverrini*.

This study investigated *in vitro* larvicidal effects of albendazole, artesunate, praziquantel and miltefosine on field-collected *O. viverrini* cercariae and metacercariae. If these drugs are effective against swimming cercariae and mature metacercariae, their use in this way could be incorporated into effective strategic control of opisthorchiasis in humans.

2. Materials and methods

2.1. Sample preparation

2.1.1. Collection of *O. viverrini*-infected *B. siamensis goniomphalos*

Snails were collected by hand from water bodies in endemic areas of Khon Kaen Province and brought back to the Malacology Laboratory, Khon Kaen University in plastic bags labeled for each locality. Snails were identified as *B. siamensis goniomphalos* based on morphology of their shells [3,12]. They were rinsed with tap-water and maintained in dechlorinated tap-water for a few days before examination for *O. viverrini* infection.

2.1.2. Collection of *O. viverrini* cercariae

Snails were placed individually in plastic cups (3 cm in diameter and 2.5 cm in height) contained dechlorinated tap-water. Cercarial shedding was induced by exposure to electric light (40 W) for 2–3 h, after which the water in the cup was examined for the presence of cercariae. Cercariae were identified as *O. viverrini* by morphology [3] and confirmed by polymerase chain reaction (PCR) using species-specific primers [13].

2.2. Life span of *O. viverrini* cercariae at various temperatures

A suitable temperature for use in the cercaricidal study was investigated. Freshly shed *O. viverrini* cercariae were maintained at various water temperatures from 4 to 52 °C (8 °C intervals, 100 cercariae in five replicates at each temperature). Their activity was observed and changes in morphology, such as tail shedding or body degeneration, were noted.

2.3. Effects of albendazole, artesunate, miltefosine, and praziquantel on *O. viverrini* cercariae

Albendazole, artesunate, praziquantel, (Sigma–Aldrich Shanghai Trading Co Ltd Shanghai, China) and miltefosine (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) were used for this study. Cercariae (n = 1380) were divided into four groups, one for each drug. Groups 1, 2, and 3 (360 cercariae/group), cercariae in those groups were divided to 6 sub-groups (60 cercariae/sub-group, 3 replicates, 20 cercariae/replicate) Group 1 and 2: cercariae were exposed to concentration of albendazole and artesunate (dissolved in 10% DMSO) of 0.0 (2 control groups, water and solvent of 10% DMSO), 0.2, 0.4, 0.6 and 0.8 ppm. Group 3: cercariae were exposed to praziquantel at 0.0000 (2 control groups, water and solvent of 10% DMSO), 0.0125, 0.0250, 0.0500, and 0.1000 ppm. Group 4 (300 cercariae), cercariae were divided to 5 sub-groups (60 cercariae/sub-group, 3 replicates, 20 cercariae/replicate) to expose to miltefosine at 0.0000 (control in water), 0.1375, 0.2750, 0.5500, and 1.1000 ppm. To study the effects of drugs, cercariae were exposed for 24 h then rinsed with dechlorinated tap water and observed for 24 h. The criteria for death of a cercaria were lack of movement and physical degeneration.

2.4. Effects of albendazole, artesunate, miltefosine, and praziquantel on *O. viverrini* metacercariae

2.4.1. Effect of drugs on mature metacercariae

This study consisted of two experiments. Experiment Ⅰ determined survival of mature metacercariae *in vitro* and Experiment Ⅱ determined infectivity and development of worms in infected hamsters. Mature *O. viverrini* metacercariae were obtained from natural infected cyprinid fish by the pepsin digestion method [14].

Experiment Ⅰ: The metacercariae (n = 3600) were divided into 4 groups (900 metacercariae/group), one for treatment with each drug. Within each group there were six sub-groups (150 metacercariae/sub-group, 3 replicates, 50 metacercariae/replicate), each exposed to praziquantel, miltefosine, artesunate and albendazole at 0 (2 control groups, water and 10% DMSO), 50, 100, 150 and 200 ppm. Criteria for death of metacercariae were no movement of larvae and degeneration.

Experiment Ⅱ: Infectivity and development of drug-treated mature metacercariae in hamsters.

Metacercariae were exposed to drugs for 24 h as in Experiment I, then washed with water and administered to hamsters (4 hamsters/sub-group, 50 metacercariae/hamster). Hamsters were housed in the Animal House, Faculty of Medicine, Khon Kaen University. Food and water were provided *ad libitum* for 30 d, after which hamsters were euthanized with diethyl ether in a fume hood and *O. viverrini* adults collected from the livers. The protocol for *O. viverrini* infection and sacrifice of hamsters was approved by the Animal Ethics Committee of Khon Kaen University, Thailand (record No. ACUC-KKU-34/2558).

2.5. Morphology and fecundity of adult worms

Morphology of adult worms from hamster livers was studied for ten adult worms from each concentration of each drug (miltefosine, artesunate and albendazole). Worms were fixed in 10% neutral buffered formalin, stained with Semichon's acetic carmine, dehydrated in an alcohol series and permanent slide mounts made. The length and width, body area, area of testes (anterior and posterior testes), and area of ovaries were determined for each worm under a microscope (Olympus Co., Tokyo, Japan) using the program DP2-BSW (Olympus Co., Tokyo, Japan). Eggs-per-worm was calculated from ten worms from each hamster of each concentration of each drug and control groups. The uterus of each worm was minced individually in a plastic tube containing a known number of drops of normal saline solution (NSS). One drop of this mixture was randomly