Transmission Electron Microscopy of *Schistosoma mansoni* Cercariae Treated with Hinokitiol (α-thujaplicin), a Compound for Potential Skin Application against Cercarial Penetration

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Chisty, M.M., Nargis, M., Inaba, T., Ishita, K., Osanai, A. and Kamiya, H. Transmission Electron Microscopy of *Schistosoma mansoni* Cercariae Treated with Hinokitiol (α-thujaplicin), a compound for Potential Skin Application against Cercarial Penetration. Tohoku J. Exp. Med., 2004, 202 (1), 63-67 — Since skin is the only route of entry of the parasite in schistosomiasis patients, intervention at the level of skin penetration should control the infection. Several compounds were screened for their ability to protect against cercarial penetration. Hinokitiol (α-thujaplicin) was found to have a significant cercaricidal effect in vitro, although there is no information on its cercaricidal mechanisms. To study the kinetics of morphological changes in *Schistosoma mansoni* associated with exposure to hinokitiol in vitro, cercariae were incubated in media containing hinokitiol at different concentrations and examined by transmission electron microscopy (TEM). TEM revealed that ultrastructural changes occurred by 15 minutes post exposure, at a concentration of 25 μg/ml. Degenerative changes involving both tegument and deeper parenchymal structures were progressive with duration of exposure at the concentration of 50 μg/ml. These structural changes may account for the inability of hinokitiol-treated cercariae to infect the host. ——— hinokitiol; *Schistosoma mansoni*; cercariae; transmission electron microscopy

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Electron Microscopy of *S. mansoni* exposed to Hinokitiol

Schistosomiasis is a life threatening tropical disease of humans. Non-immune travelers visiting endemic areas are at high risk of acquiring this disease and disseminating the parasite to non-endemic areas (Stone 1995). Since skin is the only route of entry for this parasite into humans, intervention that prevents entry of cercariae into the skin should control the infection (Stirewalt and Dorsey 1974). Several compounds were screened for their ability to confer protection against cercarial penetration following skin application. Among these, a few compounds (diethyl boramide, niclosamide, cederol, β-thujaplicin and diethyl toluamide) showed significant cercaricidal effects in vitro (Naples et al. 1992; Abu-Elyazeed et al. 1993; Nargis et al. 1997; Salafsky et al. 1998). β-thujaplicin is a tropolone-related compound found in the heartwood of several cupressaceous plants such as western red cedar (*Thuja plicata*), eastern white cedar (*Thuja occidentalis*) and hinoki cypress (*Chamaecyprais obtusa*) (Nozoe 1936; Erdtmann and Gripenberg 1948). It is known to be effective as an antimicrobial agent (Katsumura et al. 1948; Kobori and Tanabe 1993, 1994), and is being used as an anti-bacterial hand washing solution (Kobori and Tanabe 1994). However, a review of literature reveals no ultrastructural information of hinokitiol-treated cercariae. The aim of this study, therefore, was to evaluate the in vitro effects of hinokitiol on cercariae of *Schistosoma mansoni* at the level of ultrastructure.

**MATERIALS AND METHODS**

A Puerto Rican strain of *S. mansoni* was maintained in our laboratory by passage through Mongolian gerbils, *Meriones unguiculatus*, and *Biomphalaria glabrata*. Cercariae were obtained from the infected snails and used for experiments within 1 hour of shedding.

Synthesized hinokitiol powder of 100% purity was obtained from Takasago International Co. Tokyo. Hinokitiol powder dissolved (200 µg/ml) in water and serial dilutions were prepared from 25 to 50 µg/ml in distilled water (Nargis et al. 1997). Four ml of each dilution was transferred to each culture tube. Control tubes received an equal amount of water. Approximately 400 cercariae of *S. mansoni* were placed in each test tube. Samples were collected at 5, 15, 30, 60 and 120 minutes after exposure to hinokitiol at each concentration and processed for transmission electron microscopy (TEM).

Cercariae collected from each tube were transferred to a fresh tube containing 10 ml of RPMI 1640 medium plus 10% foetal calf serum (JRH Bioscience, Kansas) and concentrated into a pellet by centrifugation at 300 g for 10 minutes. The pellet was washed with the same medium, then fixed overnight at 4°C in a fixative containing 3% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer (PH 7.4). After 1 hour post-fixation in 1% osmium tetroxide the pellets were dehydrated through a series of ethanol and embedded in epoxy resin. Methylene blue stained sections were examined light microscopically and ultrathin sections were examined in a transmission electron microscope (JEOL, Tokyo) after staining with uranyl acetate and lead citrate.

**RESULTS AND DISCUSSION**

Cercariae exposed to hinokitiol at concentrations of 25 µg/ml or more showed progressive morphological changes (Figs. 1 and 2), observed earliest at 15 minutes post exposure. Gross changes were observed, including cercarial tail loss in about 50% of cercariae at 15 minutes, and 90% by 30 minutes after exposure. These observations were consistent with the report by Nargis et al. (1997), which showed that hinokitiol at the concentration of 25 µg/ml significantly affected the cercarial movement and swimming activity. Epon embedded thin sections were observed light microscopically and showed progressive degeneration of the acetabular glands (Fig. 1C), thinning of the tegument causing external protrusion with focal loss of spine (Figs. 1D and 1E), edematous swellings and other degenerative changes (Fig. 1F). Control cercariae exhibited normal morphology (Figs. 1A and 1B).
Ultrastructural changes were evident as early as 15 minutes post exposure to hinokitiol, and became more severe with increased duration of exposure. Early degenerative changes included accumulation of membranous bodies (Fig. 2B), migration of cytoplasmic granules into the tegument (Fig. 2C) and loss of external glycocalyx resulting extreme thinning of the tegument (Figs. 2C, 2D and 2E), diffuse edematous changes in the parenchyma and focal lysis of the tegument causing expulsion of sub-tegumental materials (Fig. 2D). These changes were noted in all of the parasites examined, although to a varying degree. Changes progressed in severity with increased duration of exposure (Figs. 2E and 2F).

Topically applied hinokitiol may not efficiently diffuse into the host skin and nor alter the host immune response against migrating schistosomes, although in vitro experiments suggested an immunosuppressive effect (Inamori et al. 1993). This study demonstrated that hinokitiol has a damaging effect in vitro on *S. mansoni*.
Electron Microscopy of *Schistosoma mansoni* exposed to hinokitiol (25 μg/ml) in a concentration and time dependent manner. The minimum concentration (25 μg/ml) required to produce demonstrable degenerative changes (Figs. 1 and 2) is lower than the concentrations reported for a variety of bacteria and fungi, where effective concentrations of hinokitiol were 100 or 50 μg/ml (Kobori and Tanabe 1993; Okabe et al. 1989). In previous experiments we have reported that hinokitiol (50 μg/ml) treated cercariae showed defective motility and infectivity (<1% adult worm recovery in mice vs. >60% in vehicle controls (Nargis et al. 1997). Based on the results from the present study, degenerative structural alterations prevented the cercariae from penetrating the host skin.

Apart from anti-microbial effects, various bioactivities of hinokitiol have been reported, such as repellent activity for ticks, inhibitory effect on the germination of plant seeds and the growth of roots of several plants, cytotoxic effect on tumor cells, and lymphocyte blastogenesis (Okabe et al. 1988; Inomori et al. 1991, 1993).
The mechanism underlying broad range bioactivities and the target(s) is still unrevealed. In vitro cytotoxic and immunosuppressive effects of hinokitiol have been reported; namely it prevents ultraviolet radiation-induced apoptosis in keratinocytes of mice (Baba et al. 1998), and influenza virus-induced apoptosis, replication and release from the infected Madin-Darby canine kidney cells (Miyamoto et al. 1998).

Hinokitiol is generally considered safe to host skin even at high concentrations, as it might not be absorbed through the host skin (Nargis et al. 1997). Furthermore, hinokitiol applied to the skin did not alter the migration of epidermal Langerhans‘ cells to regional lymph node of guinea pigs infected with S. mansoni (Chisty, M.M. et al.; unpublished data). Intervention may be achieved by application of hinokitiol with an appropriate base that may help it persist on the skin surface and make it more resistant to removal by contact with water.

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References


