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In vitro and in vivo efficacies of ionophores against *Cryptocaryon irritans*

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ABSTRACT

We assayed the effects of a variety of antiprotozoal compounds against trophonts of *Cryptocaryon irritans*, the causative agent of 'white spot disease of marine fish' in vitro using the double layered media that we developed previously for the culture of the parasite. In the assay, ionophores, particularly sodium salinomycin, showed apparent killing and growth-suppression effects against the parasite. As there was no mortality in Japanese flounder *Paralychthys olivaceus* that were fed a diet containing sodium salinomycin (200 ppm) for two weeks, we evaluated the efficacy of 200 ppm sodium salinomycin against *C. irritans* in Japanese flounder. We fed Japanese flounders a medicated diet for 5 d prior to and 3 d after challenge with *C. irritans.* In the experimental group, the number of protomonts recovered from the fish and the size of tomonts that were transformed from the protomonts were significantly reduced, when compared to the control group. Furthermore, in a different experiment, the fish that were fed a diet medicated with sodium salinomycin survived longer than those fed an unmedicated diet after challenge. Sodium salinomycin can be a good candidate drug for chemotherapy and control of *Cryptocaron irritans* infection.

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1. Introduction

Cryptocaryon irritans is a ciliate parasite that causes 'white spot disease in marine fish' worldwide. The development of this parasite has been classified into four stages (Colorni, 1985; Colorni and Burgess, 1997; Dickerson, 2006). The 'trophont' represents the parasitic stage, during which the parasite resides and feeds within the epithelial layer of the skin, fins, and gills. Mature trophonts leave the host fish as a free swimming 'protomont'. The protomonts settle at the bottom of the water column where they encyst, entering the 'tomont' stage. Following this, the 'theront' represents the free-swimming stage that is released from tomonts, and which is invasive to fish hosts. *C. irritans* disrupts osmoregulation and respiratory function in fish, often causing high mortality in heavily infected fishes. Most species of marine warmwater teleosts, including food fishes that are cultured in floating net cages and land-based tanks and ornamental fishes held in public and private aquaria, are susceptible to the parasite (Ogawa and Yokoyama 1998). Despite significant economic losses and high mortalities, few effective treatments and control measures have been developed against *C. irritans* infection, particularly in food fish that are cultured in net cages in open waters.

A number of variations on chemical bath treatments have been used to control *C. irritans*, including copper sulfate, formalin, methylene blue, and quinine hydrochloride (Herwig, 1987; Huff and Burns, 1981; Nigrelli and Ruggieri, 1966; Wilkie and Gordin, 1969). However, this approach has several drawbacks. These chemical agents that are effective against *C. irritans* only target the developmental stages outside the fish so are ineffective at preventing infection in fish that are held in net cages in the open sea. Moreover, the widespread use of chemicals contributes to environmental pollution and public concerns for food and environmental safety.

Oral administration of lysozyme chloride appears to be effective for treatment of *C. irritans* infections, and is approved for use on food fish in Japan (http://www.nval.go.jp/asp/asp_dbDR_idx.asp), although published data is not available. In addition, medium chain fatty acids (e.g., caprylic acid) have proven to be effective when used as a dietary additive (Hirazawa et al., 2001). However, these two treatments alone are not sufficient to control *C. irritans* infection. Hence, there is need for more effective chemotherapeutic drugs against *C. irritans.* Generally, chemotherapeutic drugs are screened using in vitro assays. Until recently there has been no method to culture the trophont or theront stages of *C. irritans* in vitro. However, Yoshinaga et al. (2007) developed a double layered medium for the in vitro culture of *C. irritans* in which inoculated theronts of the parasite transformed to trophonts and developed into protomonts. Our objective was to evaluate the efficacy of a range of anti-protozoal drugs against *C. irritans*. Using the double layered medium developed by Yoshinaga et al. (2007), we determined that ionophores, particularly sodium salinomycin, were effective at killing *C. irritans* trophonts in vitro. Given this, we evaluated the risk and efficacy of oral administration of sodium salinomycin in Japanese flounder, *Paralichthys olivaceus*.

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2. Materials and methods

2.1. Propagation and collection of parasites

We purchased ornamental fish that were infected with *Cryptocaryon irritans* from a local pet shop. The parasites were passaged and propagated on black mollies (*Poecilia* sp. hybrid, likely *P. latipinna*). We then collected protomonts from the infected black mollies following the description of Yoshinaga and Dickerson (1994).

The protomonts were washed 5 times with filter-sterilized seawater (FSS) supplemented with 100 IU ml^{-1} penicillin G potassium and 100 μg mL^{-1} streptomycin sulfate then incubated in petri dishes (10 cm^2) containing supplemented FSS (4 mL) at 25 °C. Each dish contained 100–300 protomonts, which encysted within several hours to form tomonts. The seawater was replaced daily. The tomonts typically began to release theronts after 5 d, though the numbers were initially low. We collected theronts that were released after 6–7 d incubation for use in in vitro and disease challenge assays.

2.2. Fish

We purchased Japanese flounders (*Paralichthys olivaceus*) (mean weight ~4 g) from a commercial hatchery (Marintech Co. Ltd., Aichi Prefecture) for use in the in vivo assays. The fish were held in a 1 m^3 recirculation aquarium equipped with a biological filter and fed once daily with a commercial diet until the beginning of the experiment. The commercial diet also formed the base of the medicated diets.

2.3. In vitro screening

We used the double layered medium that was developed for the in vitro culture of *C. irritans* by Yoshinaga et al. (2007) for in vitro screening of antiprotozoal compounds to find candidate drugs for chemotherapy of 'white spot disease of marine fish'. The medium consisted of a fish cell layer (FHM cells derived from embryo of fat head minnow) and an agarose gel layer containing Leibovitz's L-15 medium overlaid on the cell layer. We inoculated *C. irritans* theronts between the two layers, after which they transformed to tomonts, then developed into protomonts within 4–5 d and left host fish. Screening was carried out by inoculating theronts into media supplemented with a variety of compounds at a range of concentrations.

During the in vitro screening, the compounds were dissolved in the appropriate solvent at 1000 times their final concentration. The stock solution was then added at a ratio of 1:1000 volume to 0.5% ultralow-melting-point agarose solution containing L-15 medium, antibiotics (100 IU ml^{-1} penicillin G potassium and 100 μg ml^{-1} streptomycin sulfate), and 10% fetal calf serum. We loaded 5 ml of the medicated agarose solutions onto a layer of FHM cells that were seeded at 2.5×10^5 cells cm^{-2} on the bottom of 10 cm^2 cell culture dishes and gelatinized in a refrigerator. A theront suspension containing 50–100 theronts in 20 μl seawater was inoculated at the center of the cell layer beneath the gel with a 200 μl pipette tip and incubated at 25 °C. We observed and counted parasites in the medium with an inverted microscope immediately after inoculation and at 24 h interval for 3 d following inoculation. We obtained images of surviving trophonts using a digital camera attached to the inverted microscope and measured the length of each individual using Scion image (Scion Corporation, Frederick, ND, USA). The control medium was supplemented with the solvent used to dilute each compound and inoculated with theront suspension. The assays were carried out in triplicate in three wells. We conducted two rounds of in vitro screening. First we evaluated the effects of a range of anti-protozoal chemicals (sodium salinomycin, tricrabendazol, secnidazol, metronidazol, tinidazol, and amprolium hydrochloride), followed by screening of three ionophores (sodium monencin, narasin, and sodium salinomycin) (Table 1). Before screening, we examined the cytotoxic effects of each compound to determine the allowable highest concentrations of each compounds for the assay. In brief, we cultured FHM cells on the media supplemented with the chemicals at serial (10-fold) dilutions within the range 200–0.02 μM without inoculating the theronts.

Table 1
Antiprotozoal compounds assayed in vitro against *Cryptocaryon irritans*.

	Manufacture	Soluvent used for dissolving	Molarity (μM) in media	Weight/volume concentration (ppm) in media
Primary screening				
Tricrabendazol	WAKO	DMSO[a]	200	72
			20	7.2
			2	0.72
Sodium salinomysin (98%)	WAKO	DMSO	2	1.5
			0.2	0.15
			0.02	0.015
Secnidazol	LKT laboratories	DMSO	200	37
Metronidazol	Sigma	DMSO	200	34
Tinidazol	LKT laboratories	DMSO	200	49.5
Amprolium HCl	LKT laboratories	Methanol	200	48.7
Secondary screening				
Sodium salinomycin (98%)	WAKO	DMSO	2	1.5
			0.2	0.15
			0.02	0.015
Sodium monencin (95%)	Sigma	DMSO	2	1.4
			0.2	0.14
			0.02	0.014
Narasin (97%)	Sigma	DMSO	2	1.53
			0.2	0.153
			0.02	0.0153

[a] Dimethyl sulfoxide.

2.4. Safety of oral administration of sodium salinomycin to Japanese founder

We chose sodium salinomycin because of its efficacy in vitro and availability. Before evaluating its efficacy, we determined the safe oral dose of sodium salinomycin for Japanese flounder (*Paralychthys olivaceus*).

We were offered crude sodium salinomycin (titer, 450 mg/g) from the Kohkin Chemical Co. Ltd. (Higashi-Osaka, Japan) for use in all in vivo experiments. The compound was suspended in feed oil using a sonicator then mixed with food pellets at a ratio of 5:100 (weight/weight) in plastic bags with shaking to give final concentrations of 100, 200, and 400 ppm sodium salinomycin. The medicated diets were then kept overnight at 4 °C to ensure absorption of the drugs. The control diet was prepared in the same manner but only included feed oil.

We conducted two sets of experiments to test the effects of diets supplemented with sodium salinomycin. In each experiment, 80 fish were randomly divided into 8 groups and placed into 60 L aquaria equipped with an overhead biological filter (N = 10 fish per aquarium). The fish were acclimated in the aquaria for 3 d prior to the experiments at 20 ± 2 °C and 34 ppt salinity. Following acclimation, the fish were fed one of the medicated diets, at one of three concentrations, or the control diet for two weeks. The diets were freshly prepared and were fed ad libitum over a period of 15 min, once a day. We recorded the total amount that was fed each day. Each concentration was fed to fish in two replicate aquaria. We measured body weight and standard body length before and after the experiment. Dead fish were removed from the aquaria daily. We monitored concentrations of NH_4^+/NH_3 and NO_2

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