Effects of 3,N-methylglucamine lasalocid on *Amyloodinium ocellatum*

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ABSTRACT: The cosmopolitan nature of *Amyloodinium ocellatum* insures the constant possibility of an epizootic in high density mariculture facilities. Chemotherapeutics have shown variable results against the parasite and none are currently approved for use in food fish. The ionophorous antibiotic lasalocid is currently approved for food animal use for the treatment and prophylaxis of coccidian protozoa. The sodium salt of lasalocid was converted to a water-soluble 3,N-methylglucamine salt to evaluate its therapeutic potential for amyloodiniosis. Tomonts and trophonts were incubated in saltwater containing antibiotics (penicillin 100 IU ml⁻¹, streptomycin 100 µg ml⁻¹) and 100 to 0.001 ppm concentrations of 3,N-methylglucamine lasalocid (3NMG-lasalocid). The 24 h tomont division rate and *in vitro* trophont density decreased in a dose-dependent manner when exposed to 3NMG-lasalocid concentrations between 100 and 0.001 ppm. Motile dinospores did not excyst until the 3NMG-lasalocid concentration was reduced to 0.001 ppm, and then at a concentration 44 % that of controls. Cracked and ruptured tomonts were observed by scanning electron microscopy and degenerated internal membranous structures of trophonts were observed by transmission electron microscopy. Trophont infection on 0.5 to 1 g red drum *Sciaenops ocellatus* fry exposed to 2.5 dinospores ml⁻¹ in 6.0 l of saltwater was reduced by 80 % on gill filaments and 54 % mm⁻² surface area when treated with 0.1 ppm 3NMG-lasalocid. Infection was prevented at 1.0 ppm 3NMG-lasalocid.

KEY WORDS: Lasalocid · *Amyloodinium* · Ionophore · Red drum

INTRODUCTION

Management measures such as high water turnover rates, micro-filtration, and sanitation are used to control *Amyloodinium ocellatum* in mariculture facilities, but the ubiquitous dispersal of the parasite insures the possibility of an epizootic. Fresh water or formalin immersion bath protocols are effective management procedures for quarantined fish in laboratory and pet fish facilities (Lawler 1979, Paperna 1984a, Noga & Bower 1987), but are impractical for large production facilities. Several herbicides, disinfectants, salinity concentrations, and antiprotozoal drugs have been tested for the control of amyloodiniosis with variable results (Dempster 1955, Lawler 1977, Johnson 1984, Paperna 1984b, Lewis et al. 1988).

Dinospores have been considered the most susceptible stage of the 3-phase life cycle (sessile feeding trophont/encysted reproductive tomont/free-swimming infective dinospores) of *Amyloodinium ocellatum*. Copper compounds (0.75 ppm free copper) can be used to treat an outbreak of *A. ocellatum* by eliminating the dinospore, but this dose may be harmful to most fish species (Paperna 1984a). Most fish species will survive and amyloodiniosis can be prevented or controlled at 0.15 ppm free copper (Dempster 1955, Johnson 1984). The antimalarial drug chloroquine has been demonstrated to have therapeutic potential. Chloroquine eliminated dinospore activity *in vitro* after 24 h at 2.5 µg ml⁻¹, and oral doses of 50 mg kg⁻¹ body weight produced inhibitory tissue drug levels (>5.0 µg drug ml⁻¹) for 7 d with most of the drug disappearing from the tissue within 30 d (Lewis et al. 1988). However, due to governmental regulations and environmental concerns,

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Fig. 1. *Amyloodinium ocellatum*. Forty-eight hour cell culture trophont. Thecal opening (arrow) at bottom of trophont from which rhizoids and stomopode emerge. Also thought to be the site of ion pump for osmotic regulation at varying salinity. $\times 2000$ (scale bar = $10 \mu\text{m}$)

chloroquine is unlikely to be approved for aquatic food animal use.

Amyloodinium ocellatum has a temperature-dependent salinity tolerance range of 1 to 78 ppt (Paperna 1984b), suggesting the presence of a powerful and efficient ion pump. An opening at the base of the thecal plate shell of the trophont, from which the rhizoid complex and stomopode emerge (Fig. 1), is subtended by a thin electron-dense layer of densely staining material, which swells to a massive osmiophilic ring at the perimeter (Lom & Lawler 1973). The author theorized that this is the site of an ion pump regulating ionic equilibrium at variable salinity.

The polyether ionophorous antibiotics disrupt internal membranous organelles by the stimulation of energy-linked transport in mitochondria, increased membrane permeability, and the formation of lipophilic complexes with sodium cations and their transport across membranes into the cell (Smith et al. 1981, Mehlhorn et al. 1983, Smith & Galloway 1983, Daszak et al. 1991). The ion pump thought to be present in *Amyloodinium ocellatum* would be a likely target for ionophorous drugs with ion influx potential. The sodium salt of lasalocid is labeled for use as a feed additive (AvatecTM, poultry; BovatecTM, cattle) for the prevention and treatment of coccidian intestinal parasites. Therefore, the potential of lasalocid as a treatment or prophylactic for amyloodiniosis was evaluated.

The ionophorous antibiotics (e.g. monensin, romensin, salinomycin, lasalocid) typically have low water solubility (0.2% in water for lasalocid). Dissolving lasalocid in ethanol (2.1% solubility in ethanol) and adding to saltwater produced a flocculent precipitate. Therefore, the sodium salt of lasalocid was converted to an N-methylglucamine salt to enhance its water solubility for use in this study.

MATERIALS AND METHODS

Lasalocid conversion. Fifty grams of lasalocid provided by the manufacturer (Hoffmann-La Roche, Inc., Nutley, NJ, USA) as a sodium salt (molecular weight 612.78) was dissolved in 500 ml chloroform and placed into a separatory funnel. The sodium lasalocid was converted to lasalocid free acid by adding an equal volume of 1 N HCl to the solution, shaking and allowing the acid/chloroform layers to separate. The bottom chloroform layer was drained into another separatory funnel and the process repeated with a second 500 ml of 1 N HCl.

The chloroform layer was drained into another separatory funnel, an equal volume of deionized water added, and the contents shaken and the aqueous/chloroform layers allowed to separate. The chloroform layer was drained into another separatory funnel and a second volume of deionized water added and the process repeated. The chloroform layer was drained into a beaker and evaporated under water faucet vacuum in a heated water bath (37°C) until a thick paste formed (48 to 72 h).

One hundred grams of N-methylglucamine (molecular weight 195.2) was dissolved in 500 ml methanol and combined with the lasalocid free acid paste. The volume was reduced 90% by heating (37°C) while stirring to evaporate the methanol, forming a thick oil (18 to 24 h). The preparation was dissolved in an adequate amount of hexane (about 1.0 l) and chilled overnight at 4°C while stirring to precipitate the 3,N-methylglucamine-lasalocid crystals (3NMG-lasalocid). The 3NMG-lasalocid crystals were collected by filtration and the filter paper soaked in deionized water to dissolve the crystals. The water was filtered through a second filter paper to remove non-water-soluble particles and the filtrate lyophilized to obtain pure 3NMG-

Table 1 Saltwater media (SM) formulations for cell culture propagation. Sterilized by 0.45 μm filtration. Based on IO2/HBSS use by Noga (1987, 1989, 1992)

	SM-30 ppt (g l ⁻¹)	SM-10 ppt (g l ⁻¹)
NaCl	24.29	8.10
MgSO ₄ ·7H ₂ O	4.85	1.62
MgCl ₂ ·6H ₂ O	3.49	1.16
CaCl ₂ ·2H ₂ O	1.17	0.39
KCl	0.77	0.26
NaHCO ₃	0.38	0.13
Na ₂ HPO ₄ ·7H ₂ O	0.07	0.023
KH ₂ PO ₄	0.045	0.015
Glucose	—	1.0
Penicillin	100 IU ml ⁻¹	100 IU ml ⁻¹
Streptomycin	100 μg ml ⁻¹	100 μg ml ⁻¹
pH	7.4	7.4
Specific gravity	1.023 g ml ⁻¹	1.007 g ml ⁻¹

lasalocid. Samples of the final product, sodium lasalocid, and N-methylglucamine were submitted to the Texas A&M University chemistry department for nuclear magnetic resonance (NMR) analysis.

Tomont treatment. The protocol for collecting microbe-free *Amyloodinium ocellatum* tomonts is described elsewhere (Oestmann & Lewis 1995). In brief, tomonts were collected by osmotic shock, purified by Percoll® gradient centrifugation, enumerated, and distributed (30 000 tomonts in 30 ppt saltwater medium, SM-30; Table 1) into seven 25 cm² cell culture flask. After 1 h settling time, the SM-30 used to prepare the tomonts was aspirated from 6 of the flasks and replaced with SM-30 supplemented with 10-fold dilutions of 3NMG-lasalocid (100 to 0.001 ppm 3NMG-lasalocid in SM-30) with the seventh flask serving as a control. At 24 h incubation the tomont division rates were assessed by observing with an inverted microscope. Dinospores were enumerated at 72 h incubation by aspirating an aliquot with a 37% formalin coated Pasteur pipette and loading onto a hemocytometer (Bower et al. 1987). Samples were removed from the flasks and fixed overnight at 4°C in 5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (Karnovsky 1965). The fixative was replaced with 0.1 M sodium cacodylate buffer the next morning in preparation for scanning electron microscopy.

3NMG-lasalocid toxicity to red drum cell culture. The toxic effect of 3NMG-lasalocid was assessed on red drum *Sciaenops ocellatus* dorsal fin cell (RDFC) monolayers. Three-day-old monolayers were trypsinized, enumerated, and their viability assessed by trypan blue exclusion. Seven 25 cm² cell culture flasks were prepared using 5 ml aliquots of 1.62×10^6 RDFC ml⁻¹ in L-15 (BioWhittaker, Inc., Walkersville, MD, USA) cell culture medium supplemented with 10%

fetal bovine serum, 1% L-glutamine, and penicillin (100 IU ml⁻¹) and streptomycin (100 μg ml⁻¹). Confluent monolayers had formed after 24 h incubation at 26°C, at which time the media from 6 flasks were aspirated and replaced with L-15 containing supplements plus 10-fold dilutions of 3NMG-lasalocid (100 to 0.001 ppm 3NMG-lasalocid in L-15) with the seventh flask serving as a control. Incubation continued at 26°C for 3 d, at which time the RDFC monolayers were trypsinized, enumerated, and their viability assessed by trypan blue exclusion.

In vitro trophont treatment. The effect of 3NMG-lasalocid was evaluated on *in vitro* trophonts propagated on red drum dorsal fin cells grown as aggregates (Oestmann 1994). Dinospores emerging from microbe-free tomonts attached and transformed into trophonts on RDFC aggregates in 10 ppt saltwater media (SM-10; Table 1). Aliquots of infected RDFC aggregates were divided into 7 siliconized glass Leighton tubes. One hour after exposure to dinospores, the SM-10 from 6 tubes was aspirated and replaced with SM-10 containing 10-fold dilutions of 3NMG-lasalocid (100 to 0.001 ppm 3NMG-lasalocid in SM-10) with the seventh tube serving as a control. The tubes were then placed in a 23°C incubator on a rotary table and gently rotated to keep the aggregates suspended. At 24 and 48 h incubation trophont densities were evaluated microscopically and aggregate samples fixed as described above for tomonts and subsequently fixed in osmium tetroxide for transmission electron microscopy.

In vivo trophont treatment. Six red drum fry (0.5 to 1 g) were placed in each of 6 small plastic tanks in 6.0 l saltwater, 20 ppt (Fritz Supersalt; Fritz Chemical Co., Dallas, TX, USA) for 1 wk acclimation. Tank 1 was then treated with 2.5 dinospores ml⁻¹ (15 000 dinospores), tank 2 was treated with 2.5 dinospores ml⁻¹ plus 0.1 ppm 3NMG-lasalocid, tank 3 was treated with 0.1 ppm 3NMG-lasalocid alone. Tank 4 was treated with 2.5 dinospores ml⁻¹ plus 1.0 ppm 3NMG-lasalocid while tank 5 was treated with 1.0 ppm 3NMG-lasalocid alone. Tank 6 served as the non-treated control. After 24 h exposure, the fish were killed by cervical transection and trophont load per gill filament assessed microscopically (10 filaments per arch on each of 6 fish averaged). The surface area trophont load was also assessed on the tail, dorsal, anal, and pectoral fin clips (average mm⁻²).

RESULTS

The conversion of sodium lasalocid to an N-methylglucamine salt is very inefficient, yielding only about 50 mg of final product from 50 g of sodium lasalocid.

Fig. 2. *Amyloodinium ocellatum*. Tomonts incubated 72 h in 0.1 ppm 3NMG-lasalocid. Cracked and ruptured tomonts spilling white starch grains: irregular and non-dividing tomonts also can be seen. $\times 1000$ (scale bar = 10 μm)

Fig. 3. *Amyloodinium ocellatum*. Twenty-four hour cell culture trophont with no exposure to 3NMG-lasalocid. Cytoplasm of trophont and RDFC aggregate shows orderly cellular structure. $\times 5000$ (scale bar = 10 μm)

The sodium lasalocid was expected to convert to a 2,N-methylglucamine lasalocid. However, NMR analysis revealed 3,N-methylglucamine substitutions had taken place at the sodium and both hydroxyl group positions, yielding a final product with a molecular weight of 1176.38.

RDFC monolayers treated with 3NMG-lasalocid exhibited a dose-dependent toxic response. At 24 h the 100 ppm 3NMG-lasalocid treated monolayer had sloughed and the 10 ppm 3NMG-lasalocid treated cells had retracted, rounded edges with many floating cells. The 1.0 to 0.001 ppm 3NMG-lasalocid treated monolayers appeared healthy. After 3 d exposure to 3NMG-lasalocid the control monolayer viability was

96% while the 10 to 0.001 ppm 3NMG-lasalocid treatment viabilities were 93, 98, 98, 97, and 98% respectively. The cell count also was inversely proportional to the 3NMG-lasalocid dose. The control cell count was 6.8×10^5 RDFC ml^{-1} while the cell counts for the 10 to 0.001 ppm 3NMG-lasalocid treatment were 1.3×10^5 , 5.2×10^5 , 6.4×10^5 , 6.9×10^5 , and 6.8×10^5 RDFC ml^{-1} respectively.

The 24 h tomont division rate also decreased in a dose-dependent manner when treated with 3NMG-lasalocid. The control tomont division rate was 82% compared to 59, 69, 75, 78, 80, and 81% (average of 3 trials) observed for the 100 to 0.001 ppm 3NMG-lasalocid dilutions respectively. Irregular division, cracked and ruptured tomonts, and release of starch grains were observed by scanning electron microscopy of 3NMG-lasalocid treated tomonts (Fig. 2). Dinospores excysted but were deformed and non-motile at 1 to 0.01 ppm 3NMG-lasalocid. Motile dinospores did not excyst until the 3NMG-lasalocid concentration was reduced to 0.001 ppm, and then at a concentration 44% that of controls (27 800 dinospores ml^{-1} for control vs 12 250 dinospores ml^{-1} at 0.001 ppm 3NMG-lasalocid).

Subjectively, the *in vitro* trophont infection on RDFC aggregates was observed to decrease in a dose-dependent manner. Trophonts were not present in sufficient numbers for electron microscopic examination until the 3NMG-lasalocid concentration was decreased to 0.01 ppm. Effects of the 3NMG-lasalocid treatment observed by transmission electron microscopy were similar to effects previously described in coccidian parasites (Riley 1969, Scholtyseck et al. 1971, 1972, Vetterling et al. 1973, Mehlhorn et al. 1983, Smith & Galloway 1983, Daszak et al. 1991) (Figs. 3 & 4). Internal membranous organelles of trophonts were disrupted and degener-

Fig. 4. *Amyloodinium ocellatum*. Twenty-four hour cell culture trophont incubated in 0.01 ppm 3NMG-lasalocid. Cytoplasm of trophont and RDFC aggregate are disrupted with increased vacuolization. $\times 5000$ (scale bar = 10 μm)

Fig. 5. *Amyloodinium ocellatum*. Degenerated mitochondria (arrows) of trophonts incubated in 0.01 ppm 3NMG-lasalocid. $\times 20\,000$

ated, indicative of the actions of ionophorous antibiotics (Fig. 5) documented in the previous reports.

Trophont infection on 0.5 to 1 g red drum fry exposed to 2.5 dinospores ml^{-1} in 6.0 l of saltwater (20 ppt) was reduced by 80% on gill filaments compared to the tank treated with dinospores alone (mean 6.69 vs 1.37 trophonts per filament) when treated with 0.1 ppm 3NMG-lasalocid ($p < 0.01$). The gill lamellae of fish in tanks treated with dinospores alone exhibited severe degeneration, clubbing, and necrosis. Infection was reduced by 54% mm^{-2} surface area (mean 11.55 vs 5.34 trophonts mm^{-2}) in the 0.1 ppm 3NMG-lasalocid treated tank ($p < 0.01$). Fish in the control tank, 0.1 ppm 3NMG-lasalocid treated tank, 1.0 ppm 3NMG-lasalocid treated tank, and the tank treated with 2.5 dinospores ml^{-1} and 1.0 ppm 3NMG-lasalocid showed no sign of infection or gill necrosis. All fish survived to the experimental end point, indicating that 3NMG-lasalocid may not be toxic when used at the described dosages for 24 h.

DISCUSSION

Amyloodinium ocellatum is an important parasite in mariculture facilities on the U.S. Gulf of Mexico coast. The lack of effective treatment measures and the cosmopolitan nature of the parasite insures epizootics with

high morbidity and mortality will likely continue to occur. Lasalocid currently is labeled for use in food animals (poultry and cattle) for the prevention of coccidian intestinal protozoa. A water-soluble 3NMG-lasalocid was demonstrated in these studies to be effective against 2 stages of the life cycle (tomont and trophont) previously considered refractory to treatment (Johnson 1984, Paperna 1984a). *In vitro* trophont infection was significantly reduced at 0.1 ppm and eliminated at 1.0 ppm 3NMG-lasalocid, with motile dinospore excystment from tomonts reduced or prevented at levels as low as 0.001 ppm. Toxicity to 0.5–1.0 g red drum fry was not evident at these dosages.

This drug may be an effective therapeutant for a broad range of common protozoal diseases common in aquaculture (e.g. *Ichthyobodo*, *Ichthyophthirius multifiliis* in freshwater fish and gregarines in shrimp). Further studies on the drug's toxicity to various cultured fish species, tissue residue levels, efficacy against other protozoal parasites, and possible effluent levels are warranted. A full scale field trial to test the treatment and prophylactic potential of 3NMG-lasalocid should be undertaken for amyloodiniosis.

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