**INTRODUCTION**

*Cryptobia salmositica* Katz, 1951 is a haemoflagellate that causes disease and mortality in economically important fishes in North America (Woo & Poynton, 1995). The clinical signs of the disease include exophthalmia, splenomegaly, anaemia, general oedema and abdominal distention with ascites (Woo, 1979). Infection with the parasite results in high mortalities in natural infections and it is considered a lethal pathogen of salmon in semi-natural and intensive culture facilities (Bower & Thompson, 1987). Pacific salmon (*Oncorhynchus* spp.) are anadromous fish species. Experimentally infected fish retain the infection and mortalities continue when experimentally infected hosts are transferred from fresh-water to salt-water (Bower & Margolis, 1984; Li & Woo, 1997). Also, significant mortalities are associated with post-spawning rainbow trout (*Oncorhynchus mykiss*) and pre-spawning chinook salmon (*Oncorhynchus tshawytscba*) (Woo & Poynton, 1995). Adult Pacific salmon had detectable infections as early as five days after they returned to fresh-water and parasitaemias in many fish were very high when they spawned (Bower & Margolis, 1984).

One method of control is vaccination. The pathogenic strain of *C. salmositica* has been attenuated and is used as a live vaccine to protect fish against experimental cryptobiosis (Woo & Li, 1990; Ardelli et al., 1994; Li & Woo, 1995; Sitja-Bobadilla & Woo, 1994). The strategy is to immunize presmolts as these fish would be protected while in fresh-water and when they return from the marine environment (Woo, 1992). Li & Woo (1995) have shown that vaccinated trout are protected for at least two years, and that protection is not affected when vaccinated fish are transferred to salt-water (Li & Woo, 1997). There is a need for chemotherapy as there is about 50-60% annual mortality in chinook, *Oncorhynchus tshawytscba*, broodfish in the Soleduck hatchery in Washington State, USA (L. Peck personal communication, 1994). Low concentrations of crystal violet (20-50 μM) cause swelling of the mitochondria and the uncoupling of oxidative phosphorylation in *Trypanosoma cruzi* (Gadelha et al., 1989). *C. salmositica* is phylogenetically related to *T. cruzi* and it also has a large mitochondria. The purpose of the present study was to examine the *in vitro* effects of crystal violet on...
C. salmositica. This is part of an ongoing project on the development of chemotherapy against salmonid cryptobiosis.

MATERIALS AND METHODS

IN VITRO CULTURE OF CRYPTOBIA SPP.

A cloned strain of pathogenic Cryptobia salmositica was used to infect rainbow trout held at 11°C. The strain was initially isolated from Piscicola salmositica and details of the cloning of the parasite and fish maintenance have been described earlier (Woo, 1979). Blood from an infected trout was aseptically inoculated into sterile culture flasks containing Minimum Essential Medium (MEM) supplemented with Hank’s salts, L-glutamine, 25 mM Hepes buffer and 25% heat-inactivated foetal bovine serum, and cultured. The nonpathogenic vaccine strain of C. salmositica was cloned and has been maintained in MEM since 1989 (Woo & Li, 1990).

Cryptobia bullocki was originally isolated from the blood of a southern flounder, Paralichthys lebostigma (Woo & Thomas, 1991). It has also been maintained in MEM. Cryptobia catostomi was isolated from the blood of a white sucker Catostomus commersoni and maintained in TDL-15 medium supplemented with heat-inactivated white sucker plasma (Li & Woo, 1996).

IN VITRO EFFECTS OF CRYSTAL VIOLET

Approximately 1,500 cultured individuals of an avirulent strain of C. salmositica (washed three times in cold blooded vertebrate Ringer’s (CBVR) solution) in 25 μl of CBVR were incubated for three hours at 11°C (in a microtitre plate) with 25 μl of crystal violet at concentrations of 0.1 μM, 1.0 μM, 10.0 μM and 100.0 μM. Control wells contained parasites incubated with CBVR, phosphate buffered saline (PBS) and MEM. The crystal violet was dissolved in PBS at pH 7.2. Each concentration of crystal violet was replicated 24 times. After three hours the wells were examined using an inverted microscope (ocular 10 X and objective 10 X) for living parasites. The endpoint was the examination period when there was an absence of living parasites.

LONG TERM EFFECTS OF CRYSTAL VIOLET

ON PATHOGENIC AND NONPATHOGENIC STRAINS OF C. SALMOSITICA

MEM and crystal violet were added to a culture flask such that the final volume was 20 ml with a final concentration of 0.0 μM (Group A and Group E; n = 10/group), 0.05 μM (Group B and Group F; n = 10/group), 0.01 μM (Group C and Group G; n = 10/group) and 0.001 μM (Group D and Group H; n = 10/group). Approximately 250,000 pathogenic (Groups A, B, C, D) and nonpathogenic (Groups E, F, G, H) C. salmositica were inoculated aseptically into each of the culture flasks and incubated in a slanted position at 11°C. Flasks were sampled (50 μl) every three days and the number of parasites determined using a haemacytometer (Archer, 1965).

INFECTIVITY TEST FOR ASSESSING IN VITRO SENSITIVITY OF C. SALMOSITICA TO CRYSTAL VIOLET

Blood was withdrawn from the caudal vein of an infected rainbow trout and diluted (with uninfected whole trout blood) such that 25 μl of blood contained approximately 1,500 C. salmositica. Crystal violet in PBS (100 μM-Group J, 200 μM-Group K, and 500 μM-Group L) was added to the blood. Controls (Group I) were whole blood with Cryptobia incubated without crystal violet. Experimental and control groups were incubated for three hours at 11°C (in a microtitre plate) and then examined under an inverted microscope (ocular 10 X and objective 10 X) for living parasites. After incubation, each well in the microtitre plate (with 25 μl of crystal violet and C. salmositica) was rinsed with 0.1 ml of Alsever’s solution and the contents inoculated intraperitoneally into a juvenile rainbow trout. Fish were bled (0.1 ml/fish) at one, two, three and four weeks postinoculation and parasitaemias were determined using a haemacytometer for high parasitaemias or the haematocrit centrifuge technique for low parasitaemias (Woo, 1969).

The experiment was repeated. Blood was withdrawn from the caudal vein of an infected rainbow trout (1,500 individuals of Cryptobia in 25 μl of blood) and treated as described earlier. Crystal violet (100 μM, 200 μM, 500 μM, 2,500 μM, 5,000 μM, 1 mM, 100 mM, 200 mM, 300 mM and 1 M) was added to the blood. Controls were parasites incubated without crystal violet. Treatment and control groups were incubated for three hours at 11°C. After incubation, the contents of each well were inoculated into a juvenile rainbow trout. Fish were bled at one and two weeks postinoculation and parasitaemias determined as described earlier.

IN VITRO EFFECTS OF CRYSTAL VIOLET

ON PATHOGENIC AND NONPATHOGENIC CRYPTOBIA SPP.

Three species of Cryptobia (pathogenic and nonpathogenic strains of C. salmositica; nonpathogenic C. catostomi; pathogenic C. bullocki) were used. Approximately 1,500 individuals of each strain from culture (washed three times in CBVR) in 25 μl of CBVR were incubated (three hours at 11°C) with 0.1 μM, 1.0 μM, 10.0 μM and 100.0 μM of crystal violet in a microtitre plate kept on ice. Controls were parasites incubated
in PBS, CBVR, MEM and TDL-15 without crystal violet. After incubation, the microtitre plates were examined using an inverted microscope (ocular 10 X and objective 10 X) for living parasites.

**PREPARATION AND FIXATION OF C. SALMOSITICA FOR TRANSMISSION ELECTRON MICROSCOPY**

Pathogenic *C. salmositica* were concentrated directly from culture flasks by centrifugation and washed in CBVR. Parasites (1.0 × 10^6) were incubated at 11°C in crystal violet (0.1 μM, 1.0 μM and 10.0 μM) and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate at 15 minutes intervals for 240 minutes. Postfixation was in 2% osmium tetroxide. Samples were serially dehydrated to 70% ethanol and then treated with 0.5% p-phenylenediamine (Ledingham & Simpson, 1972) in 70% ethanol. Dehydration was continued to 100% ethanol and samples were embedded in Spurr’s resin and polymerized. Ultrathin sections corresponding to a gold interference colour were cut using a Reichert microtome equipped with a diamond knife. Ribbons were collected from the water surface on cleaned, uncoated copper mesh grids and allowed to dry for 60 minutes. Sections attached to grids were stained using saturated uranyl acetate (7.7%) and acetone (1:1) for five minutes and lead citrate for one minute. Sections were examined using a JEOL 100CX electron microscope operating at 80 kV.

**STATISTICAL ANALYSIS**

Data were analyzed using the Statistical Analysis System (SAS Institute Inc., 1985). Analysis of Variance (ANOVA) was used to determine significant differences in the number of parasites between control and experimental groups. Results were considered significant if p ≤ 0.05.

**RESULTS**

**EFFECTS OF CRYSTAL VIOLET ON CRYPTOBIASALMOSITICA**

Pathogenic and nonpathogenic strains of *C. salmositica* were still active after three hours in wells which contained 0.1 μM of crystal violet but were lysed at concentrations of 1.0 μM, 10.0 μM and 100.0 μM. Parasites remained active in control wells with PBS, MEM or CBVR. Crystal violet inhibited multiplication of the pathogenic strain of *C. salmositica* in 0.05 μM (Group B), 0.01 μM (Group C) and 0.001 μM (Group D) and most of the parasites were lysed in 12 days. The parasites in the controls (Group A) without crystal violet multiplied readily. Significant differences were detected between the number of parasites in control and treatment groups between 25-55 days after incubation in crystal violet (Fig. 1).

The nonpathogenic strain of *C. salmositica* in 0.05 μM (Group F) of crystal violet did not multiply and the parasites were lysed, while incubation in 0.01 μM

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**Fig. 1.** – Long term effects of crystal violet on a pathogenic strain of *Cryptobia salmositica*. ● Group A: not incubated in crystal violet; ▼ Group B: incubated in 0.05 μM of crystal violet; ▲ Group C: incubated in 0.01 μM of crystal violet; □ Group D: incubated in 0.001 μM of crystal violet.

**Fig. 2.** – Long term effects of crystal violet on a nonpathogenic strain of *Cryptobia salmositica*. ● Group E: not incubated in crystal violet; ▼ Group F: incubated in 0.05μM of crystal violet; ▲ Group G: incubated in 0.01 μM of crystal violet; □ Group H: incubated in 0.001 μM of crystal violet.
(Group G) and 0.001 μM (Group H) of crystal violet did not inhibit in vitro multiplication. Nonpathogenic *C. salmositica* in 0.001 μM of crystal violet multiplied more readily than those infected with non-exposed parasites. The numbers of parasites were significantly lower in Group I (500 μM) at three (p = 0.0022) and four (p = 0.0013) weeks postinoculation, in Group K (200 μM) at three (p = 0.0400) weeks postinoculation, and in Group J (100 μM) at four (p = 0.0362) weeks postinoculation (Table I).

Low concentrations (≤ 2,500 μM) of crystal violet reduced the infectivity of the parasite, as some fish were infected and parasitaemias were significantly lower than controls. At high concentrations (2,500 μM-1 M) of crystal violet, the parasite was not infective as no parasites were detected in fish (Table II).

**Effects of Crystal Violet on Cryptobia spp.**

Crystal violet was more toxic to pathogenic (pathogenic strain of *C. salmositica* and *C. bullocki*) than to nonpathogenic (nonpathogenic strain of *C. salmositica* and *C. catostomi*) Cryptobia spp. At low concentrations (0.1 μM) of crystal violet, all Cryptobia spp. were active while at high concentrations (100 μM) of crystal violet, only the nonpathogenic parasites were motile. Parasites in the medium with no crystal violet were active (Table III).

### Target of Crystal Violet Toxicity

Pathogenic *C. salmositica* had a large kinetoplast-mitochondria complex which contained a compact mass of kinetoplast DNA. The mitochondria was bound by a double membrane and it had many cristae (Fig. 3). After exposure to crystal violet, the first morphological change was the condensation of kinetoplast DNA (Fig. 4). Fifteen minutes after exposure the condensation began as distinct linear masses of DNA distributed throughout the kinetoplast (Fig. 4) and continued until the DNA had condensed into a single mass (Figs. 5 and 7). After 45 minutes, vacuoles began to form in the kinetoplast, and often pushed the cristae to the outer edges of the mitochondria (Fig. 6). At higher concentrations, the cristae often appeared distorted (Fig. 8) and the kinetoplast was fragmented and swollen (Fig. 9) after longer exposure to crystal violet.

The mitochondrial membranes were characterized by a double membrane which had a trilaminar appearance (Figs. 10 and 11). After incubation in 1.0 μM of crystal violet for 30 minutes, the outer membrane was disrupted and was no longer discernible as having a trilaminar appearance (Figs. 12 and 13) with extensive swelling of the kinetoplast.

The nucleus was bound by a double membrane and had dense chromatin (Fig. 14). After incubation in 1.0 μM of crystal violet for 120 minutes the outer nuclear membrane began to swell and was separated from the inner membrane (Figs. 15 and 16).

### Table I. Infectivity of Cryptobia salmositica after in vitro exposure to crystal violet in fish blood

<table>
<thead>
<tr>
<th>Concentration (moles/L)</th>
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<th>Week 4</th>
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<td>(11.9)</td>
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<td>(7.0)</td>
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</table>

1 Number of wells containing living parasites/number of replicates.
2 Number of fish with detectable infection/number of fish inoculated.
3 Mean parasitaemia.
4 Parasitaemia not determined.

### Table II. Effects of crystal violet on pathogenic Cryptobia salmositica in fish blood and infectivity after exposure

<table>
<thead>
<tr>
<th>Concentration (moles/L)</th>
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</table>

1 Number of wells containing living parasites/number of replicates.
2 Number of fish with detectable infection/number of fish inoculated.
3 Mean parasitaemia.
4 Parasitaemia not determined.

Table I. Infectivity of Cryptobia salmositica after in vitro exposure to crystal violet in fish blood.

Table II. Effects of crystal violet on pathogenic Cryptobia salmositica in fish blood and infectivity after exposure.
In vitro effects of crystal violet on Cryptobia

Figs. 3-9. - Fig. 3. Transmission electron micrograph of Cryptobia salmositica without exposure to crystal violet. k: kinetoplast; m: mitochondria × 28,000. Fig. 4. Electron micrograph of Cryptobia salmositica showing condensation of kinetoplast DNA after exposure to 1.0 μM of crystal violet for 30 minutes × 30,000. Fig. 5. Electron micrograph of Cryptobia salmositica showing condensation of kinetoplast DNA after exposure to 10.0 μM of crystal violet for 15 minutes × 16,000. Fig. 6. Electron micrograph of Cryptobia salmositica after exposure to 1.0 μM of crystal violet for 45 minutes. Notice the vacuole formation in the kinetoplast region × 52,000. Fig. 7. Electron micrograph of Cryptobia salmositica showing fragmentation of the kinetoplast DNA × 26,000. Fig. 8. Electron micrograph of Cryptobia salmositica after exposure to 10.0 μM of crystal violet for 120 minutes. Note the distorted shape of the cristae × 30,000. k: kinetoplast. Fig. 9. Electron micrograph of Cryptobia salmositica after exposure to 10.0 μM of crystal violet for 120 minutes. Note the distorted kinetoplast. k: kinetoplast × 26,000.

Figs. 10-13. — Fig. 10. Electron micrograph of Cryptobia salmositica without exposure to crystal violet. Note the compact kinetoplast (k), well developed cristae, and the double membrane surrounding the mitochondria x 57,000. Fig. 11. Enlargement of Fig. 10 showing the double membrane x 87,000. Fig. 12. Electron micrograph of Cryptobia salmositica incubated in 1.0 μM of crystal violet for 30 minutes. Note the swollen kinetoplast x 57,000. Fig. 13. Enlargement of Fig. 12 showing the distortion of the double membrane surrounding the mitochondria (m) x 87,000.
Figs. 14-16. – Fig. 14. Electron micrograph of *Cryptobia salmositica* incubated in the absence of crystal violet. Note the double membrane surrounding the nucleus × 47,000. Fig. 15. Electron micrograph of *Cryptobia salmositica* incubated in 1.0 μM of crystal violet for 120 minutes. Note the swelling of the outer nuclear membrane. n: nucleus × 40,000. Fig. 16. Electron micrograph of *Cryptobia salmositica* incubated in 1.0 μM of crystal violet for 120 minutes. Note the outer nuclear membrane beginning to swell (n) × 100,000.
Crystal violet (μM) & C. salmositica (nonpathogenic strain) & C. salmositica (pathogenic strain) & C. catostomi (nonpathogenic) & C. bullocki (pathogenic)

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1 Number of wells containing living parasites/number of replicates.

Table III. – The in vitro effects of crystal violet on Cryptobia spp.

DISCUSSION

In Brazil, crystal violet is used in bloodbanks to prevent transmission of Trypanosoma cruzi via blood transfusion (Gadelha et al., 1989); however, it has not been used in chemotherapy. Since there is no effective drug against C. salmositica (Woo & Poynont, 1995), we investigated the possibility that crystal violet may have similar effects against the pathogen. Crystal violet was cryptobiacidal under in vitro conditions. At low concentrations (0.1 μM), C. salmositica was active, but was lysed at higher concentrations (10.0 μM-100.0 μM). There were differences in susceptibility to crystal violet between the pathogenic and nonpathogenic strains of C. salmositica. Low concentrations (0.001 μM and 0.01 μM) of crystal violet did not inhibit in vitro multiplication of the nonpathogenic strain of C. salmositica, while multiplication of the pathogenic strain was inhibited.

Free radicals produced metabolically are important in the toxicity of a number of trypanocidal compounds (Docampo & Moreno, 1984). Either the free radical metabolites of the trypanocidal agents themselves or the superoxide anion, which results from the reduction of oxygen by the radicals, can initiate processes that lead to cell damage. Both T. cruzi cells and homogenates can enzymatically reduce crystal violet to a carbon-centred free radical (Docampo et al., 1983). Free radical formation may cause the toxicity of crystal violet to T. cruzi. Living cells are protected from the damaging effects of free radicals by protective enzymes, which metabolise the radicals to harmless products, or by certain free radical scavenging compounds, termed antioxidants (e.g. superoxide dismutase, catalase and glutathione reductase) (Bryant & Behm, 1989). If a cell’s capacity to detoxify free radicals is exceeded, membrane damage will ultimately lead to cell lysis. Free radical damage to nucleic acids and proteins will also interfere with the cell’s ability to grow and multiply (Bryant & Behm, 1989). Thus, the absence of a protective enzyme, like catalase, would make a parasite susceptible to lysis by free radicals. The pathogenic C. salmositica has undetectable catalase activities, while the nonpathogenic strain has detectable activities of the enzyme (unpublished observations). Thus, we suggest that pathogenic C. salmositica may be more susceptible to crystal violet because it lacks catalase to protect it from free radicals formed by the reduction of crystal violet.

Parasitic protozoa have unique mechanisms to avoid drug toxicity. Leishmania is thought to resist cytotoxic drugs by amplifying either target genes which encode for membrane proteins that function as ATP-dependent extrusion pumps or genes involved in alternate metabolic pathways (Ouellette & Papadopoulos, 1993). In some cases, Leishmania is capable of avoiding toxicity of methotrexate and arsenite by decreasing drug accumulation. If the drug is taken up, it may be inactivated, excreted, modified and excreted, or routed into vacuoles. Furthermore, interaction of the drug with the target may be made less effective by increasing the level of competing substrates or by altering the target to make it less sensitive to the drug (Borst & Ouellette, 1995). Amprolium inhibits the transport of thiamine across the cell membrane of Eimeria tenella, E. acervulina and E. maxima. The mechanism of resistance to this anticoccidial is thought to involve modification of a target receptor so that its sensitivity to inhibition is decreased (Chapman, 1993).

The infectivity of pathogenic C. salmositica in fish blood was altered after in vitro exposure to crystal violet. Parasitaemias were significantly lowered in fish which received the inoculum exposed to crystal violet. Parasitaemias were significantly lowered in fish which received the inoculum exposed to crystal violet. Infectivity of T. b. brucei was prevented after incubation for four hours in plasma from cattle treated with diminazene aceturate. Similarly, isometamidium at 1 ng/ml was sufficient to completely prevent the infectivity of the sensitive T. b. brucei stocks, T. b. evansi and T. vivax (Kaminsky et al., 1990). In the present study, crystal violet might have reduced the number of C. salmositica in the inoculum or it reduced the multiplication rate of the parasite or both, as control fish had significantly higher parasitaemias.

The mechanism of crystal violet toxicity is not understood, but the results of the electron microscopy study suggested that the target in C. salmositica is mainly the
mitochondria-kinetoplast complex. In *T. cruzi* the dye causes swelling of the mitochondria in trypomastigotes and epimastigotes with an uncoupling and inhibitory action on oxidative phosphorylation. After incubating pathogenic *C. salmositica* in crystal violet the most consistent early morphological change was condensation of kinetoplast DNA. Swelling of the mitochondrial and nuclear membranes as well as disruption of the kinetoplast were detected. Pentamidine is thought to preferentially fragment the kinetoplast rather than nuclear chromatin material in *Trypanosoma brucei rhodesiense* blood forms (Macadam & Williamson, 1969). This same effect produced by Berenil is thought to reflect the selective action of Berenil on kinetoplast DNA synthesis and on the buoyant density of trypanosome kinetoplast DNA (Newton & LePage, 1967; 1968). The trypanocidal drug Isometamidium is known to linearize minicircle DNA in the kinetoplast of trypanosomes (Robinson & Gull, 1991) and disruption of the kinetoplast structure has been implicated in its trypanocidal action (Chitambo *et al.*, 1992). It is thought that the drug is effective due to an interaction of isometamidium with DNA and a topoisomerase (Sutherland *et al.*, 1991). The decantation of kinetoplast DNA and disruption of the organelle suggest that the mechanism of crystal violet toxicity may be similar to that of isometamidium.

It is not clear how crystal violet enters cells. The parasite may actively uptake the dye, which leads to the cellular damage, or as suggested by Hoffman *et al.* (1995), crystal violet may bind actively to sites on membranes, causing perturbation of membrane structure. The disruption of the membrane results in a cation intrusion accompanied by a phosphate translocation which causes swelling and dissipation of membrane potential leading to uncoupling of oxidative phosphorylation. The damage to membranes in *C. salmositica* caused by crystal violet suggests that it actively binds to sites on the membrane and causes the distortion as indicated in the electron micrographs.

The results of the *in vitro* study suggest that crystal violet may be a potential therapeutic agent against salmonid cryptobiosis. In low dosage, the dye inhibits multiplication of *C. salmositica*, alters infectivity of the parasite, and causes lesions on mitochondrial and nuclear membranes. The *in vitro* effective dose of crystal violet has not been determined, but our preliminary results suggest that the curative dose must be at about 200 mM to eliminate the infection which may be toxic to some fish.

**ACKNOWLEDGEMENTS**

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