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Antiprotozoals effective *in vitro* against the scuticociliate fish pathogen *Philasterides dicentrarchi*

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ABSTRACT: The histophagous ciliate *Philasterides dicentrarchi* causes fatal scuticociliatosis in farmed turbot *Scophthalmus maximus* and sea bass *Dicentrarchus labrax*. The present study screened 52 candidate antiprotozoals for activity against this pathogen *in vitro*. Of these compounds, 14 were effective (i.e. killed all ciliates within a 24 h assay period). In descending order of efficacy (minimum lethal concentration 100 to 0.8 ppm), these were niclosamide, oxyclozanide, bithionol sulfoxide, toltrazuril, N-(2′-hydroxy-5′-chloro-benzoyl) 2-chloro-4-nitroaniline, furaltadone, doxycycline hyclate, formalin, albendazole, carnidazole, pyrimethamine, quinacrine hydrochloride and quinine sulfate. Administration in filtered seawater rather than phosphate-buffered saline inactivated doxycycline hyclate and albendazole, and markedly reduced that of bithionol sulfoxide and toltrazuril, suggesting that these compounds may not be effective in bath administration. In view of these findings, we discuss the potential utility of chemotherapy as a strategy for the control of scuticociliatosis in farmed turbot and sea bass.

KEY WORDS: Turbot · Scuticociliatosis · *Philasterides dicentrarchi* · Chemotherapy · *In vitro* assay

INTRODUCTION

The term scuticociliatosis covers the diseases of fishes, crustaceans and molluscs caused by histophagous ciliates of the order Scuticociliatida. The most important pathogenic species of this order include *Uronema marinum*, *U. nigricans*, *Anophryoides haemophila* and *Mesanophrys* spp. (Cheung et al. 1980, Morado & Small 1995, Cawthorn et al. 1996, Munday et al. 1997). Recently, there have been reports of various outbreaks of systemic scuticociliatosis due to *Philasterides dicentrarchi* in farmed sea bass and turbot (Dragesco et al. 1995, Iglesias et al. 2001). In the case of turbot, the incidence of this disease appears to have increased in recent years to the extent that it has become one of the major parasite infections in turbot farms (alongside microsporidiosis due to *Tetramicra brevifilum* and ichthyobodosis due to *Ichthyobodo necator*) and a cause of significant commercial losses. To date, however, no effective control measures have been described.

In the present study, we performed *in vitro* trials to investigate the sensitivity of *Philasterides dicentrarchi* to a wide range of chemotherapeutic compounds, with the aim of identifying compounds that may be of use in treating scuticociliatosis caused by this species.

MATERIALS AND METHODS

Isolation and culture of *Philasterides dicentrarchi*. Ciliates were harvested by collecting ascitic fluid from the body cavity of naturally infected turbot *Scophthalmus maximus* and were then maintained under the culture conditions described by Bernard & Fenchel (1996), but with autoclaved *Vibrio anguillarum* as food. *P. dicentrarchi* cultured in this way retains the capacity to induce scuticociliatosis in experimentally infected turbot.

Candidate antiprotozoals. The candidate antiprotozoals tested are listed in Table 1. Stock solutions or sus-

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Table 1. List of candidate antiprotozoals assayed in the present study against *Philasterides dicentrarchi*. pp: pure product

Candidate antiprotozoal	Brand name	Form	Manufacturer
Acaprin	pp	Powder	Bayer
Albendazole	pp	Powder	Sigma
Amoxycillin	pp	Powder	Antibióticos
Amphotericin B	Fungizona	Powder	Squibb
Ampicillin	pp	Powder	Antibióticos
Amprolium	Prolsal	Powder	Esteve
Azithromycin	Zentavion	Powder	Vita
Benzylpenicillin/benzathine	Benzetacil	Injectable suspension	Antibióticos FARMA
Bithionol sulfoxide	pp	Powder	SYVA
Carnidazole	Spartrix	Tablets	Esteve
Cephalexine	pp	Powder	Antibióticos
Chloramphenicol	pp	Powder	Gonmisol
Chloroquine diphosphate	Cidanchin	Powder	Cidan
Ciprofloxacine	Cetraxal	Oral suspension	Salvat
Closantel	Flukiver	Injectable solution	Janssen Pharmaceutica
Dimetridazole	Vibriozol	Powder	IQF
Doramectin	Dectomax	Injectable solution	Pfizer
Doxycycline hyclate	Doxidol	Powder	Uriach
Febantel	Rintal	Granulate	Bayer
Florfenicol	Nuflor	Injectable solution	Schering-Plough
Flubendazole	pp	Powder	Esteve
Formalin	pp	Solution	Panreac
Furaltadone	pp	Powder	Sigma
Gentamycin	Gevramycin	Injectable solution	Schering-Plough
Ivermectin	Ivomec	Injectable solution	Merck Sharp & Dohme
Mebendazole	Lomper	Oral suspension	Esteve
Metronidazole	Flagyl-250	Tablets	Rhône-Poulenc Rorer
N-(2′-hydroxy-5′-chloro-benzoyl) 2-chloro-4-nitroaniline	Fugo-tenil	Tablets	Uriach & Cía
Niclofolan	Bilevon	Injectable solution	Bayer
Niclosamide	pp	Powder	Virbac
Nitrofurantoin	Furantoína	Tablets	Uriach & Cía
Oxibendazole	pp	Powder	SYVA
Oxyclozanide	pp	Powder	Mallinckrodt
Oxytetracycline	pp	Powder	Sanagro
Paromomycin	Humatin	Oral solution	Parke-Davis
Penicillin G	Penilevel	Injectable solution	ERN
Penicillin V	pp	Powder	Antibióticos
Piperazine dichlorhydrate	Piperso	Powder	Sobrino
Praziquantel	Droncit	Tablets	Bayer
Pyrimethamine	Daraprim	Tablets	Wellcome Farmaceutica
Quinacrine hydrochloride	Atabrine	Tablets	Sanofi-Winthrop
Quinine sulfate	pp	Powder	Gonmisol
Ronidazole	pp	Powder	Sigma
Spiramycin	Rovamycine	Tablets	Rhône-Poulenc Rorer
Sulfadiazine	Sulfadiazina Reig Jofré	Tablets	Reig Jofré
Sulfaquinoxaline	Lapinsan Lamons Forte	Powder	Lamons
Tinidazole	Tricolam	Tablets	Farmasierra
Toltrazuril	Baycox	Oral solution	Bayer
Trichlorfon	Neguvon	Powder	Bayer
Triclabendazole	Fasinex 10%	Oral suspension	Novartis
Trimethoprim + sulfadiazine	Triglobe	Tablets	Astra

Table 2. List of antiprotozoals effective against *Philasterides dicentrarchi* in the present study. MLC: minimum lethal concentration in the 24 h assay. Values in parentheses are MLCs when the antiprotozoal solution/suspension was made up in filtered seawater rather than physiological phosphate-buffered saline (PBS) for antiprotozoals for which this had an appreciable effect. (–): null result in seawater solution/suspension

Antiprotozoal	MLC (ppm)
Niclosamide	0.8
Oxyclozanide	0.8
Bithionol sulfoxide	3.1 (25)
Toltrazuril	6.2 (50)
N-(2′-hydroxy-5′-chloro-benzoyl) 2-chloro-4-nitroaniline	6.2
Furaltadone	25
Doxycycline hyclate	50 (–)
Formalin	62
Albendazole	100 (–)
Carnidazole	100
Pyrimethamine	100
Quinacrine hydrochloride	100
Quinine sulfate	100

pensions of powder compounds (tablets were previously powdered in a mortar) were prepared in distilled water or dimethylsulfoxide (DMSO; Sigma Chemical). The resulting stocks, or commercially purchased solutions, were diluted in physiological phosphate-buffered saline (PBS; pH 7.2) or 0.2 µm-filtered seawater (salinity 28‰) to the final concentrations used in the screening (see following subsection). Tests performed in filtered seawater allow automatic exclusion of the possibility of partial or total inactivation of the test substance under marine conditions. A substance found to be effective *in vitro* by this procedure can thus be expected to be effective in bath administration to infected fish.

Determination of lethal activity. Ciliates in the late exponential phase or early plateau phase of culture were concentrated by centrifugation at 650 × *g* for 5 min and then resuspended in PBS or filtered seawater. After counting in a haemocytometer, 10 µl of ciliate suspension containing 10^4 ciliates were added to each well of 96-well microtitre polystyrene plates containing 90µl well^{-1} of the candidate antiprotozoal at the required dose in PBS or filtered seawater. Final doses tested were: (1) 250, 125, 62, 31, 16, 8.4, and 2 ppm for formalin, and (2) 100, 50, 25, 12.5, 6.2, 3.1, 1.5 and 0.8 ppm for the remaining test substances. Each determination was performed in duplicate. Wells containing ciliates in assay solution (PBS or filtered seawater) without chemicals were also assayed as negative controls. To rule out possible effects of the solvent in DMSO-dissolved compounds, duplicate wells with PBS or filtered seawater containing the highest concentration of DMSO used (up to 2.5%) were also included. Plates were incubated at 18°C for 24 h.

Ciliate motility after incubation was checked using an inverted microscope with phase-contrast illumination. Prior to scanning, each culture plate was gently rocked to ensure uniform distribution of ciliates throughout the medium. The minimal lethal concentration (MLC) for each drug was defined as the highest drug dilution at which 100% of the ciliates were lysed or not motile (there was no evidence of cilia movement using a 40× objective).

RESULTS AND DISCUSSION

The antiprotozoals showing lethal activity against *Philasterides dicentrarchi* after 24 h are listed in Table 2. Although effects on cell morphology were varied, most of the effective substances induced cell rounding and vacuolization changes before eventual lysis (Fig. 1). In assays with formalin and niclosamide, all ciliates were dead after 2 h with doses of 62 and 0.8 ppm respectively.

In similar *in vitro* studies, formalin has likewise proved effective against other causal agents of scuticociliatosis such as *Uronema nigricans* and *Anophryo-*

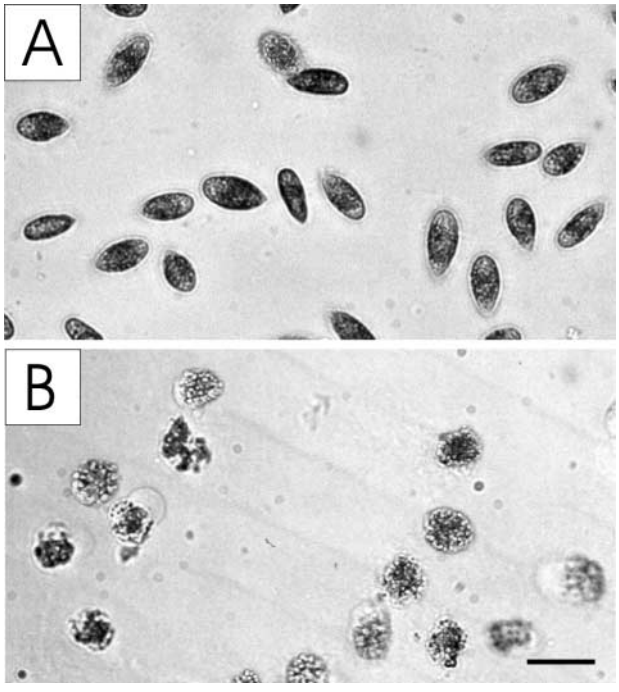

Fig. 1. *Philasterides dicentrarchi.* Typical appearance of ciliates (A) after incubation in physiological phosphate-buffered saline (PBS) or filtered seawater and (B) in response to lethal doses of an antiprotozoal. Dead ciliates are rounded in appearance or exhibit lysis. Scale bar = 40 µm

ides haemophila (Novotny et al. 1996, Crosbie & Munday 1999). This compound is routinely used in aquaculture to control ectoparasitic diseases (Treves-Brown 2000). The recommended procedure is typically a daily bath for 30 to 60 min in 167 to 250 ppm of formalin. In the present study the ciliates died rapidly at lower doses (MLC = 62 ppm). However, we consider formalin to be inappropriate for the treatment of this disease in view of the endoparasitic location of *Philasterides dicentrarchi*. When an outbreak of scuticociliatosis due to *P. dicentrarchi* is first diagnosed in a turbot farm, many fish in the affected tank already show systemic infection characterized by the presence of numerous ciliates in the branchial capillaries (Iglesias et al. 2001). In such cases, the respiratory capacity of the fish will be seriously compromised and treatment with formalin will not only be ineffective, but may in fact accelerate death by reducing oxygen availability. Nevertheless, given its effectiveness for killing free forms of the ciliate, and given that it is approved for chemotherapeutic use in aquaculture, it may be worth considering the possibility of periodic formalin baths at relatively low doses (perhaps 60 to 70 ppm), as a prophylactic measure.

Of all the effective products, the salicylanilides niclosamide and oxyclozanide were those that showed greatest activity against *Philasterides dicentrarchi* (in both cases MLC = 0.8 ppm). These products are proton ionophores that inhibit electron transport associated with oxidative phosphorylation, and thus deprive the cell of its primary source of energy (Swan 1999). Niclosamide is likewise lethal *in vitro* to *Tetrahymena pyriformis*, another pathogenic ciliate (Griffin 1989). However, and despite its *in vitro* efficacy, a series of factors need to be taken into account in carrying out assays aimed at determining *in vivo* therapeutic capacity. First, bath administration must be performed with caution, since this compound is highly toxic by this route (Schmahl et al. 1989). Second, niclosamide shows poor intestinal absorption in comparison with oxyclonazide (Swan 1999), so that tolerance of oral administration in food may be greater than tolerance of bath administration. The recommended dosages for the treatment of cestodes, including the turbot cestode *Bothriocephalus scorpii*, range between 5 and 40 mg kg^{-1} d^{-1} (Sanmartín et al. 1989, Schmahl et al. 1989).

The lack of activity of closantel, another salicylanilide that acts as proton ionophore, may indicate either (1) that niclosamide and oxyclonazide have a specific mechanism of action (other than the blockade of electron transport), or (2) that *Philasterides dicentrarchi* is resistant to closantel.

Bithionol sulfoxide is another anthelmintic that, like the salicylanilides, uncouples electron transport (Rew 1978). This compound has been shown to be effective against other fish ciliates, such as *Tetrahymena pyriformis* (*in vitro*: Griffin 1989) and *Trichodina jadranica* (in eel culture: Madsen et al. 2000). Madsen et al.'s results suggest that it is indeed effective under marine conditions. However, we found that effectiveness was markedly lower in seawater (MLC= 25 ppm) than in PBS (3.1 ppm), suggesting that *in vitro* assays of this compound should take into account the possibility of partial inactivation by seawater.

Except the anthelmintic N-(2′-hydroxy-5′-chlorobenzoyl) 2-chloro-4-nitroaniline, the remaining products that showed effectiveness against *Philasterides dicentrarchi* have known activity against certain protozoans and/or fish microsporidians. Of these, the symmetric trianizone toltrazuril may be a good candidate for the control of scuticociliatosis caused by *P. dicentrarchi*, since it has been shown to be effective not only against coccidians such as *Eimeria* spp. and *Haemogregarina* spp., but also against other tissue-dwelling protozoans (Schmahl et al. 1989). In the case of the microsporidian *Glugea anomala*, administration of this compound at very low doses (2 ppm) in periodic prolonged baths (three 24 h baths at 2 d intervals) has been observed to give rise to nuclear alterations, inhibition of nuclear division, destruction of uni- and multinucleate meronts and of the xenoma wall, fragmentation of the sporogonial plasmodium, and even alteration of large numbers of mature spores (Schmahl et al. 1990). Baths with this compound have also been reported to cause severe damage to all developmental stages (except mature spores) of the gill-histozoic myxosporeans *Myxobolus* sp. and *Henneguya* sp. (Schmahl et al. 1991). The mechanism of action of toltrazuril against these parasites is not clear, although it has been suggested that they may act on enzymes, affecting the respiratory chain and pyrimidine synthesis. In any case, and despite the fact that bath administration has been shown to be highly effective against certain tissue and intracellular parasites of freshwater fishes, our results indicate that its effectiveness was markedly lower in seawater (MLC = 50 ppm) than in PBS (6.2 ppm). As a result, oral administration should not be ruled out in future evaluations of *in vivo* effectiveness.

Toltrazuril and bithionol sulfoxide are not the only compounds that showed lower effectiveness in seawater: albendazole and doxycycline hyclate were ineffective when administered in seawater. Albendazole administered by the oral route delays the formation of xenomas in the microsporidian *Loma salmonae* (Speare et al. 1999), and is effective for the treatment of hexamitosis caused by *Hexamita salmonis* (Tojo & Santamarina 1998). It has likewise been demonstrated that periodic albendazole baths damage all developmental stages of *Glugea anomala*, including mature

spores (Schmahl & Benini 1998). In the present study, the lack of efficacy when administered in seawater argues strongly against its *in vivo* utility for treatment of scuticociliatosis.

Doxycycline shows antiprotozoal activity against various human pathogens, including the ciliate *Balantidium coli*. This compound acts on bacterial ribosomes to inhibit protein synthesis; however, its mechanism of action against protozoa is not known. The fact that oxytetracycline, the other tetracycline assayed, was not effective against *Philasterides dicentrarchi* may be due to its lower lipophilicity (Edlind 1989). These tetracyclines, especially oxytetracycline, have been used in aquaculture as bacteriostats, even though their efficacy is markedly reduced in seawater (Herwig 1979, Treves-Brown 2000), as noted in the present study for doxycycline. Nevertheless, and despite its high lipophilicity and thus strong capacity for penetrating tissues, it may be of interest to assay the efficacy of this compound administered orally.

Dragesco et al. (1995) report that outbreaks of scuticociliatosis due to *Philasterides dicentrarchi* in sea bass farms in the Mediterranean have been successfully controlled with dimetridazole. However, these authors do not provide any details of the treatment procedures used. Nitroimidazole, administered orally with food, has also been used successfully against *Ichthyophthirius multifiliis* and *Hexamita salmonis* infections in salmonids (Schmahl et al. 1989, Rapp 1995). In our *in vitro* assays, however, the only nitroimidazole that proved effective was carnidazole, and this was only at high doses (MLC = 100 ppm). The fact that the nitroimidazoles are practically ineffective against *P. dicentrarchi* is not surprising in view of the principal mechanism of action of these drugs, which are basically effective against anaerobes. All protozoa sensitive to these drugs (i.e. *Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis* and *H. salmonis*) lack mitochondria, and their energy metabolism is largely based on the pathway controlled by pyruvate:ferredoxin oxyreductase (PFOR) and the electron-carrying protein ferredoxin. Nitroimidazoles are toxic to these anaerobic protozoans because of effects on this enzyme. *P. dicentrarchi*, unlike the protozoans sensitive to nitroimidazoles, is probably microaerobic, like other similar scuticociliates, and shows numerous mitochondria clustered at the periphery of the cilia (data not shown). It is thus not surprising that the drugs found to be most effective in the present study were salicylanilides and bithionol sulfoxide, which act on the mitochondrial respiratory chain.

Of the quinine derivatives, and despite the proven antiprotozoal activity of many of these compounds, only quinine sulfate and quinacrine hydrochloride killed ciliates within 24 h, and only at very high doses (MLC = 100 ppm in both cases). Numerous authors have reported the efficacy of quinine baths for treating ectoparasitic and subepidermal protozoal infections of fishes (see e.g. Herwig 1979, Stoskopf 1993). Furthermore, the continuous administration of quinine-containing feed to ornamental fishes appears to be effective for eliminating the skin-inhabiting trophozoites of the ciliate *Ichthyophthirius multifiliis*, responsible for white spot disease (Schmahl et al. 1996). In this case, the compound provokes rupture of the external alveolar membrane and alteration of intracellular digestion. Quinine administered orally also appears to delay the formation of xenomas of the microsporidian *Loma salmonae* (Speare et al. 1998). The antimalarial quinacrine likewise shows *in vitro* activity against *Tetrahymena pyriformis*, and against tomonts of *I. multifiliis*, although in the latter case only 50% mortality was observed after 2 h of incubation at 200 ppm (Griffin 1989, Tojo et al. 1994). The precise antiprotozoal mechanism is not known, and appears to differ from one organism to another. The general antiprotozoal activity of quinacrine has been attributed to inhibition of the oxidation of succinate and to interference in electron transport, while its antimalarial properties are probably attributable to inhibition of adenosine uptake by infected blood cells, and to effects on the DNA and RNA of *Plasmodium* spp. (Zipper et al. 1995). The antimalarial activity of quinine is thought (like chloroquine) to be probably due to complexation with ferritoprotoporphyrin IX (thus preventing hemazoin sequestration and resulting in cell lysis), and not to interference in DNA and RNA synthesis, as was initially thought (see Khaw & Panosian 1995). However, in other protozoa such as *Tetrahymena pyriformis*, quinine acts to inhibit cell division and mitochondrial oxidative phosphorylation (Conklin et al. 1971).

Both in the present study and in other assays with *Tetrahymena pyriformis*, chloroquine did not show short-term antiprotozoal activity (Griffin 1989, Nilsson 1989). Nevertheless, it should be stressed that this compound may inhibit *in vitro* the proliferation and mitochondrial oxidative phosphorylation activity of *T. pyriformis* (Conklin et al. 1971). However, its efficacy is much greater at an alkaline pH (Nilsson 1989), which may explain the absence of activity in our assays.

Despite the scant capacity of these quinine derivatives to kill *Philasterides dicentrarchi in vitro*, their possible effects on cell division in similar protozoans suggests that their *in vivo* efficacy should be assayed independently, especially in the case of quinine in view of its lower toxicity than quinacrine.

The dihydrofolate reductase (DHFR) inhibitor pyrimethamine likewise proved effective in the present study, at high doses (MLC = 100 ppm). This compound, combined with sulfaquinoxaline, provokes the lysis *in*

vitro of *Anophryoides haemophila*, the scuticociliate responsible for bumper car disease in lobsters (Novotny et al. 1996). The joint use of these 2 compounds in fishes has only been reported in salmonids infected with *Loma salmonae*, in which delayed xenoma formation was observed (Speare et al. 1999).

In conclusion, only 13 of the 52 tested candidate antiprotozoals proved effective against *Philasterides dicentrarchi in vitro*. We cannot of course rule out the possibility that compounds which are ineffective *in vitro* may prove to be effective *in vivo*, although this seems unlikely. Of the 13 compounds found to be effective, the most effective were those acting on the mitochondrial respiratory chain. In view of these results, the *in vivo* efficacy of some of these compounds clearly merits attention. However, it should be borne in mind that various factors can be expected *a priori* to influence the success of chemotherapeutic measures to control scuticociliatosis in farmed turbot and sea bass. First, *P. dicentrarchi* is a highly virulent species which divides rapidly (by binary fission) and which migrates via the bloodstream and connective tissues to various organs and tissues (blood, gills, brain, liver, intestine, etc.), where it feeds actively on cells and tissue components (Iglesias et al. 2001). Second, only a few chemotherapeutic agents have been accepted for use in aquaculture by the legislative bodies of various countries, which makes the choice of drugs for treatment of infectious fish diseases, including scuticociliatosis, difficult. Finally, the fact that some of the most effective compounds in the present study showed reduced activity in seawater may mean that they are not sufficiently effective in bath administration, and oral administration is difficult in severe cases because, when the disease is in an advanced stage, the turbots exhibit anorexia.

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