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In vitro efficacy of new antiprotozoals against *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida)

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ABSTRACT: *Philasterides dicentrarchi* is a histiophagous ciliate that causes severe losses in turbot and sea bass farming. This study investigated the *in vitro* efficacy against *P. dicentrarchi* of 85 newly synthesized compounds and 12 commercial compounds, of which 2 are fluoroquinolones (norfloxacine and lomefloxacine) with known antibacterial activity. Seventeen of the newly synthesized compounds (2 naphthyridines, 2 pyridothienodiazines and 13 pyridothienotriazines) and the fluoroquinolone norfloxacin showed good activity. The most promising compound was the pyridothienotriazine 12k, with activity similar to that of the salicylanilides niclosamide and oxiclozanide (MLC 0.8 mg l^{-1} in PBS, 1.5 mg l^{-1} in seawater; MLC = minimum 24 h lethal concentration).

KEY WORDS: *Philasterides dicentrarchi* · Turbot · Scuticociliatosis · *In vitro* assay · Naphthyridines · Pyridothienotriazines · Pyridothienodiazines

INTRODUCTION

Philasterides dicentrarchi is a histiophagous scuticociliate that provokes significant economic losses in aquaculture, through systemic infection of gilthead bass (Dragesco et al. 1995) and turbot (Iglesias et al. 2001). In turbot farms, the incidence of this disease appears to have increased in recent years, to the extent that it is now one of the most significant parasite pathologies. No effective control measures are currently available. This parasite may be readily and rapidly eliminated while it remains outside the host body by formalin baths (Iglesias et al. 2002); however, once it has penetrated the body and initiated endoparasitic infection, this treatment is no longer effective.

One of the major problems in aquatic parasitology is the need for chemotherapeutical agents to be harmless for the aquatic environment and the host at doses effective against the parasite. This, together with the high mutation and genetic recombination rates of parasitic protozoans (Wang 1984), is possibly the reason why these protozoans develop drug resistance so quickly. This makes the identification of effective chemotherapeutical compounds very difficult (Wang 1984), slowing down the development of new antiprotozoal agents and making their modes of action difficult to study. We have recently reported a study of the *in vitro* activity against *Philasterides dicentrarchi* of a series of known antiprotozoal drugs (Iglesias et al. 2002), but to date there are still no fully effective con-

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trol measures against this ciliate *in vivo*. Activity studies of new drugs must thus continue.

In the present study, we investigated the susceptibility of *Philasterides dicentrarchi* to 85 newly synthesized antiprotozoals and 12 commercial compounds, of which two (norfloxacin and lomefloxacin) are commercial antibiotics with known antibacterial activity. This approach facilitated the identification of structure-function relationships. Preliminary activity data for compounds 2f, 5o, 12d, 12f, 12h, 12k, 12m, 7h, 9 and the antibacterials, together with synthesis routes and structural characteristics for many of the new drugs tested here, have been published recently (Quintela et al. 1999, 2003a).

MATERIALS AND METHODS

Isolation and culture of *Philasterides dicentrarchi*. Ciliates were harvested by collecting ascitic fluid from the body cavity of naturally infected turbot and were maintained under the culture conditions described by Bernard & Fenchel (1996), with autoclaved *Vibrio anguillarum* as food source. The cultured ciliates maintained infectivity in experimental infection trials.

Drugs. The drugs tested were 85 newly synthesized compounds and 12 commercial compounds belonging to the following chemical groups: (1) simple piperazines: PI-1, PI-2, PI-3, PI-4, PI-5, PI-6 (synthesized as per Raviña et al. 1996), PI-8, PI-9 (Raviña et al. 1995), PI-10 (Raviña et al. 1999), PI-12 (Estévez et al. 1998) and the commercial simple piperazines PCP (piperazine citrate pentahydrate, FLUKA), PDH-1 (piperazine dihydrochloride hydrate, FLUKA), PCA-1 (S-2 piperazine carboxylic acid dihydrochloride, FLUKA), PNP (1-phenylpiperazine, SIGMA), P2C (1-(2-chlorophenyl) piperazine monohydrochloride, SIGMA), P3C (1-(3-chlorophenyl) piperazine hydrochloride, SIGMA), P4C (1-(4-chlorophenyl) piperazine dihydrochloride, SIGMA), PBF (1-(α,α,α-trifluoro-m-tolyl) piperazine, SIGMA), PAC (4′-piperazinoacetophenone, SIGMA), CNP (trans-1-cinnamylpiperazine, SIGMA), norfloxacin (PN in Table 1) and lomefloxacin (PL in Table 1) (SIGMA, ALDRICH). (2) Naphthyridines 2b, 2d, 2e, 2f, 2g, 2i, 2j, 2k, 5o (synthesized as per Quintela et al. 2003a). (3) Pyridothienodiazines 3a, 4e, 4g, 4i, 4l, 8a, 9e, 9f, 9g, 9j, 9l and 7 (Quintela et al. 1998a), and compound H-26 with the same skeleton as 9g but differing in that its ethoxy substituent has been replaced by a phenyl group and the N-4′-acetyl piperazino substituent by a N-piperazine group. (4) Pyridothienotriazines: compounds 6a, 7a, 7d, 7f, 7g, 7h, 7i, 8a, 8b and 9 (synthesized as per Quintela et al. 1998b), compounds 12d, 12i, 12l, 14 and 16 (as per Quintela et al. 1999), and compounds 12c, 12e, 12f, 12g, 12h, 12k and 12m (synthesized as per Quintela et al. 2003a). (5) Pyridines: compounds 8, 22, 23, 24, 25, 28 and 31 (synthesized as per Quintela et al. 1997). (6) Pyrazolopyrimidines: compounds 3c, 4b, 4c, 6c, 9b and 9c (synthesized as per Quintela et al. 2003b). (7) Isoxazolpyrimidines: compounds IOP-2 and IOP-5 (synthesized as per Vidal et al. 2000), and other compounds with the same skeleton as IOP-2 but with N-morpholino (BR-46), N-thiomorpholino (BR-47), thiol (BR-100), N-4′-acetylphenylpiperazino (BR-101) and N-diethyl (BR-102) substituents in place of the N-propyl group. (8) Pyrimidines: compound 6 (synthesized as per Quintela et al. 2003a), compounds 35, 37, 39, 41 and 45 (synthesized as per Quintela et al. 1997), compound PPC-19 with the same skeleton as 35 but differing in that its phenyl substituent has been replaced by a 4-chlorophenyl group and the N-4-methylpiperidino by a N-4-aminopyridine group. Compounds LLC-62, LLC-63, LLC-66 and LLC-72 present the same skeleton as compound 6 but LLC-62 has an N-morpholino substituent in place of the N-thiomorpholino, LLC-72 has an N-morpholino in place of the N-piperazine substituent, LLC-63 has an N-morpholino instead of the N-thiomorpholino substituent and a N-4-methylpiperazino instead of the N-piperazine substituent, and LLC-66 has a thiomorpholino group instead of the piperazino and a morpholino instead of the thiomorpholino of compound 6.

Susceptibility testing. Stock solutions of the compounds were made up at 0.1 mg ml^{-1} in dimethyl sulfoxide (DMSO; Sigma Chemical) and serial dilutions were prepared in physiological phosphate-buffered saline (PBS, pH 7.2) or 0.2 µm filtered seawater (salinity 28‰) to give the final concentrations tested (100, 50, 25, 12.5, 6.2, 3.1, 1.5 and 0.8 mg l^{-1}). The compounds were tested in seawater with the aim of evaluating whether or not they maintain their activity in this medium.

For testing, ciliates in late exponential or early plateau phase were concentrated by centrifugation at 650 × *g* for 5 min and resuspended in PBS or filtered seawater. After counting in a haematocrit, 10 µl of the suspension containing 10^4 ciliates was added to each well of 96-well polystyrene microtitre plates containing 90 µl per well of the candidate antiprotozoal at the test dose, in PBS or filtered seawater. The plates were incubated at 18°C for 24 h.

Each dilution was assayed in duplicate and compared with a negative control containing the ciliates in the test solution (PBS or filtered seawater) without the test drug. To rule out possible toxic effects of the solvent DMSO, we also included duplicate wells containing the highest concentration of DMSO (2.5%) but no test drug. DMSO was observed not to cause significant mortality or any other obvious toxic effect.

The effects of each test drug were assessed using an inverted microscope with phase-contrast illumination. Results are expressed as minimum lethal concentration (MLC), i.e. the minimum concentration required to cause 100% ciliate mortality.

RESULTS AND DISCUSSION

Some of the compounds analyzed caused noticeable reductions in ciliate motility and morphological alterations (cell rounding, vacuolization, eventual cell lysis, see Fig. 1). Drugs effective against *Philasterides dicentrarchi* must be able to eradicate all the ciliates present because fecundity and invasiveness of surviving ciliates (Iglesias et al. 2001) will give way to a new infection within a few days.

The antiparasitic activity of compounds with a piperazine ring is well documented and in fact, piperazine and its salts are widely used in veterinary medicine. We thus performed additional assays to assess the susceptibility of *Philasterides dicentrarchi* to newly syn-

Table 1. *In vitro* activities of the compounds tested against *Philasterides dicentrarchi* for 24 h dissolved in PBS or seawater. Results are expressed as MLC (minimum lethal concentration in mg l^{-1})

	Compound	PBS	Seawater	Compound	PBS	Seawater
Simple piperazines	PN	50	50	PI-1	–	–
	PL	–	–	PI-2	–	–
	PCP	–	–	PI-3	–	–
	PDH-1	–	–	PI-4	–	–
	PCA-1	–	–	PI-5	–	–
	PNP	–	–	PI-6	–	–
	P2C	–	100	PI-8	100	50
	P3C	–	100	PI-9	–	–
	P4C	100	–	PI-10	–	–
	PBF	–	100	PI-12	–	–
	PAC	–	–			
	CNP	–	100			
Naphthyridines	2b	–	–	2i	–	–
	2d	–	–	2j	–	–
	2e	100	–	2k	–	–
	2f	50	12.5	5o	25	12.5
	2g	–	–			
Pyridothienodiazines	3a	–	–	9e	–	–
	4e	–	–	9f	–	–
	4g	–	–	9g	–	–
	4i	–	–	9j	–	–
	4l	–	–	9l	–	–
	8a	–	–	7	12.5	12.5
	H-26	50	–			
Pyridothienotriazines	6a	3.1	–	12 d	50	12.5
	7a	–	–	12 e	50	-
	7d	–	–	12 f	25	12.5
	7f	–	–	12 g	100	50
	7g	25	–	12 h	25	25
	7h	6.2	3.1	12 i	100	50
	7i	–	–	12 k	0.8	1.5
	8a	–	–	12 l	–	–
	8b	–	–	12 m	25	25
	9	25	25	14	–	–
	12c	50	100	16	–	–
Isoxazolpyrimidines	IOP-2	–	–	BR-100	–	–
	IOP-5	–	–	BR-101	–	–
	BR-46	–	–	BR-102	100	100
	BR-47	–	–			
Pyrimidines	6	100	-	PPC-19	100	100
	35	–	–	LLC-62	–	–
	37	–	–	LLC-63	–	–
	39	–	–	LLC-66	–	–
	41	–	–	LLC-72	–	–
	45	–	–			

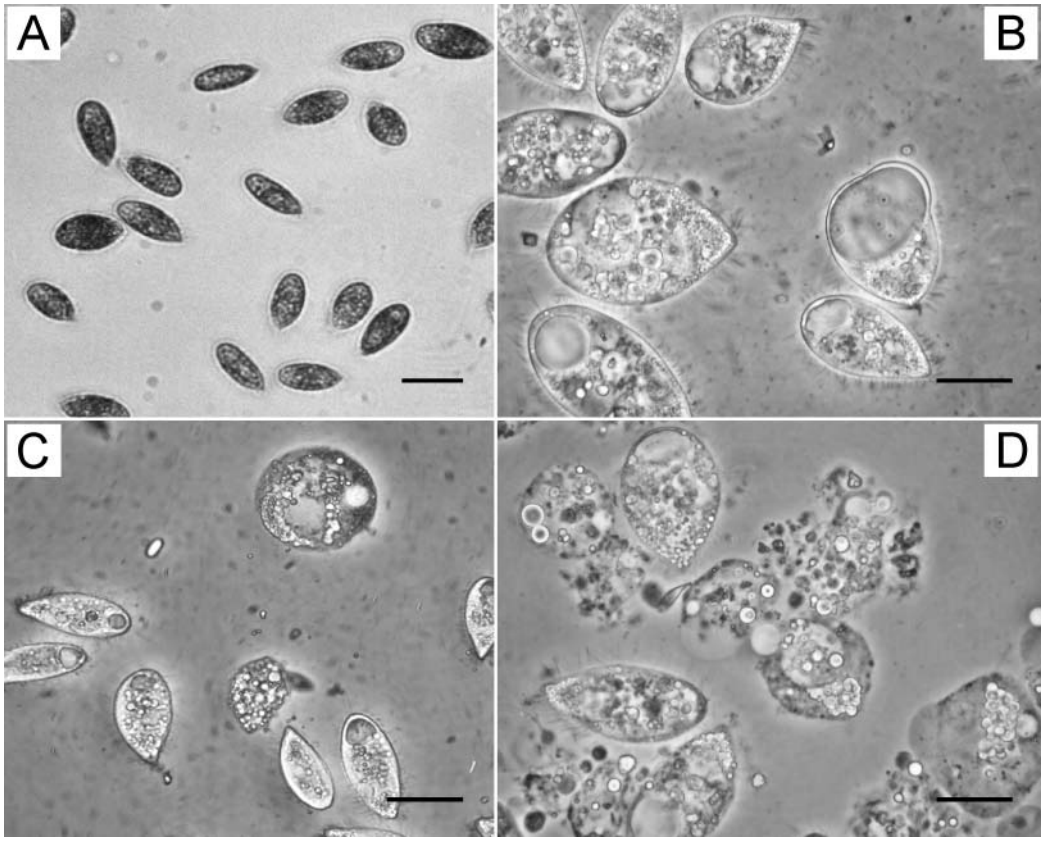

Fig. 1. *Philasterides dicentrarchi*. Micrographs showing effects of the active compounds on morphology: (A) normal culture, no drug (scale bar = 40 µm); (B) drug-induced vacuole formation (scale bar = 15 µm); (C) drug-induced cell rounding (scale bar = 24 µm); (D) drug-induced lysis (scale bar = 15 µm)

thesized and commercial simple piperazines. Previous studies indicate that various pathogenic freshwater protozoans and metazoans infecting trout are not effectively killed by piperazines (*Icthyobodo necator*, Tojo & Santamarina 1998a; *Gyrodactylus* spp., Tojo & Santamarina 1998b; *Hexamita salmonis*, Tojo & Santamarina 1998c). Our results (Table 1) show that several of these compounds are effective, though in some cases at very high concentrations (i.e. MLC 100 mg l^{-1}; see Table 1).

Norfloxacin and lomefloxacin are fluoroquinolone (fluorated quinolone) derivates of the piperazine chemical group. These compounds are widely used, in view of their high bioavailability after oral administration and relatively minor adverse effects in humans (Céspedes & Portal 1998). Fluoroquinolones act by inhibiting topoisomerase II, a key enzyme in DNA transcription and replication in both eukaryotes and prokaryotes (Drlica & Franco 1988), and have a wide antibacterial spectrum (Montay et al. 1984, Brun-Pascaud et al. 1992). In spite of the chemical structure of fluoroquinolones being very similar, these small structural changes give rise to differences in their biological characteristics; so norfloxacin is effective against *Philasterides dicentrarchi* at a dose of 50 mg l^{-1} in both PBS and seawater, while lomefloxacin does not show activity in any of the assay solutions (Quintela et al. 2003a). Similarly, the fluoroquinolone ciprofloxacin did not show any activity in a previous study (Iglesias et al. 2002). The resistance to these antibiotics may be due to alterations in subunit A of the parasite's DNA gyrase, resulting from chromosomal mutations, and/or to alterations in cell permeability provoked by excessive intracellular concentrations of the antibiotic (Fuentes 1990). The activity of the tetracyclines, used in aquaculture as antibacterials, is markedly reduced in seawater (Herwig 1979, Iglesias et al. 2002). The same may occur with lomefloxacin, and indeed previous studies have noted that the bioavailability of quinolones may be reduced in seawater, through reduced drug uptake as a result of the presence of bivalent cations such as Mg^{2+} (Burka et al. 1997). Note, however, that lomefloxacin was similarly ineffective in PBS, so that the

observed ineffectiveness may simply reflect a lack of activity against *P. dicentrarchi*.

Of all the studied naphthyridines, only compounds 2f and 5o caused 100% mortality at 50 mg l^{-1} or less in both assay solutions: MLC in PBS was 50 and 25 mg l^{-1} respectively, while MLC in seawater was 12.5 mg l^{-1} in both cases. No other naphthyridine derivate was active in either of these assay solutions, except for 2e, which was effective in PBS at the high dose of 100 mg l^{-1} (see Table 1).

Of the pyridothienodiazines studied, compound 7 was the most effective, with an MLC of 12.5 mg l^{-1} in both PBS and seawater (Quintela et al. 2003a). Compound H-26 was effective only in PBS (50 mg l^{-1}, see Table 1), so it may be considered for *in vivo* oral administration, but its non-effectivity in seawater should be borne in mind when considering bath treatment.

Some of the pyridothienotriazines tested also showed good activity in both PBS and seawater (compounds 7h, 9, 12d, 12f, 12m, 12h and 12k; see Table 1). Compounds 7h and 9 showed MLCs of 6.2 mg l^{-1} and 25 mg l^{-1} in PBS, and 3.1 and 25 mg l^{-1} in seawater, respectively (Quintela et al. 2003a). Compound 12k was the most active product detected, with MLCs of only 0.8 mg l^{-1} in PBS and 1.5 mg l^{-1} in seawater (Quintela et al. 2003a), comparable to the MLC of 0.8 mg l^{-1} in seawater displayed by the salicylanilides niclosamide and oxiclozanide (Iglesias et al. 2002). Other pyridothienotriazines were ineffective in seawater, but very active in PBS (7g and 6a were effective at doses of 25 and 3.1 mg l^{-1}, respectively, in this assay solution), so they may be considered for oral administration when testing their activity *in vivo*.

The pyridines and pyrazolopyrimidines tested in this study were ineffective in both assay solutions and are thus not included in Table 1. Previous studies have shown that some pyrazolopyrimidines are effective *in vitro* against *Leishmania* and *Trypanosoma cruzi* (Fish et al. 1985) or block trypomastigote-amastigote transformation in *T. cruzi* (Avila & Avila 1987). Isoxazolpyrimidines were also ineffective, except for compound BR-102, which was active at high doses (100 mg l^{-1}) in both PBS and seawater.

Pyrimidinic derivates showed low efficacy against this ciliate, with an effective dose of 100 mg l^{-1} in both PBS and seawater for compound PPC-19 and 100 mg l^{-1} only in seawater for compound 6 (see Table 1).

In conclusion, 21 of the 85 newly synthesized compounds tested in the present study showed *in vitro* activity against *Philasterides dicentrarchi* in seawater and/or PBS, and 17 were effective as defined by 24 h MLC ≤ 50 mg l^{-1}. The lowest MLC was obtained for the pyridothienotriazine 12k. Low MLCs were also obtained for the pyridothienotriazines 7h, 9, 12d, 12f, 12m and 12h, for the pyridothienodiazine 7, and for naphthyridines 2f and 5o. Of the 12 commercial compounds, 6 were effective in PBS or seawater at a dose of 100 mg l^{-1}, except for the antibiotic norfloxacin that was effective at 50 mg l^{-1} in both PBS and seawater. All these compounds contain a piperazine ring in their chemical structure. In spite of the non-efficacy of simple piperazine against this parasite, the presence of this ring in a compound seems to enhance its antiprotozoal activity against *P. dicentrarchi*. If this proves to be true, a knowledge of the structure-activity relationships for these compounds would facilitate the development of effective drugs for the control of these pathogenic protozoans. Further trials are now underway to comprehensively evaluate the efficacy of these compounds *in vivo*.

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