Nematocide activity of 6,7-diarylpteridines in three experimental models

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Abstract

The *in vitro* nematocide activity of seventeen 6,7-diarylpteridines has been tested using three different experimental models, *Caenorhabditis elegans*, *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. The method of evaluation of inhibition in the secretion of acetylcholinesterase by *H. polygyrus* seems to be the most indicated to avoid false positives. The *in vivo* activities, against *Trichinella spiralis*, of the most *in vitro* active pteridines have been assayed. All pteridine derivatives bearing 6,7-di-*p*-bromophenyl substituents have shown *in vitro* nematocide activites in the three experimental models used. Amongst all the pteridines tested *in vivo*, only 2,4-pteridinedithione derivatives exhibited moderate activity.

Introduction

Parasitic helminths cause serious infectious diseases in humans and domestic animals. Control of these infections relies mostly on chemotherapeutics (the anthelmintics), but resistance has developed against most of these broad-spectrum drugs in many parasite species (Roos, 1997). Thus, the common use of anthelmintics in farm animals has resulted in the appearance of drug resistant strains of worms, mainly nematodes (Waller *et al*., 1996). On the other hand, the currently used anthelmintic agents, belonging to the benzimidazole family, have proved to be poorly effective in the treatment of some systemic parasitic diseases produced by helminths in man (Cook, 1990). For these reasons, new classes of anthelmintic compounds are urgently needed. Following our interest in this field (Herrero *et al*., 1993), we found that some 6,7-diarylpteridines were a class of nematocides, not previously known, which show *in vitro* activity against the free living nematode *Caenorhabditis elegans* (Ochoa *et al*., 1996, 1997; Martínez *et al*., 1997). Most of these compounds show significant nematocide activity against *C. elegans* but not against eggs of *Heligmosomoides polygyrus* (Ochoa *et al*., 1996). With the aim of knowing more about nematocide properties of pteridines and comparing the efficacy of several test models, we are now using three different experimental models to evaluate the *in vitro* nematocide activity of seventeen 6,7-diarylpteridines (fig. 1) selected among a pool of pteridines previously synthesized (Ochoa *et al*., 1997). We have selected the following assays: (i) a test of the inhibition of *C. elegans* population growth; (ii) a test of the lethal dose for *Nippostrongylus brasiliensis* L4; and (iii) the effect on acetylcholinesterase (AChE) secretion by adult *H. polygyrus*. These two last models have been recently used for determining nematocide activities (Alonso-Villalobos & Martínez Grueiro, 1999; Gordon *et al*., 1998). For the study of *in vivo* activity, preadults of *Trichinella spiralis* have been employed.

Materials and methods

In vitro *tests*

These tests were carried out in tissue-culture 24-well

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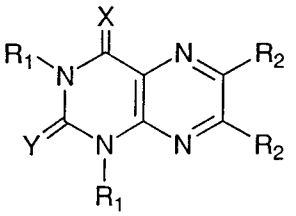
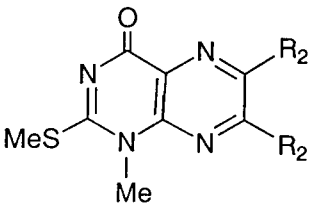
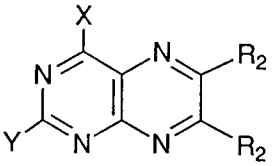


1, R_1 = H, R_2 = *p*-MeO-phenyl, X = Y = O
2, R_1 = H, R_2 = 3-thienyl, X = Y = O
3, R_1 = Me, R_2 = *p*-F-phenyl, X = Y = O
4, R_1 = Me, R_2 = *p*-Br-phenyl, X = Y = O
5, R_1 = H, R_2 = *p*-MeO-phenyl, X = O, Y = S
6, R_1 = H, R_2 = *p*-F-phenyl, X = O, Y = S
7, R_1 = H, R_2 = *p*-Cl-phenyl, X = O, Y = S
8, R_1 = H, R_2 = *p*-Br-phenyl, X = O, Y = S
9, R_1 = H, R_2 = phenyl, X = Y = S
10, R_1 = H, R_2 = *p*-F-phenyl, X = Y = S
11, R_1 = H, R_2 = *p*-Br-phenyl, X = Y = S



12, R_2 = *p*-F-phenyl
13, R_2 = *p*-Br-phenyl



14, R_2 = 2-thienyl, X = Y = H
15, R_2 = 2-thienyl, X = Y = NH_2
16, R_2 = 2-thienyl, X = NH_2, Y = H
17, R_2 = *p*-F-phenyl, X = Y = SMe

Fig. 1. Chemical structures of the 6,7-diarylpteridine derivatives studied.

plates. In all cases, water-insoluble compounds were dissolved in dimethyl sulfoxide (DMSO) and used immediately. Control worms were placed in test medium alone and containing 0.5% (*C. elegans* and *N. brasiliensis* assays) or 0.25% (acetylcholinesterase assay) DMSO (v/v). Albendazole, levamisole, morantel and mebendazole were used as reference drugs.

Caenorhabditis elegans *assay*

Determination of activity against the free-living nematode *C. elegans* was performed following the technique of Simpkin & Coles (1981) with slight modifications (Martínez Grueiro & Martínez Fernández, 1988). To each well, 1.5 ml of culture medium was added followed by 10 μl of the appropriate compound solution. A further 0.5 ml sample of culture medium containing 10–15 *C. elegans* larvae (L2 or L3 obtained from synchronous cultures) was then added to each well giving a final volume of 2 ml. Six wells were set up for each concentration. The effects of compounds on the development and reproductve capacity of *C. elegans* were determined by comparing the population levels attained in the control and test wells after an incubation period of 7 days at 20 ± 1°C.

Nippostrongylus brasiliensis *assay*

The effect of compounds on fourth-stage larvae (L4) of *N. brasiliensis* was evaluated as previously described (Gordon *et al*., 1998). A complete culture medium (1.8 ml) was placed into each well, followed by 50 L4 suspended in 0.2 ml of complete culture medium. The L4 were recovered from male Wistar rats on the third day post-infection (p.i.) with L3 of *N. brasiliensis*. Appropriate dilutions in DMSO were prepared for each compound in order to obtain the desired concentration after the addition of 10 μl into each well. Six wells were set up for each concentration. The percentage of dead worms was determined on day 5 and corrected by the controls in which only vehicle was used.

In vitro *effect on acetylcholinesterase (AChE) secretion by adult* Heligmosomoides polygyrus

Assays were performed according to previous protocols (Alonso-Villalobos & Martínez Grueiro, 1998). Adult worms obtained from experimentally infected mice (ten male and ten female per well) were incubated with the desired concentration of the compound at 37°C in RPMI 1640 medium containing 20 mM Hepes (pH 7.2) and antibiotics for 24 h. Three wells were used for each experimental group. After the incubation period, media containing excretion/secretion products were collected, centrifuged and the supernatants stored at –20°C until the enzyme assay. AChE activity was measured by microplate adaptation of the procedure of Ellman *et al*. (1961). Results were expressed as the percentage decrease in absorbance in the medium with drug treated worms relative to appropriate control wells.

In vivo *test*

Anthelmintic activity was tested *in vivo* against preadults of *T. spiralis*. The parasitological procedures were as described previously (Denham & Martínez Fernández, 1970). Compounds were tested at the unique dose of 100 mg kg^{-1}.

CD-1 mice were each infected by oral inoculation with 300 larvae of *T. spiralis*. Water-insoluble products were prepared as homogeneous suspensions in 1% aqueous

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carboxymethylcellulose solution and administered to the mice by gavage at 24 h p.i. The volume of suspension was adjusted so that each test animal received the specified dosage in a 0.4 ml final suspension. Untreated control mice received vehicle only. Animals were sacrificed, under ether anesthesia, on day 7 p.i. and adult worms were counted. Anthelmintic efficacy was assessed by comparing worm burdens in treated animals with those in the untreated controls (%R). Statistical comparisons between groups (8–10 mice per group) were done using the Student's t-test. A probability greater than 0.05 was not considered significant.

Parallel sets were run to examine the efficacy of mebendazole and levamisole.

Results and discussion

The *in vitro* nematocide activity of pteridine derivatives 1–17 were tested using the three experimental models mentioned above. The concentration inhibiting 50% of the *C. elegans* population growth (IC_{50}), the lethal concentration for 50% of the *N. brasiliensis* L4 population (LD_{50}) and the concentration inhibiting 50% of acetylcholinesterase (AChE) secretion in *H. polygyrus* adults (IC_{50}) are shown (table 1). A number of compounds exhibited nematocide activity in the three models used, and only compound 12 did not present any activity in the three models. Twelve compounds showed some activity against *C. elegans*, 12 against *N. brasiliensis* and only ten against *H. polygyrus*. However, the most active compound against one model is not always the most active against the others. Thus, against *C. elegans*, compounds 3, 4, 7, 8, 10, 11 and 13 demonstrated an IC_{50} lower than 10 μM, the IC_{50} of compound 11 being lower than those of albendazole and mebendazole and similar to that of levamisole used as standards. However, the compounds that exhibit a LD_{50} lower than 10 μM against L4 *N. brasiliensis* are 1, 4, 14, 15 and 16. On the other hand, the most active compounds in the test of inhibition of AChE for *H. polygyrus* were 8, 11 and 14, while compounds 1, 4, 5, 6, 7, 10 and 13 showed only moderate activity. None of the compounds tested were more active than the standards against *N. brasiliensis* and *H. polygyrus*.

Compounds 1, 4, 7, 8, 11, 13 and 14 were selected to be tested *in vivo* against preadults of *T. spiralis*, due to the fact that all of them showed some activity against the three experimental *in vitro* models. Compounds 3 and 15, which only presented *in vitro* activity against *C. elegans* and *N. brasiliensis*, and compound 10 which exhibited activity against *C. elegans* and *H. polygyrus* were also selected for *in vivo* experiments. Unfortunately, only compounds 10 (%R = 38.3, $P < 0.05$) and 11 (%R = 37.8, $P < 0.01$), which were both active *in vitro* against *C. elegans* and *H. polygyrus*, showed a significant reduction in the growth of the nematodes. Mebendazole (%R = 74, $P < 0.01$ at 10 mg kg^{-1}) and morantel (%R = 73, $p < 0.01$ at 25 mg kg^{-1}) were used in this test as standards.

From these results, the model of inhibition of AChE secereton by *H. polygyrus* seems to be the best to avoid false positives. Taking into account the structure of the

Table 1. *In vitro* nematocide activity of 6,7-diarylpteridines against three experimental nematode models.

Compound	*Caenorhabditis elegans* IC_{50} [a]	*Nippostrongylus brasiliensis* LD_{50} [b]	*Heligmosomoides polygyrus* IC_{50} [c]
1	++	+++	⊕
2	—	++	∅
3	+++	++	∅
4	+++	+++	⊕
5	+	—	⊕
6	—	—	⊕
7	+++	++	⊕
8	+++	++	⊕⊕
9	++	—	∅
10	+++	—	⊕
11	++++	+	⊕⊕
12	—	—	∅
13	+++	+	⊕
14	+	+++	⊕⊕
15	++	+++	∅
16	—	+++	∅
17	—	++	∅
Albendazole	+++	++++	⊕⊕⊕⊕⊕
Levamisole	++++	++++	⊕⊕⊕⊕
Morantel	ND	ND	⊕⊕⊕
Mebendazole	+++	ND	⊕⊕⊕⊕

[a]Concentration inhibiting 50% of *C. elegans* population growth.
[b]50 Lethal concentration for *N. brasiliensis* L4: —>100 μM; + 100–50 μM; ++50–10 μM; +++10–1 μM; ++++1–0.1 μM; ND not determined.
[c]Concentration inhibiting 50% of *in vitro* AChE secretion: ∅ >1000 μM; ⊕ 1000–100 μM; ⊕⊕ 100–10 μM; ⊕⊕⊕10–1 μM; ⊕⊕⊕⊕ 1–0.1 μM; ⊕⊕⊕⊕⊕ < 0.1 μM.

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most *in vitro* active compounds, compounds bearing 6,7-di-*p*-bromophenyl substituents seemed to be the best. But, only the two 2,4-pteridinedithione derivatives tested exhibited *in vivo* activity, which must be enhanced. These derivatives might therefore be considered as new lead compounds for chemotherapeutic agents against parasitic nematode infections.

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