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Sensitivity *in vitro* of *Giardia intestinalis* to dyadic combinations of azithromycin, doxycycline, mefloquine, tinidazole and furazolidone

A. A. Crouch*, W. K. Seow, L. M. Whitman and Y. H. Thong *Immunobiology Laboratory, Department of Child Health, University of Queensland, Mater Children's Hospital, South Brisbane, Australia 4101.*

Abstract

The new macrolide antibiotic, azithromycin, produced significant growth inhibition of *Giardia intestinalis* at 100 μg/ml, but adherence inhibition was significant at concentrations as low as 1 μg/ml for the two strains used in these experiments. The dyadic combinations of azithromycin-furazolidone, doxycycline-mefloquine, doxycycline-tinidazole and mefloquine-tinidazole were synergistic for inhibition of adherence. These results suggest that these dyadic combinations may be worthy of consideration for chemotherapy of recalcitrant giardiasis.

Introduction

The protozoan parasite *Giardia intestinalis* (also known as *G. lamblia*) is a significant cause of acute diarrhoea and chronic malabsorption in most regions of the world (KNIGHT, 1980). Although treatment with tinidazole, metronidazole, mepacrine (quinacrine) or furazolidone is generally effective, therapeutic failures have been well documented (MCINTYRE *et al.*, 1986). There is therefore a need to identify new drugs as well as to investigate the potential of combination chemotherapy for the treatment of giardiasis.

In a previous paper (CROUCH *et al.*, 1986) we reported that doxycycline and mefloquine had significant anti-*Giardia* properties *in vitro*. In this study, we describe the sensitivity *in vitro* of *G. intestinalis* to azithromycin, a newly developed macrolide-like antibiotic (WISE, 1989), as well as to dyadic combinations of azithromycin, doxycycline, mefloquine, tinidazole and furazolidone. These studies were conducted using 2 *in vitro* assays developed in our laboratory (SEOW *et al.*, 1985; CROUCH *et al.*, 1986).

Materials and Methods

Axenic culture of G. intestinalis

The strains of *G. intestinalis* used in these experiments were a clinical isolate designated BRIS/82/HEPU/41 (previously cited as GC41) and the standard Portland-1 strain, both kindly provided by Dr Peter Boreham, Queensland Institute of Medical Research (BOREHAM *et al.*, 1984). They were grown in Diamond's TYI-S-33 medium (DIAMOND *et al.*, 1978) as modified by BELOSEVIC *et al.* (1982), supplemented with 10% heat-inactivated foetal calf serum (Flow Laboratories, Virginia, USA). The organisms were grown to confluence in 12 ml polycarbonate tubes at 37°C. Trophozoites were harvested by chilling the tubes in ice-water for 5 min, and then washed 3 times and resuspended in TYI basal medium.

*Present address: Australian Red Cross, Kampong Speu Hospital, Cambodia.
Correspondence to Professor Y. H. Thong.

Chemotherapeutic agents

Azithromycin, tinidazole and doxycycline were obtained from Pfizer Ltd; furazolidone from Norwich Laboratories; mefloquine from Walter Reed medical Center, USA. The drugs were dissolved in TYI basal medium, except for furazolidone, which was used as a suspension.

Adherence inhibtion assay

This assay was performed as previously described (SEOW *et al.*, 1985). After pre-incubation of the trophozoite suspension ($6–10\times10^6$/ml) and appropriate concentrations of chemotherapeutic agent for 2 h at 37°C, 130 μl volumes were pipetted into dacron fibre microcolumns, which were placed in a high humidity incubator at 37°C. The microcolumns were removed after 30 min and placed in a specially constructed appartaus which applied a suction pressure of approximately 250 millibars for one min. The effluents were collected in disposable test tubes and chilled in ice before enumeration of trophozoite concentrations with a Coulter counter or haemocytometer. Experiments were performed in triplicate and the results calculated as follows:

$$\text{Percentage adherence} = 100 - \left(\frac{C_e}{C_o} \times 100\right)$$

where C_e is the concentration of *Giardia* in the effluent, and C_o the concentration in the original suspension. Adherence inhibition was calculated by the following formula:

$$\text{Percentage adherence inhibition} = 100 - \left(\frac{\text{Adherence in drug-treated suspension}}{\text{Adherence in untreated suspension}} \times 100\right)$$

Growth inhibition assay

For these experiments, 48 h cultures of *G. intestinalis* were harvested as previously described, and adjusted to a concentration of 2×10^6/ml. 0·5 ml aliquots of this suspension were added to 4·5 ml of TYI-S-33 medium containing 10% foetal calf serum and the appropriate concentration of chemotherapeutic agents. Control cultures were the same except for the absence of antimicrobial agents.

These cultures were kept at 37°C for 48 h, after which they were chilled and the trophozoite concentration determined with the aid of a Coulter counter. Experiments were performed in triplicate, and the results calculated as follows:

$$\text{Percentage growth inhibition} = 100 - \left(\frac{\textit{Giardia} \text{ concentration in drug-treated culture}}{\textit{Giardia} \text{ concentration in untreated culture}} \times 100\right)$$

Table 1. *In vitro* effects of azithromycin on growth and adherence of *Giardia intestinalis*

Azithromycin (μg/ml)	Growth inhibition (%)[a] Portland-1 strain	Growth inhibition (%)[a] BRIS/82/HEPU/41 strain	Adherence inhibition (%)[a] Portland-1 strain	Adherence inhibition (%)[a] BRIS/82/HEPU/41 strain
1	6·2±2·3	3·9±0·3	9·8±1·4	10·9±0·4***
10	3·5±2·2	1·8±0·7	14·4±2·8**	15·4±0·9***
100	7·1±2·6*	8·9±1·8*	19·5±3·4***	19·8±1·5***

[a]Results represent mean ±standard error of the mean of 3 separate experiments, each performed in triplicate. *$P<0·05$; **$P<0·01$; ***$P<0·001$.

Table 2. Effect of dyadic combinations of five drugs on growth *in vitro* of *Giardia intestinalis*

Drug[a]	Growth inhibition (%)[b] Portland-1 strain	Growth inhibition (%)[b] BRIS/82/HEP/41 strain
Azithromycin (20)	7·6±1·6	5·0±0·8
Doxycycline (20)	31·0±3·3	29·8±1·5
Mefloquine (5)	24·4±3·3	22·9±2·0
Tinidazole (0·2)	28·4±2·2	21·2±2·2
Furazolidone (0·2)	37·0±1·2	23·0±0·7
Azithromycin (20) +doxycycline (20)	28·3±0·4	29·8±1·9
Azithromycin (20) +mefloquine (5)	20·3±2·1	26·0±2·8
Azithromycin (20) +tinidazole (0·2)	27·8±3·5	24·5±2·7
Azithromycin (20) +furazolidone (0·2)	28·8±1·8	27·5±2·5
Doxycycline (20) +mefloquine (5)	55·7±1·8	57·9±1·9
Doxycycline (20) +tinidazole (0·2)	47·0±3·8	38·9±6·2
Doxycycline (20) +furazolidone (0·2)	40·8±1·4	40·9±4·6
Mefloquine (5) +tinidazole (0·02)	51·7±0·2	38·5±4·6
Mefloquine (5) +furazolidone (0·2)	40·9±6·0	45·5±6·1
Tinidazole (0·2) +furazolidone (0·2)	46·4±4·3	31·2±3·3

[a]Figures in parentheses are concentrations in μg/ml.
[b]The results represent mean ±standard error of the mean of 3 experiments performed on separate occasions, each in triplicate.

Table 3. Effect of dyadic combinations of five drugs on the adherence of *Giardia intestinalis*

Drug[a]	Adherence inhibition (%)[b] Portland-1 strain	Adherence inhibition (%)[b] BRIS/82/HEPU/41 strain
Azithromycin (20)	9·5±0·02	7·9±0·6
Doxycycline (20)	10·8± 0·5	12·8±0·3
Mefloquine (10)	16·7± 0·9	22·4±1·1
Tinidazole (5)	20·3± 0·8	23·8±1·3
Furazolidone (1)	10·3± 0·9	8·1±0·4
Azithromycin (20) +doxycycline (20)	12·4± 1·6	21·8±4·5
Azithromycin (20) +mefloquine (10)	16·3± 2·9	19·6±2·8
Azithromycin (20) +tinidazole (5)	34·1± 2·8[c]	17·0±2·1
Azithromycin (20) +furazolidone (1)	26·3± 2·5[c]	20·2±0·4[c]
Doxycycline (20) +mefloquine (10)	28·5± 4·8[c]	32·3±9·1[c]
Doxycycline (20) +tinidazole (5)	36·5± 0·1[c]	39·7±2·0[c]
Doxycycline (20) +furazolidone (1)	12·3± 3·1	15·5±4·1
Mefloquine (10) +tinidazole	45·7± 5·6[c]	45·7±6·3[c]
Mefloquine (10) +furazolidone (1)	25·7± 1·2	28·1±2·7
Tinidazole (5) +furazolidone (1)	22·9± 1·7	16·9±3·2

[a]Figures in parentheses are concentrations in μg/ml.
[b]The results represent mean ±standard error of the mean of 3 to 12 experiments performed on separate occasions, each in triplicate.
[c]Synergism.

Statistical analysis

Student's *t* test was used for statistical analysis.

Results

Effect of azithromycin

The sensitivity *in vitro* of *G. intestinalis* to azithromycin is presented in Table 1. Azithromycin significantly inhibited growth in axenic culture at 100 μg/ml, and significantly inhibited adherence at concentrations of 1–100 μg/ml. The inhibition of adherence was dose-dependent for both strains of *G. intestinalis* (Table 1).

Effect of dyadic combinations on growth

In the next set of experiments, we investigated the effects of dyadic combinations of the 5 drugs on growth of *G. intestinalis in vitro*. The results are summarized in Table 2. The Portland-1 strain was generally more susceptible to the 5 drugs than the BRIS/82/HEP/41 strain. Also, dyadic combinations of the drugs were additive but not synergistic in their inhibitory effects on growth.

Effect of dyadic combinations on adherence

Finally, we examined the effects of dyadic combinations of the 5 drugs on adherence of *G. intestinalis*. The results (Table 3) show that the two strains of *G. intestinalis* were approximately equal in terms of sensitivity to the 5 drugs.

In contrast to the growth inhibition studies, some dyadic combinations of the 5 drugs had synergistic effects for both strains of *G. intestinalis*. These were: azithromycin-furazolidone, doxycycline-mefloquine, doxycycline-tinidazole and mefloquine-tinidazole combinations (Table 3).

Discussion

Azithromycin is derived from erythromycin by expansion of the lactone ring to incorporate a methyl-substituted nitrogen. This novel azalide compound has four times greater activity than erythromycin against *Haemophilus influenzae*, a long serum half-life of 12–14 h and superior tissue penetration properties (GIRARD *et al.*, 1987; WISE, 1989). Reports of its efficacy *in vitro* against protozoan parasites such as *Entamoeba histolytica* (RAVDIN & SKILOGIANNIS, 1989) prompted the inclusion of azithromycin in this study. The results showed that azithromycin had minimal inhibitory effects on axenic growth, but appreciable and significant inhibitory effect on adherence of *G. intestinalis* in the concentration range of 1–100 μg/ml. *E. histolytica* is also sensitive to azithromycin in the same concentration range (RAVDIN & SKILOGIANNIS, 1989).

Other drugs with potential for the treatment of giardiasis have been identified by *in vitro* techniques. Using a radiometric assay, BOREHAM *et al.* (1984, 1985) found several drugs with high anti-*Giardia* activity, including niridazole and some of the newly synthesised 5-nitroimidazole compounds such as satranidazole, ronidazole and S750400A. Our own *in vitro* assays (SEOW *et al.*, 1985; CROUCH *et al.*, 1986) also found some drugs with potent anti-*Giardia* activity, including doxycycline and mefloquine. However, no information is currently available with regard to the synergistic effects of anti-*Giardia* drugs *in vitro*. Now that drug resistance has been convincingly demonstrated for *G. intestinalis* (MCINTYRE *et al.*, 1986), information on synergism *in vitro* would be particularly useful as a guide to combination chemotherapy, which is a well-established strategy for the treatment of infectious diseases. However, the results of our growth inhibition experiments showed that none of the dyadic combinations had any synergistic effect on *G. intestinalis*.

In contrast, the results of the adherence inhibition experiments show synergism for *G. intestinalis* in a number of dyadic combinations, notably azithromycin-furazolidone, doxycycline-mefloquine, doxycycline-tinidazole and mefloquine-tinidazole. The significance of these findings is unclear, but the propensity to adhere to intestinal mucosa appears to be an important requisite of pathogenicity of *G. intestinalis*, as it is for other infectious organisms such as bacteria and fungi. Also, we have previously shown that all conventional anti-*Giardia* drugs exhibit inhibitory effects on the adherence of *Giardia* (CROUCH *et al.*, 1986), and we surmised that one of the major modes of action of these drugs may be interference with the adherence mechanism, either by compromising the integrity of the *Giardia* cell membrane (ketoconazole, mefloquine) or its anaerobic metabolic pathways (tinidazole, metronidazole and furazolidone). These results suggest that dyadic combination chemotherapy with drugs shown here to be synergistic *in vitro* may be worthy of further investigation.

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