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# Anticryptosporidial activity of ranalexin, lasalocid and azithromycin alone and in combination in cell lines

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**The *in vitro* anticryptosporidial activities of ranalexin, lasalocid and azithromycin alone and in combination were investigated against four clinical isolates of *Cryptosporidium parvum*. Susceptibility was tested by inoculating the isolates on to cell monolayers and determining the parasite count after 48 h incubation at 37°C. The culture medium was supplemented with Dulbecco's modified Eagle's medium containing serial dilutions of the above-mentioned compounds. Ranalexin showed moderate anticryptosporidial activity: at a concentration of 64 mg/L it reduced parasite counts by 33.8%. Azithromycin at a concentration of 8 mg/L gave inhibition comparable to that observed with the highest concentration of ranalexin. Lasalocid showed the highest activity, with a 70.3% reduction in parasite counts at 2 mg/L. The combination of ranalexin 64 mg/L and lasalocid 2 mg/L completely suppressed parasite growth without harming the monolayer.**

## Introduction

*Cryptosporidium parvum* is a major cause of diarrhoeal disease in a wide range of mammals. Several antimicrobial agents have been used *in vitro*, in animals or in humans without success.[1] Ranalexin is a cationic peptide isolated from bullfrog (*Rana catesbeiana*) skin and is structurally related to the bacterial antibiotic polymyxin.[2,3] It is thought to function through the formation of ion channels, pores spanning the membranes of bacteria.[2] Azithromycin is a macrolide antibiotic recently introduced as a potential anticryptosporidial drug. It has been reported to be effective *in vivo* in the treatment of cryptosporidiosis in steroid-immunosuppressed rodents and is currently in clinical trials as an anticryptosporidial agent.[4,5] Lasalocid, a polyether carboxylic acid ionophore, was isolated from *Streptomyces lasaliensis*. It is used as an anticoccidial drug in farm animals. It disrupts membrane potential and stimulates ATPase activity in mitochondria.[6,7] The aim of the present study was to investigate the activity of the above-mentioned drugs alone and in combination against *C. parvum*.

## Materials and methods

### *Organisms and cell cultures*

Oocysts of *C. parvum* were derived from stool specimens of four different AIDS patients. Stool specimens were stored at 4°C in 2.5% (w/v) potassium dichromate (Sigma-Aldrich, Milan, Italy) for up to 4 months until processing. Stools were homogenized in physiological saline and filtered through a metal sieve to remove coarse debris. Fatty material was removed by ether sedimentation and the supernatant and fatty plug were discarded. Oocysts were then purified and concentrated by flotation in Sheather's sugar solution. The upper layer was removed and collected. Contaminating bacteria were eliminated by three washes in sterile distilled water followed by two washes in 0.05% (v/v) sodium hypochlorite and incubation in phosphate-buffered saline (PBS) containing penicillin G 2000 U/mL, streptomycin 2000 mg/L and amphotericin B 10 mg/L for 4 h at 37°C. Excystation of sporozoites was achieved by incubating oocysts in PBS containing 0.25% (w/v) trypsin and 0.75% (w/v) sodium taurocholate for 60 min at 37°C. Free sporozoites were pelleted by centrifugation (200**g** for 20 min) and resuspended in Dulbecco's modified Eagle's medium (DMEM; Bio-Whittaker, Walkersville, USA).

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Finally, the four isolates were pooled and counted in a haemocytometer, and samples were used for culture. A549 cells (Bio-Whittaker) were maintained in 25 $cm^2$ tissue culture flasks. The medium consisted of DMEM with 10% fetal calf serum (Bio-Whittaker), 1% L-glutamine (Bio-Whittaker), 20 mM *N*-2-hydroxyethylpiperazine *N*-ethanesulphonic acid (HEPES) (Sigma-Aldrich), penicillin G (100 U/mL), streptomycin (100 mg/L) and amphotericin B (0.5 mg/L). Cells were removed from the surface of flasks using a solution of 0.25% (w/v) trypsin and 0.53 mM EDTA in PBS; they were then counted using a haemocytometer. Forty-eight hours before parasite inoculation, A549 cells were plated on to 35 mm diameter tissue culture plates at a concentration of $10^5$ viable cells in a total volume of 5 mL. Viability was assessed by Trypan Blue exclusion. Infection of the cell monolayer was initiated by adding $10^4$ pooled sporozoites in 50 μL of medium. After incubation for 4 h at 37°C in 5% $CO_2$ to allow attachment and penetration of sporozoites, the monolayers were washed with DMEM to remove non-invasive sporozoites, residual oocysts and non-adherent epithelial cells, and 5 mL of new growth medium with or without antimicrobial agents was added. Infected cell cultures were kept at 37°C in 5% $CO_2$ throughout the study.

### *In vitro studies*

Ranalexin (Sigma-Aldrich) was solubilized in PBS, pH 7.2, yielding 1 mg/mL stock solution. Lasalocid (Sigma-Aldrich) was dissolved in dimethylsulphoxide (DMSO) and was then further diluted to a final concentration of 1 mg/L in culture medium. Azithromycin (Pfizer/Roerig, Rome, Italy) was dissolved in methanol–acetone (1:1 v/v) at a concentration of 1 mg/mL. The following concentrations of each agent were tested singly: ranalexin, 4, 16 and 64 mg/L; lasalocid, 0.125, 0.50 and 2 mg/L; azithromycin, 0.5, 2 and 8 mg/L. In experiments to test drug interactions, they were tested at the highest concentrations. Antibiotic-free plates were used as controls in the study. Experiments were performed in triplicate. The monolayers were incubated for 72 h at 37°C in 5% $CO_2$. Following four washes in PBS to remove free oocysts and non-adherent epithelial cells, 5 mL of new growth medium was added and the monolayers were observed under Nomarski interference contrast optics at 1000×. Parasite growth was assessed 48 h after infection in 50 random fields. Only meronts and gamonts were enumerated, in order to avoid counting inviable, but adherent, sporozoites or merozoites.[8]

The cytotoxicities of the drugs and their combinations were determined by the CellTiter 96 AQ cell proliferation assay (Promega, Lyon, France). Controls for each cytotoxicity assay included: (i) uninfected cells incubated in DMEM; (ii) infected cells incubated in DMEM; and (iii) cells exposed to a freeze–thaw lysate containing $10^4$ oocyst equivalents in DMEM.

### *Analysis of results*

The anti-cryptosporidial activity of each compound and combination was evaluated by comparing the number of parasites from plates of antimicrobial-supplemented medium with that from control plates without antimicrobials. The average number of parasites per millilitre was calculated by counting 50 random fields (×1000 magnification) of each of three monolayers. The activity of each agent and combination was expressed by calculating the ratio of the parasite numbers in drug-treated cultures to the parasite numbers in control cultures after 48 h incubation. For the agents and combinations that were toxic to the cell monolayer, the peak ratios were calculated by considering the concentration below the one showing the toxic effects.

## Results

In control plates without drugs, the average number of parasites in 50 random fields was 43.8 ± 8.8 (range 34.6–52.2). There was a high preponderance of meronts over microgamonts; macrogamonts were not seen 48 h after infection. The ratios of the peak organism counts in drug-treated cultures to the peak organism counts in control cultures showed that the agents very slightly inhibited parasite growth at the lowest concentration tested. Lasalocid 2 mg/L had the highestactivity *in vitro* (peak ratio 0.30), with a 70.3% reduction in parasite counts. Ranalexin 64 mg/L (0.66) had an *in vitro* activity comparable to that of azithromycin (0.67) at a concentration of 8 mg/L with a 33.8% and 33.1% reduction in parasite count, respectively. Ranalexin 64 mg/L and azithromycin 8 mg/L produced a 69.2% reduction in parasite count with a peak ratio of 0.30. Interestingly, ranalexin 64 mg/L and lasalocid 2 mg/L almost completely suppressed parasite growth without harming the monolayer (peak ratio 0.03). The activities of the drug combinations are presented in the Table. The cytotoxic effect was practically absent at all concentrations of drugs tested alone (percentage cytotoxicity, –3.9 to 9.5) and at all combinations (percentage cytotoxicity, –1.5 to 10.4).

## Discussion

In this study a quantitative system was used to evaluate the *in vitro* anticryptosporidial activity of three agents. Technically, the system is relatively simple to use, and from the time of monolayer preparation, a drug test can be completed in about 48 h. One disadvantage is that gamont development was comparatively rare, and hence, only the effect on asexual development could be readily measured.

Ranalexin may perturb membrane functions responsible for osmotic balance in susceptible target organisms. It has been suggested that it associates with membranes by electrostatic forces: several molecules associate to form a water-filled pore which then serves as an ion-conducting,

**Table.** Inhibitory effects of ranalexin, azithromycin and lasalocid on *Cryptosporidium parvum*

| Agent (mg/L) | Parasite count[a] | Peak ratio[b] |
|---|---|---|
| Control | 43.8 ± 8.8 | – |
| Ranalexin (4) | 40.7 ± 7.5 | 0.93 |
| Ranalexin (16) | 36.0 ± 8.5 | 0.82 |
| Ranalexin (64) | 29.0 ± 8.5 | 0.66 |
| Azithromycin (0.5) | 40.7 ± 10.4 | 0.93 |
| Azithromycin (2) | 38.3 ± 5.5 | 0.87 |
| Azithromycin (8) | 29.3 ± 6.5 | 0.67 |
| Lasalocid (0.125) | 38.7 ± 7.5 | 0.88 |
| Lasalocid (0.50) | 31.7 ± 6.0 | 0.72 |
| Lasalocid (2) | 13.0 ± 2.0 | 0.30 |
| Ranalexin (64) + azithromycin (8) | 13.3 ± 0.6 | 0.30 |
| Ranalexin (64) + lasalocid (2) | 1.3 ± 1.1 | 0.03 |
| Azithromycin (8) + lasalocid (2) | 7.3 ± 2.5 | 0.17 |

[a]Mean number of parasites (±S.D.)/50 fields (×1000).
[b]Ratio of peak parasite number in treated cultures to peak parasite number in control culture.

anion-selective, channel.[2,3] The antimicrobial activity of azithromycin is known to result from its ability to inhibit protein synthesis by binding to the transpeptidation site of the larger ribosomal subunit. Lasalocid is a lipophilic, membrane-interacting molecule. Its effect on cells is thought to be related to direct or indirect alterations of cell membranes: it forms specific complexes with cations such as $Ca^{2+}$ and transports these cations across membranes.[6,7] Our results suggest that these drugs slightly inhibit parasite growth at concentrations that are non-toxic for the cell monolayer. The most active drug was lasalocid.

Additive effects were observed with several combinations. The mechanism of the positive interaction between ranalexin, azithromycin and lasalocid appears to be complex. The combination of ranalexin and lasalocid may be additive or synergic, since their mechanisms of action are similar, involving interaction with the phospholipids of the cell membrane. The increased permeabilization may lead to severe perturbation of the intracellular ionic balance and to cell death. These compounds could perturb biological membrane function as a result of the combined effect of two or more different mechanisms. Proof of clinical benefits is lacking, however. Recent reports demonstrate that polymyxin-like peptides such as ranalexin interact positively with lipophilic and amphiphilic agents such as rifampicin, macrolides, fusidic acid and novobiocin. They have been shown to maximize the entry of several hydrophobic substrates, such as macrolides, into the cell.[9,10] Overall, general conclusions concerning these compounds as possible anticryptosporidial agents are favourable: the additive effects of several combinations make these molecules potentially useful agents. Further investigations are needed before firm conclusions can be drawn.

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