**In vitro** anti-cryptosporidial activity of cationic peptides alone and in combination with inhibitors of ion transport systems

Andrea Giacometti*, Oscar Cirioni, Francesco Barchiesi, Fausto Ancarani and Giorgio Scalise

Institute of Infectious Diseases and Public Health, University of Ancona, Piazza Cappelli 1, 60121 Ancona, Italy

The anti-cryptosporidial activity of four cationic peptides alone and in combination with five ion transport system (ITS) inhibitors was investigated for six clinical isolates of *Cryptosporidium parvum* recovered from stools of AIDS patients. The susceptibility tests were performed by inoculating the protozoa on to cell monolayers and determining the parasite count after 48 h incubation at 37°C. The culture medium was supplemented with serial dilutions of cecropin P1, magainin II, indolicidin and ranalexin alone or in combination with amiloride and its analogues. No agent was able to inhibit parasite growth completely. The peptides had some inhibitory effect on parasite growth: cecropin P1, magainin II, indolicidin and ranalexin at a concentration of 50 µM produced 30.6, 33.2, 38.5 and 42.1% reductions, respectively, in schizont count. Conversely, the ITS inhibitors were scarcely effective. Positive interaction was demonstrated when the peptides were tested in combination with ITS inhibitors.

**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. The discovery of vertebrate polycationic peptides isolated from the ventral skin of toads and frogs and subsequently the identification of other peptides in the giant silk moth and in pig intestine have opened a new area of research into antimicrobial agents. The amino acid sequences of several cationic peptides and the structural requirements for their biological activity have been determined. These compounds could perturb membrane functions responsible for osmotic balance in susceptible target organisms. It has been suggested that the mode of action of these molecules on the membranes of bacteria, fungi, protozoa and artificial lipid bilayers may be similar and involves the formation of ion-channel pores spanning the membranes without requiring a specific target receptor. Association of several such peptides would form a water-filled pore which would serve as an ion-conducting, anion-selective channel.

The ion transport system (ITS) is the target of amiloride, a substituted pyrazinoyl guanidine therapeutically useful as a potassium-sparing diuretic, and its analogue bearing substituents either on the 5-amino nitrogen or on a terminal guanidine nitrogen atom. Amiloride analogues such as 5-(N,N-dimethyl)amiloride (DMA), 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and, particularly, 5-(N-methyl-N-isobutyl)amiloride (MIBA) are selective inhibitors of Na+/H+ antiport. These agents are able to affect the mechanisms that regulate the intracellular pH and therefore cause intracellular acidification. Moreover, benzamil, a Na-benzyl derivative of amiloride, is a selective and potent blocker of Na+/H+ and Na+/Ca2+ channels. The ability of these drugs to suppress the Na+/H+ antiport in tumour cells and their efficiency in causing cell killing have yet to be evaluated. Little information is available about their toxicity, pharmacokinetics and mechanisms of interaction with other molecules, either *in vitro* or *in vivo*.

Cationic peptides and amiloride analogues act primarily on the structure or function of biological membranes. Therefore one can speculate about a possible synergic interaction between these two different groups of molecules. In the present study we have investigated the in vitro activity of four cationic peptides, tested alone and in combination with amiloride and its analogues, on the growth of *C. parvum* in the A549 cell line.

---

*Corresponding author. Tel: +39-071-596-3467; Fax: +39-071-596-3468; E-mail: cmalinf@popcsi.unian.it

© 2000 The British Society for Antimicrobial Chemotherapy
Materials and methods

Organisms

Oocysts of *C. parvum* were isolated from stools of six different patients with AIDS. 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.

Drugs

Cecropin P1, magainin II, indolicidin and ranalexin (all from Sigma-Aldrich) were solubilized in phosphate-buffered saline (PBS, pH 7.2) (BioWhittaker, Walkersville, USA), yielding 1 mM stock solutions. Amiloride, DMA, EIPA, MIBA and benzamil (all from Sigma-Aldrich) were dissolved in 2% dimethylsulphoxide (DMSO) and then brought to the final concentration of 1 mM in distilled water.

Parasite preparation

These procedures have been described in detail previously. Briefly, stools were homogenized in physiological saline and filtered through a metal sieve to remove coarse debris. Fatty material was removed by ether sedimentation: stools were separated into 9 mL aliquots and ether (1 mL) was mixed with each aliquot, which was then centrifuged (200 g for 20 min). The supernatant with the fatty plug was discarded. Oocysts were successively purified and concentrated by flotation in Sheather’s sugar solution (500 g sucrose and 6.5 g phenol in 320 mL of distilled water). 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% sodium hypochlorite and finally by incubation in PBS containing penicillin G (2 MU/L), 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% trypsin and 0.53 mM EDTA 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) (BioWhittaker). Finally, the six isolates were pooled, counted for 60 min at 37°C. Free sporozoites were pelleted by centrifugation (200 g for 20 min) and resuspended in DMEM, (ii) infected cells incubated in DMEM and (iii) cells exposed to a freeze–thaw lysate containing 10⁴ oocyst equivalents in DMEM. The cytotoxicity levels were indicated as no (0–5%), mild (6–25%), moderate (26–50%) and severe (51–100%) cytotoxicity.

Cell cultures

Cells (BioWhittaker) were maintained in 25 cm² tissue culture flasks. The medium consisted of DMEM with 10% fetal calf serum (BioWhittaker), 1% L-glutamine (BioWhittaker), 20 mM N-2-hydroxyethylpiperezine-N-2-ethanesulfonic acid (HEPES) (Sigma-Aldrich), penicillin G (100 mU/L), streptomycin (100 mg/L) and amphotericin B (0.5 mg/L). Cells were lifted from the surface of flasks using a solution of 0.25% trypsin and 0.53 mM EDTA in PBS and quantified with a haemocytometer. Forty-eight hours before parasite inoculation, A549 cells were plated into 35 mm diameter tissue culture plates at a concentration of 10⁵ 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 10⁶ pooled sporozoites in 50 μL of medium. After incubation for 4 h at 37°C in 5% CO₂ 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% CO₂ throughout the study.

In vitro studies

Cecropin P1, magainin II, indolicidin and ranalexin were examined singly at concentrations of 0.5, 5 and 50 μM. In experiments to test drug interactions, the three above-mentioned concentrations of each cationic peptide were tested in combination with each ITS inhibitor at concentrations of 5 and 50 μM. Antibiotic-free plates were used as controls. Experiments were performed in triplicate. *C. parvum* pooled sporozoites were added at a concentration of 10⁵ sporozoites per plate. The monolayers were incubated for 72 h at 37°C in 5% CO₂. 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 magnification. Parasite growth was assessed at 48 h after infection in 50 random fields. Only meronts and gamonts were enumerated, to avoid counting non-viable, but adherent, sporozoites or merozoites.

Cytotoxicity assay

The cytotoxicities of drugs and their combinations were determined by the CellTiter 96 AQ cell proliferation assay (Promega Corp., 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 10⁴ oocyst equivalents in DMEM. The cytotoxicity levels were indicated as no (0–5%), mild (6–25%), moderate (26–50%) and severe (51–100%) cytotoxicity.

Analysis of results

The anti-cryptosporidial activity of each compound and combination was evaluated by comparing the parasite count from plates with antimicrobial-supplemented medium with that from control plates without antimicrobials. The number of parasites was calculated as the mean of the number of organisms observed in three monolayers exposed to the same concentration of drug, by microscopic
Table. Anti-cryptosporidial activity of cationic peptides alone and in combination with ITS inhibitors against *C. parvum* (per cent reduction in the number of parasites compared with control plates)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cecropin P1</th>
<th>Magainin II</th>
<th>Indolicidin</th>
<th>Ranalexin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 µM</td>
<td>5 µM</td>
<td>50 µM</td>
<td>0.5 µM</td>
<td>5 µM</td>
</tr>
<tr>
<td>No drug</td>
<td>0</td>
<td>1.1</td>
<td>11.8</td>
<td>30.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Amiloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>2.3</td>
<td>3.6</td>
<td>15.8</td>
<td>38.5a</td>
<td>4.4</td>
</tr>
<tr>
<td>50 µM</td>
<td>6.1</td>
<td>10.7</td>
<td>22.3</td>
<td>44.3a</td>
<td>10.2</td>
</tr>
<tr>
<td>DMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>1.8</td>
<td>3.6</td>
<td>19.0</td>
<td>39.5a</td>
<td>3.9</td>
</tr>
<tr>
<td>50 µM</td>
<td>5.8</td>
<td>8.4</td>
<td>23.4</td>
<td>43.8a</td>
<td>9.0</td>
</tr>
<tr>
<td>EIPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>2.2</td>
<td>5.0</td>
<td>17.9</td>
<td>36.7a</td>
<td>5.9</td>
</tr>
<tr>
<td>50 µM</td>
<td>6.5</td>
<td>11.1</td>
<td>24.4</td>
<td>45.1a</td>
<td>12.1</td>
</tr>
<tr>
<td>MIBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>2.6</td>
<td>5.3</td>
<td>18.2</td>
<td>34.3a</td>
<td>6.3</td>
</tr>
<tr>
<td>50 µM</td>
<td>7.2</td>
<td>9.8</td>
<td>23.8</td>
<td>41.3a</td>
<td>11.3</td>
</tr>
<tr>
<td>Benzamil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µM</td>
<td>2.5</td>
<td>5.5</td>
<td>18.6</td>
<td>36.7a</td>
<td>5.4</td>
</tr>
<tr>
<td>50 µM</td>
<td>5.7</td>
<td>11.9</td>
<td>26.9</td>
<td>44.9a</td>
<td>11.7</td>
</tr>
</tbody>
</table>

*P < 0.05.
Results and discussion

Cationic peptides, because of their small size and antimicrobial potency, may have therapeutic potential in the treatment of infections. In our study an in vitro culture system for C. parvum was used to measure the anticytospordial activity of four biologically active peptides alone and in combination with agents known as selective calcium channel blockers or selective inhibitors of Na+/H+ antiport.

In control plates without drugs the average number of parasites in 50 random fields was 36.8 (range 29.7–48.1). A high preponderance of meronts over microgamonts was observed. Macrogamonts were not seen at 48 h after infection. A significant inhibitory effect on parasite growth was noted for each peptide at the concentrations of 5 and 50 μM (Table). All peptides were similarly effective, although ranalexin exhibited the highest activity with a >40% at 50 μM. Cecropin P1, magainin II, indolicidin and ranalexin at concentrations of 5 μM produced a decrease in parasite counts of 11.8, 13.7, 12.2 and 14.7%, respectively. The same peptides at concentrations of 50 μM produced a decrease in parasite counts of 30.6, 33.2, 38.5 and 42.1%, respectively. No peptide was able to inhibit parasite growth completely. Amiloride, DMA, EIPA, MIBA and benzamil had no significant inhibitory effects on C. parvum (data not shown).

The activity of the peptides remained virtually unchanged when they were tested in combination with ITS inhibitors at a concentration of 5 μM. In contrast, their activity was significantly improved when they were combined with agents known as selective calcium channel blockers or selective inhibitors of Na+/H+ antiport.

Each drug concentration was defined as inhibitory if it caused a significant decrease in parasite count when compared with control plates. The significance of differences was evaluated by Student’s t test. To assess the efficacy of drug combinations, the significance of differences between results obtained by testing the peptides in combination with amiloride and its analogues were compared with those of control plates by Student’s t test. A P value of =0.05 was considered significant.

References


Received 28 January 1999; returned 6 August 1999; revised 1 September 1999; accepted 27 November 1999