In-vitro activity of lytic peptides, inhibitors of ion transport systems and ionophorous antibiotics against *Pneumocystis carinii*

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The in-vitro activity of two vertebrate lytic peptides, two ion transport system inhibitors and two polyether ionophores was investigated against four clinical isolates of *Pneumocystis carinii* recovered from bronchoalveolar lavages of AIDS patients. The susceptibility tests were performed by inoculating the isolates on to cell monolayers and determining the parasite count after 72 h incubation at 37°C. The culture medium was supplemented with Dulbecco’s modified Eagle’s medium containing serial dilutions of cecropin P1, magainin II, benzamil, 5-(N-methyl-N-isobutyl)amiloride (MIBA), lasalocid and nigericin. The two vertebrate lytic peptides showed high activity against trophozoites and cysts. At a concentration of 66.77 mg/L, cecropin P1 produced a 93.3% and 98.1% reduction in cyst and trophozoite counts, respectively, while magainin II at a concentration of 49.33 mg/L produced a 90.6% and 98.7% reduction in cyst and trophozoite counts, respectively. The IC₅₀ values of benzamil and MIBA were observed at the highest concentrations tested, 35.62 and 29.98 mg/L, respectively. However, 90% inhibition was not achieved. Lasalocid and nigericin at 0.05 mg/L gave inhibition comparable to that observed with the highest tested concentrations of cecropin P1 and magainin II, but significant injury to the cell monolayer was also observed when nigericin was tested at this concentration. Lasalocid 0.05 mg/L produced a reduction of 91.3% and 92.0% in cyst and trophozoite counts, respectively. Our results suggest that lytic peptides and lasalocid may be effective in inhibiting *P. carinii* growth at concentrations which are not toxic for the cell monolayer.

**Introduction**

The well-publicized limitations of currently available anti-*Pneumocystis carinii* agents in AIDS patients have emphasized the need for new therapeutic approaches.¹⁻³ New strategies for producing molecules with novel mechanisms of action are essential for the therapy or prophylaxis of pneumocystosis.

Recently, the identification of lytic peptides in the ventral skin of toads and frogs,⁴,⁵ in the giant silk moth,⁶ in pig intestine⁷ and in mammalian granulocytes⁸ has opened a new area of research into antimicrobial agents. These compounds are thought to function through the formation of ion channel pores spanning the membranes of bacteria, erythrocytes, artificial lipid bilayers and protozoa.⁹ The discovery of naturally occurring antimicrobial compounds without toxicity for mammalian cells would be beneficial. Cecropins and magainins lyse bacteria and do not kill mammalian cells.

A miloride inhibits Na⁺ transporters and is therapeutically useful as a potassium-sparing diuretic. Its target is the ion transport system (ITS).⁹ A miloride analogues, such as 5-(N,N-dimethyl)amiloride (DMA), 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and 5-(N-methyl-N-isobutyl)amiloride (MIBA), are selective inhibitors of Na⁺/H⁺ antiport. By affecting the mechanisms that regulate intracellular pH (pHᵢ), they lead to intracellular acidification.¹⁰,¹¹ Benzamil, a Na-benzyl derivative of amiloride, is a selective and potent blocker of Na⁺/H⁺ and Na⁺/Ca²⁺ channels.¹² Ionophores are lipid-soluble compounds that transport polar cations, such as Ca²⁺, across cell membranes.¹³ Lasalocid and nigericin are polyether carboxylic acid ionophores, isolated from *Streptomyces lasaliensis* and *Streptomyces hygroscopicus*, respectively. Lasalocid is used as an anticoccidial drug in farm animals, while nigericin disrupts membrane potential and stimulates ATPase activity in mitochondria.¹⁴,¹⁵

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All these compounds act primarily on the structure or function of biological membranes. The aim of the present study was to investigate their efficacy against *P. carinii*.

**Materials and methods**

**Organisms**

Four clinical isolates of *P. carinii* were isolated from bronchoalveolar lavages of four AIDS patients.

**Drugs**

Cecropin P1 (Sigma–Aldrich, Milan, Italy) and magainin II (Sigma–Aldrich) were dissolved in phosphate-buffered saline (PBS), pH 7.2 (Bio-Whittaker, Walkersville, MD, USA) yielding stock solutions of, respectively, 3338.9 mg/L (1 mM) and 2466.9 mg/L (1 mM). MIBA (Sigma–Aldrich) and benzamil (Sigma–Aldrich) were dissolved in 2% dimethylsulphoxide (DMSO) and then diluted to a final concentration of 299.8 mg/L (1 mM) and 356.2 mg/L (1 mM), respectively, in culture medium. Lasalocid (Sigma–Aldrich) and nigericin (Sigma–Aldrich) were dissolved in DMSO and then further diluted to a final concentration of 1 mg/L in culture medium. Atovaquone (Wellcome Italia, Rome, Italy), sulphamethoxazole and trimethoprim (Sigma–Aldrich) were dissolved in 50% methanol/50% acetone at a concentration of 10 g/L and then diluted to a concentration of 100 mg/L in culture medium.

**Parasite preparation**

These procedures have been described in detail previously. Briefly, specimens were homogenized and centrifuged (at 1800 g for 15 min). The pellet was resuspended in physiological saline. Contaminating bacteria were eliminated by four washes in 10 mL of physiological saline followed by incubation in PBS containing ampicillin (2000 mg/L) and streptomycin (2000 mg/L) for 4 h at 37°C. Organisms were pelleted by centrifugation (at 1800 g for 20 min), resuspended in Dulbecco's modified Eagle's medium (DMEM) (Bio-Whittaker), counted following methenamine silver stain to detect cysts and with Giemsa stain for counting *P. carinii* trophozoites, and finally aliquoted for culture. The final inoculum was $10^2$–$10^3$ *P. carinii* organisms/mL.

**Cell cultures**

The A 549 cell line was maintained in 96-well plates in medium containing DMEM, 10% fetal calf serum, 1% L-glutamine, 20 mM N-2-hydroxyethylpiperazine-N-ethanesulphonic acid (HEPES), penicillin G (200,000 U/L), streptomycin (200 mg/L) and amphotericin B (0.5 mg/L). Cultures were initiated by adding 0.1 mL of inoculum to an adherent layer of 50–70% confluent cells. 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 cysts and non-adherent epithelial cells, and 0.2 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**

The following concentrations of each agent were tested: cecropin P1, 0.66, 6.67 and 66.77 mg/L; magainin II, 0.49, 4.93 and 49.33 mg/L; MIBA, 0.29, 2.99 and 29.98 mg/L; benzamil, 0.35, 3.56 and 35.62; lasalocid and nigericin, 0.0005, 0.005 and 0.05 mg/L. Two control agents, atovaquone and trimethoprim-sulphamethoxazole, were tested at concentrations of 2, 8 and 32 mg/L, and 4, 8 and 16 mg/L, respectively. A antibiotic-free plates were used as controls in the study. Experiments were performed in triplicate.

*P. carinii* were added at a concentration of $10^2$–$10^3$ organisms per well. The medium pH was verified during the experiment. The monolayers were incubated at 37°C in a 5% CO$_2$ atmosphere. After 72 h the supernatant was withdrawn from each triplicate well and *P. carinii* trophozoites and cysts counted following Giemsa and methenamine silver staining.

**Analysis of results**

The activity of each compound was evaluated by parasite count from plates with antimicrobial-supplemented medium compared with parasite count from control plates without antimicrobials. The average number of *P. carinii* parasites/mL was calculated by counting 50 oil immersion fields ($\times$1000 magnification) of each of three slides.

The 50 and 90% inhibitory concentrations (IC$_{50}$ and IC$_{90}$, respectively) of the drug were defined as the concentrations required to produce 50 and 90% reduction, respectively, in the mean cyst or trophozoite counts compared with controls without drug, after 72 h incubation in the presence of drugs.

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 the Student’s t-test. A P value of $\leq 0.05$ was considered significant.

**Results**

In control plates without drugs, at least a three-fold increase in the number of *P. carinii* nuclei for each of the four clinical isolates occurred over 3 days. The average number of parasites grown in the absence of antibiotic was
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38.3 (range 22–59) when calculated by counting 50 oil immersion fields. Both trimethoprim–sulphamethoxazole and atovaquone inhibited parasite growth. In wells containing atovaquone 8 mg/L or trimethoprim–sulphamethoxazole 8 mg/L the number of countable trophozoites was less than half of that of control plates without drugs, while the concentrations of atovaquone and trimethoprim–sulphamethoxazole required to produce 50% reduction in the mean cyst counts were 8 and 4 mg/L, respectively (Table I).

The activities of lytic peptides showed some differences on the basis of the development stage detected. Cecropin P1 and magainin II showed high activity against trophozoites, while they were slightly less effective against cysts (Table I). The IC\textsubscript{50}s of benzamil and MIBA were observed at the highest concentrations tested (35.62 and 29.98 mg/L, respectively). However, 90% inhibition was not achieved (Table I).

At a concentration of 0.05 mg/L, lasalocid and nigericin gave inhibition comparable to that observed with either cecropin P1 and magainin II at concentrations of 66.77 and 49.33 mg/L, respectively, but significant injury to the cell monolayer was also observed when nigericin was tested at this concentration (Table I). Overall, lasalocid and lytic peptides were as effective as control drugs, but only at the highest concentrations tested.

On the basis of the statistical analysis, cecropin P1 at concentrations of 6.67 and 66.77 mg/L, magainin II at concentrations of 4.93 and 49.33 mg/L, benzamil at concentrations of 3.56 and 35.62 mg/L, MIBA at concentrations of 2.99 and 29.98 mg/L, and polyether ionophores at concentrations of 0.005 and 0.05 mg/L were inhibitory (P < 0.05).

D\textbf{iscussion}

In our study a quantitative system for evaluating the in-vitro anti-P. carinii activity of several membrane effectors has been developed. The A549 line and 96-well plates were used to evaluate the activity of low concentrations of several agents.

The cecropins, a family of amphiphilic peptides,

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (mg/L)</th>
<th>% Reduction in no. of parasites</th>
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<tr>
<td></td>
<td></td>
<td>cysts</td>
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<tr>
<td><strong>Trimethoprim–sulphamethoxazole</strong></td>
<td>4</td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>83.1</td>
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<tr>
<td></td>
<td>16</td>
<td>97.5</td>
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<tr>
<td><strong>A tovaquone</strong></td>
<td>2</td>
<td>6.6</td>
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<tr>
<td></td>
<td>8</td>
<td>71.1</td>
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<tr>
<td></td>
<td>32</td>
<td>91.9</td>
</tr>
<tr>
<td><strong>Cecropin P1</strong></td>
<td>0.66</td>
<td>7.3</td>
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<tr>
<td></td>
<td>6.67</td>
<td>41.6</td>
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<td></td>
<td>66.77</td>
<td>93.3</td>
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<tr>
<td><strong>Magainin II</strong></td>
<td>0.49</td>
<td>7.0</td>
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<tr>
<td></td>
<td>4.93</td>
<td>39.9</td>
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<td></td>
<td>49.33</td>
<td>90.6</td>
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<tr>
<td><strong>Benzamil</strong></td>
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<td>2.0</td>
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<td></td>
<td>3.56</td>
<td>29.8</td>
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<td></td>
<td>35.62</td>
<td>62.9</td>
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<tr>
<td><strong>MIBA</strong></td>
<td>0.29</td>
<td>1.6</td>
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<tr>
<td></td>
<td>2.99</td>
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<td></td>
<td>29.98</td>
<td>58.7</td>
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<tr>
<td><strong>Lasalocid</strong></td>
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<td></td>
<td>0.005</td>
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<td></td>
<td>0.05</td>
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<tr>
<td><strong>Nigericin</strong></td>
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<td>2.1</td>
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<td></td>
<td>0.005</td>
<td>32.1</td>
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<tr>
<td></td>
<td>0.05</td>
<td>100.0\textsuperscript{a}</td>
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\textsuperscript{a}Presence of cytolytic effect on cell monolayers.

Table. Inhibitory effects of drugs against P. carinii: percent reduction in the number of parasites relative to that in control plates without drugs

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represent a major, inducible, antibacterial defence system in insects. Recent reports described a family of antimicrobial peptides present in the ventral skin of the toad X. enopus laevis. These peptides, named magainins, may be the vertebrate counterpart of the cecropins. Because of their small size and antimicrobial potency, they may have therapeutic potential for the treatment of bacterial, fungal and protozoan infections in humans. M. agnin and cecropin peptides could perturb membrane functions responsible for osmotic balance in susceptible target organisms. It has been hypothesized that the lytic peptides associate with the membrane by electrostatic forces. The association of several molecules would form a water-filled pore which would serve as an ion-conducting, anion-selective, channel.

The cytotoxic effect of amiloride analogues are very dependent on extracellular pH ($p_{\text{H}}$). They are not toxic to cultured cells when used alone for up to 6 h at $p_{\text{H}}$ 6.0–7.4. These compounds give a plateau level of cell killing, such that there is little additional effect at higher doses. Recently their efficacy has been shown in reducing the growth of P. carinii in in-vitro models.

No studies have been reported to delineate the mechanism of antimicrobial activity of lasalocid. It has been suggested that it catalyses potassium–hydrogen exchange differences in the bacterial cell. Nigericin is another polyether ionophore which exerts significant activity against all Plasmodium falciparum developmental stages in vitro. It acts by disrupting membrane potential and stimulating ATPase activity in mitochondria.

A n in-vitro drug screening system is important for the initial identification of candidate anti-P. carinii compounds before in-vivo testing, but a continuous in-vitro culture system for P. carinii remains elusive. We determined the differences in anti-P. carinii activity between molecules known as membrane effectors and evaluated their effects on the cell monolayer in a short-term cell monolayer system.

In our experiments, lytic peptides and lasalocid were as effective as control drugs, but only at the highest concentration tested, while MIBA and benzamil were much less effective. These results suggest that lytic peptides and polyether ionophores are effective in inhibiting parasite growth at concentrations which are not toxic for the cell monolayer. Cecropin P1 and magainin II showed some effectiveness as control drugs, but only at the highest concentration tested. Only two of the molecules known as membrane effectors showed some activity against trophozoites was marginally greater than that against cysts.

Further research is needed to characterize in more detail the mode of action of vertebrate lytic peptides and their in-vivo toxicity, pharmacokinetics and the mechanisms of interaction with other molecules. The fact that lytic peptides are found in mammals and in insects is an important indication that antimicrobial peptides may be a universal means for defence against infections. Further investigations involving more compounds, used individually and in combination, and with additional statistical techniques are needed before firm conclusions about effectiveness can be drawn. Further investigations are also needed to detect differences between in-vitro and in-vivo efficacy.

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