Antisense phosphorodiamidate morpholino oligomer inhibits viability of *Escherichia coli* in pure culture and in mouse peritonitis

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**Objectives:** Antisense phosphorodiamidate morpholino oligomers (PMOs) are synthetic DNA mimics that specifically inhibit gene expression in pure cultures of *Escherichia coli*. Previously, an 11 base PMO targeted to an essential gene (*acpP*) for phospholipid biosynthesis was shown to inhibit growth of a pure culture of *E. coli* AS19, which has an abnormally permeable outer membrane. The objectives of experiments in this report are to show that the AcpP PMO significantly inhibits growth of strain SM105, which has a normal, intact outer membrane, both in pure culture and in infected mice.

**Methods:** In pure culture, SM105 was grown in rich broth supplemented with 20 μM AcpP PMO, and growth was monitored by optical density and viable cell count. Mice were infected by intraperitoneal injection with a non-lethal inoculum of either *E. coli* AS19 or SM105. Following infection, mice were treated intraperitoneally with 300 μg of the 11 base antisense PMO targeted to *acpP*, a scrambled sequence PMO or PBS.

**Results:** Growth of SM105 was slower and viable cells were significantly reduced by up to 61% in pure cultures supplemented with AcpP PMO compared with untreated cultures or cultures supplemented with a scrambled sequence PMO. A single dose of AcpP PMO reduced peritoneal cfu of *E. coli* AS19 about 39- to 600-fold compared with controls at 2, 7, 13 and 23 h after treatment. The same PMO significantly reduced cfu of *E. coli* SM105 75% compared with controls at 12 h after treatment. However, there was no difference in cfu at 2, 7 or 24 h. A second dose at 24 h again reduced SM105 cfu about 10-fold by 48 h post-infection. In other experiments with infected mice, multiple doses of AcpP PMO sustained the ~10-fold reduction in SM105 cfu at 6, 12 and 24 h post-infection. Compared with equivalent (micromolar) doses of ampicillin, AcpP PMO was significantly more effective at all time points. Specificity of PMO inhibition was shown in other experiments by treating infected mice with a PMO targeted to a non-essential reporter gene for luciferase. A luciferase-specific PMO reduced both the amount and activity of luciferase to the same extent, whereas scrambled PMO had no effect.

**Conclusions:** An 11 base antisense PMO targeted to *acpP* significantly inhibited viability of a strain of *E. coli* with a normal, intact outer membrane both in pure culture and in infected mice. Inhibition by PMOs was sequence-specific.

Keywords: PMO, antibiotics, gene-specific, bacterial growth inhibition

**Introduction**

Antibiotic-resistant bacteria are increasing the need for additional antibacterial agents. A fundamentally different concept from traditional antibiotics is to inhibit gene expression in a sequence-specific manner. Antisense mechanisms of gene regulation occur naturally in all kingdoms of life, from bacteria to plants and animals.1,2 Synthetic antisense oligomers have been shown to inhibit gene expression and growth of bacteria in pure culture and in infected tissue culture.1,3

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Phosphorodiamidate morpholino oligomers (PMOs) inhibit bacterial targets by an antisense mechanism. In *Escherichia coli*, the outer membrane restricts the entry of PMOs, and this significantly reduces the effectiveness of the PMO. Significant inhibition of targeted gene expression has been shown only in strains with an abnormally permeable outer membrane, or by covalently attaching to the PMO a peptide that facilitates uptake across the outer membrane. Recently, we have shown that the size of the PMO can be reduced from 20 to about 10 bases without compromising specificity or inhibition of target expression. The reduction in size greatly improves the efficacy of PMOs in strains with an abnormally permeable outer membrane.

In this report, the efficacy of an 11 base PMO targeted to acpP is demonstrated first in pure cultures of a strain with a normal outer membrane. *acpP* codes for acyl carrier protein, which is the carrier on which new acyl chains are biosynthesized, and is essential for membrane lipid synthesis. Following this, the same PMO is administered to mice infected with *E. coli*, and found to decrease viable bacteria. In addition, sequence-specific inhibition of bacterial gene expression is demonstrated in mice.

**Materials and methods**

**Antisense oligomers**

PMOs were synthesized, purified and analysed at AVI BioPharma (Corvallis, OR, USA) as described previously. The sequences of the PMOs (5′ to 3′) are: AcPp, CTT CGA TAG TG2; AcPp scrambled control, TCT CAG ATG GT; Luc, ACG TTG AGG G2; and Luc scrambled control, TCC ACT TGC C. Superscripts indicate the nucleotide position of the targeted coding sequence. All PMOs were prepared for injection in PBS and sterile filtered through 0.2 μm filters.

**Bacterial cultures**

*E. coli* AS19 was a gift from Peter Nielsen (University of Copenhagen, Denmark), and SM105 was from *E. coli* Genetics Stock Center (Yale University, New Haven, CT, USA). AS19 was chosen for use because it has an abnormally permeable outer membrane and grows at 37°C. SM105 was chosen for use because it has a normal outer membrane and has been previously used to characterize PMOs in pure culture. In previous work, SM105 was used because it is the parental strain of SM101, which is another abnormally permeable strain that is better characterized than AS19, and has been used previously to characterize PMOs in pure culture. However, SM101 is a conditional lethal mutant that cannot grow at 37°C. Bacteria were grown at 37°C in LB broth (supplemented with 100 mg/L ampicillin for transformants that expressed luciferase). For experiments in pure culture, overnight cultures were diluted 2 × 10^-2, mixed with or without 20 μM PMO, and growth measured as described previously. For experiments in mice, overnight cultures were diluted 2 × 10^-2, grown to OD<sub>600</sub> = 0.12 [measured by placing 100 μL of culture in a 96-well, low binding microtitre plate (Corning Costar, Corning, NY, USA)], centrifuged (4000 g, 10 min, 20°C), and resuspended in 5% (w/v) mucin (type III; Sigma Chemical Co., St Louis, MO, USA) in PBS to final concentrations as follows: AS19, 1.5 × 10<sup>6</sup> cfu/mL [standard deviation (SD) = 5.6 × 10<sup>5</sup>, n = 2]; SM105, 5.7 × 10<sup>6</sup> cfu/mL (SD = 1.9 × 10<sup>5</sup>, n = 2) for experiments shown in Figure 3 and 6.8 × 10<sup>5</sup> cfu/mL (SD = 3.4 × 10<sup>4</sup>, n = 4) for those shown in Figure 4; AS19 (pT7myc-luc), 7.0 × 10<sup>9</sup> cfu/mL (SD = 2.8 × 10<sup>8</sup>, n = 2).

**Luciferase reporter**

pT7myc-luc that expresses luciferase was constructed as described previously, and transformed into AS19 by standard procedures.

**Animals**

Female, 6- to 8-week-old Swiss Webster mice (Simonsen Labs., Inc., Gilroy, CA, USA) were used in all but one experiment, but identical results were obtained for each strain as described previously. Briefly, groups of mice were injected intraperitoneally with various concentrations of each strain in 5% mucin in PBS. Peritoneal lavages were collected at intervals over 48 h and plated to determine cfu/mL. From these data, one or two specific amounts of each strain were selected for use in subsequent PMO treatment studies. The selected amounts of each strain produced a sustainable infection over 48 h without causing death or any apparent distress to the animals. The total cfu in the inocula was as follows: AS19, 1.5 × 10<sup>7</sup> cfu; SM105, 5.7 × 10<sup>6</sup> cfu for experiments shown in Figure 3, and 8.1 × 10<sup>6</sup> cfu for those shown in Figure 4; AS19 (pT7myc-luc), 7.2 × 10<sup>6</sup> cfu.

Each mouse was injected intraperitoneally with 0.1 mL of bacteria resuspended in 5% mucin/PBS, then immediately injected intraperitoneally with 0.1 mL of PMO (3.0 mg/mL) or PBS. Where indicated, additional doses (300 μg of PMO in 0.1 mL of PBS) were injected intraperitoneally. At various times after infection (as indicated in figures), groups (n = 3–5) of mice were injected intraperitoneally with 2.0 mL of PBS, and their abdomens gently massaged for 2 min. Peritoneal lavage was removed, diluted in PBS and plated in triplicate on LB to determine cfu.

The lack of effect of mucin on AcpP PMO efficacy was assessed in pure culture by diluting 2 × 10^-2 an overnight culture of AS19 into 5% mucin/50% LB broth with 20 μM AcpP PMO or scrambled PMO. After 15 h at 37°C with aeration, each culture was diluted and plated on LB plates. The mean cfu/mL was 7.3 × 10<sup>6</sup> (SEM = 3.7 × 10<sup>5</sup>, n = 3) for the cultures treated with AcpP PMO and 5.2 × 10<sup>5</sup> (SEM = 5.8 × 10<sup>4</sup>, n = 3) for the cultures treated with scrambled PMO.

All procedures involving animals complied fully with United States Federal and Oregon state laws, and were evaluated and approved by the Oregon State University Institutional Animal Care and Use Committee (approval number 2947, approved 28 August 2003).

**Luciferase and western blot**

Peritoneal lavages (1.00 mL) from mice infected with AS19 (pT7myc-luc) were centrifuged (10000 g, 2 min, 4°C) and the supernatants discarded. The pellets were resuspended in 50 μL of PBS. An aliquot of resuspended cells was mixed with an equal volume of 2 × cell culture lysis reagent (Promega, Inc., Madison, WI, USA) and frozen at −85°C. Frozen lysates were thawed and luciferase light production was measured in duplicate in a luminometer as described previously. A secondary aliquot of the cell suspension was mixed with 2 × SDS sample buffer and analysed by western blot using 4–20% gradient Gene Mate Express Gels (ISC BioExpress, Inc., Kaysville, UT, USA). Blots were prepared with primary antibody to luciferase (Cortex Biochemical, San Leandro, CA, USA) or antisera to OmpA, secondary goat anti-rabbit IgG-horse radish peroxidase conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and ECL Western Blotting Reagent (Amersham Biosciences, Buckinghamshire, UK). Film negatives were scanned and digitized on a Kodak Image Station 440 CF. The net intensity of each band was calculated by subtracting the mean background intensity.
Luciferase protein was normalized to OmpA by dividing the net intensity of the luciferase band by the net intensity of the OmpA band in the same sample. The percentage inhibition was calculated by subtracting the mean luciferase/OmpA of Luc PMO-treated mice from the mean luciferase/OmpA of scrambled PMO-treated mice, dividing the difference by the mean luciferase/OmpA of scrambled PMO-treated mice, then multiplying by 100%.

Statistical analysis
Individual mouse cfu/mL values were transformed logarithmically for statistical analysis using InStat statistical software (GraphPad Software, San Diego, CA, USA). Differences in treatment group means were analysed with unpaired t-test, not assuming equal variances, with Welch correction. Treatment group values were analysed for Gaussian distributions using the method of Kolmogorov and Smirnov, which confirmed in all analyses the normality test of the data. A one-tailed t-test was applied to differences in means between AcpP PMO and either PBS or scrambled PMO treatment groups, whereas a two-sided t-test was applied in all other analyses.

Results

In vitro effects of PMO
Growth of *E. coli* was monitored by optical density in LB broth with 20 μM antisense PMO complementary to the region around the start codon of *acpP*, with 20 μM scrambled sequence PMO or without PMO. The AcpP PMO inhibited growth, which was apparent at 4 h, but diminished by 8 h (Figure 1a). The growth rate from 2 to 4 h of the culture with AcpP PMO was significantly (*P* < 0.01) reduced from 0.93 (SEM = 0.01, *n* = 3) to 0.72 (SEM = 0.02, *n* = 3) generations/h compared with the untreated culture. The viable cell count at 4 and 8 h (Figure 1b) was significantly (*P* < 0.01) reduced at both time points by 61% and 18%, respectively, in the culture treated with AcpP PMO compared with the untreated culture. There was no significant inhibition of growth by optical density or viable cell count in the culture treated with scrambled PMO compared with the untreated culture. Increasing the concentration of PMO from 20 to 100 μM did not significantly increase the inhibition (data not shown).

Strain AS19 in vivo
PMO was first tested in mice infected with *E. coli* AS19, which has an outer membrane that is abnormally permeable to high molecular weight solutes and is ultra-sensitive to antibiotics. AS19 has been used previously in pure culture to assess AcpP PMO and other antisense oligomers. Groups of mice were injected intraperitoneally with a non-lethal amount of *E. coli* AS19. Immediately following infection, each mouse was injected intraperitoneally with 300 μg (76 nmol) of AcpP PMO, scrambled sequence PMO or PBS. Peritoneal lavages were collected at 2, 7, 13 and 23 h post-infection, and plated for bacteria. The results show that at all times analysed, the difference in cfu/mL was highly significant (*P* < 0.01) between mice treated with AcpP PMO and mice treated with either scrambled PMO or PBS (Figure 2). Mean cfu/mL in the AcpP PMO treatment group ranged from 1.4% (at 2 h) to 0.3% (at 7 h) of the cfu/mL in PBS treated mice. There was no significant difference in group means between scrambled PMO and PBS treatment group means at any time point.

Strain SM105 with normal outer membrane
The same PMOs were again tested with a non-lethal inoculum in mice, except using *E. coli* SM105, which has a normal outer membrane. SM105 has been used previously in pure culture to assess antisense AcpP PMOs. AcpP PMO significantly (*P* = 0.02) reduced cfu/mL by 75% compared with PBS at 12 h post-infection. There was no significant reduction in cfu/mL (*P* > 0.05) at 2, 6 or 24 h (Figure 3). There was no difference (*P* > 0.05) between PBS and scrambled PMO treatment group means at any time point.
Mice were injected with a second dose at 24 h post-infection. By 48 h post-infection, there were significant differences in cfu/mL between the AcpP PMO treatment group and either of the other treatment groups. AcpP PMO significantly reduced cfu/mL by 89% ($P < 0.01$) compared with PBS, and 70% ($P < 0.05$) compared with scrambled PMO (Figure 3). There was no mortality in any of the groups.

Multiple doses of PMO during the first 24 h of infection

The results shown in Figure 3 suggested that multiple doses of AcpP PMO during the first 24 h after infection might further reduce cfu. Mice were infected with a 10-fold lower dose of *E. coli* SM105, then treated at 0, 6, 12 and 18 h post-infection with equal molar amounts (76 nmol/dose) of AcpP PMO, scrambled PMO or ampicillin. Peritoneal lavages were collected at 6, 12 and 24 h post-infection and plated to determine bacterial cfu/mL.

The results show AcpP PMO treatment significantly ($P < 0.02$) reduced cfu/mL at 6, 12 and 24 h, compared with scrambled PMO (Figure 4). The percentage reduction in mean cfu/mL compared with the scrambled PMO-treated mice was progressively greater after each dose of AcpP PMO, increasing from 46% at 6 h, to 58% at 12 h and 68% at 24 h.

AcpP PMO treatment significantly ($P < 0.01$) reduced cfu/mL at all time points compared with ampicillin treatment. The reductions at 6, 12 and 24 h were 68%, 45% and 66%, respectively.

There was no statistically significant ($P > 0.05$) difference in means between scrambled PMO and ampicillin treatment groups at either 12 or 24 h. At 6 h, cfu/mL was significantly ($P < 0.01$) lower by 46% in scrambled PMO-treated mice compared with ampicillin-treated mice. There was no mortality in any of the groups.

Target sequence-specific inhibition

The above results with AcpP and scrambled PMOs suggest that inhibition was sequence specific. To demonstrate directly a sequence-specific effect, mice were infected with *E. coli* AS19 that expresses firefly luciferase, then treated at 0 and 13 h post-infection with a PMO (Luc) complementary to the region around the start codon of the luciferase transcript or a scrambled PMO. Peritoneal lavages were collected at 13 and 22 h post-infection and analysed for cfu, luciferase enzymic activity, and luciferase and OmpA protein (western immunoblot). As expected, the results show no significant ($P > 0.05$) inhibition of growth with Luc PMO treatment compared with scrambled PMO treatment (Table 1). Luciferase activity in samples from Luc PMO-treated mice was inhibited 53% and 46% at 13 and 22 h, respectively, compared with samples from scrambled PMO-treated mice (Table 1).

Western-blot analysis agreed closely with the results of luciferase activity. In samples from Luc PMO-treated mice, there was a 68% and 47% reduction in the amount of luciferase protein at 13 and 22 h, respectively, compared with samples from scrambled PMO-treated mice (Table 1).
The results show that in the mouse model of *E. coli* peritonitis, intraperitoneal treatment with AcpP PMO significantly reduced cfu/mL compared with treatment with scrambled sequence PMO or PBS. This result demonstrates for the first time that antisense DNA analogues inhibit bacterial growth in animal infections.

The magnitude of reduction in cfu/mL was dependent on the strain used for infection. The outer membrane is known to be a major barrier to PMOs and other DNA analogues in pure culture. The *E. coli* AS19 is abnormally permeable to high molecular weight solutes because of an apparent defect in the outer membrane. The AcpP and scrambled PMOs both have a molecular weight of 3958. Strains of *E. coli* with a normal outer membrane, such as SM105, restrict entry of solutes greater than about 600 Da. Nevertheless, PMOs enter SM105, apparently because of their linear shape.

Multiple doses (300 μg/dose = 76 nmol/dose) of PMO sustained reductions in cfu over the entire 24 h of the experiment. The percentage reductions in cfu in mice were greater than the percentage reductions in cfu seen in pure culture supplemented with 20 μM AcpP PMO (Figure 1). We have also found that equivalent (76 nmol) doses of ampicillin, which at this dose is known to be borderline sub-therapeutic, were significantly less effective than AcpP PMO (Figure 4). Based on conventional pharmaceutical methods of converting animal dosage, we

### Table 1. Gene-specific inhibition

<table>
<thead>
<tr>
<th>PMO treatment</th>
<th>Viable cell count</th>
<th>Western blot</th>
<th>Luciferase activity</th>
</tr>
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<tbody>
<tr>
<td>Luc</td>
<td>6.46 (0.20, 8)</td>
<td>6.54 (0.629, 8)</td>
<td>6.19 (0.773, 8)</td>
</tr>
<tr>
<td>Scrambled</td>
<td>6.27 (0.18, 8)</td>
<td>5.83 (0.755, 8)</td>
<td>8.12 (1.94, 5)</td>
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Discussion

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calculate that 100–200 mg/day of PMO may be a feasible therapeutic dose in humans. Although the AcpP PMO inhibited growth, these results do not prove sequence-specific inhibition. It was conceivable that the AcpP PMO sequence was generally toxic, whereas the scrambled control was not. To show sequence-specific gene inhibition, we used a reporter gene (luciferase) whose expression would not affect growth, and measured luciferase directly by two independent criteria, luciferase activity and luciferase protein abundance. We showed that a PMO complementary to the luciferase mRNA inhibited luciferase expression at two different times after administration of the PMO. Moreover, inhibition was quantitatively similar with both methods of measurement. These results show directly that PMO inhibits target expression in a sequence-specific manner.

We are optimistic that improvements can be made in PMO efficacy. In pure culture, PMO efficacy is increased by attaching a short peptide to the PMO, which facilitates entry through the outer membrane of E. coli. Different targets may be more sensitive to modest reductions in expression compared with acpP, which encodes one of the more abundant proteins in E. coli that can be severely reduced in concentration without affecting normal function. Additional doses and other routes of administration of the PMO may also improve efficacy. Work is currently in progress to improve PMO efficacy.

Acknowledgements

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