Supplementary Materials: Bi-Functional Alginate Oligosaccharide–Polymyxin Conjugates for Improved Treatment of Multidrug-Resistant Gram-Negative Bacterial Infections

Joana Stokniene, Lydia C. Powell, Olav A. Aarstad, Finn L. Aachmann, Philip D. Rye, Katja E. Hill, David W. Thomas and Elaine L. Ferguson

1. Methods

NMR Spectroscopy

Samples (10 mg) were dissolved in 99.9 atom % D$_2$O (600 µL) and analysed by NMR at 25 °C on a Bruker AVIII-HD 800 spectrometer equipped with CP-TCI 5 mm cryoprobe (Bruker BioSpin AG; Fällanden, Switzerland). Diffusion-ordered spectroscopy (DOSY) was used to measure the diffusion of the OligoG conjugate products. A 2D DOSY was measured using a Bruker BioSpin stimulated echo pulse sequence with bipolar gradients (STEBPGP). Gradient pulses of 2 ms duration (δ) and 32 different strengths varying linearly from 0.03 to 0.57 Tm$^{-1}$ were applied and the diffusion delay (Δ) was set to 80 ms. The spectra were recorded using the TopSpin software (version 3.5pl7) and analysed with the TopSpin software (version 4.0.7) (Bruker BioSpin AG; Fällanden, Switzerland).

2. Results

Figure S1. Size exclusion chromatography with multi-angle light scattering detection (SEC-MALS) analysis of OligoG-conjugates (three different batches of OligoG-A-colistin (OAC) and one batch of OligoG-E-colistin (OEC)), showing overlaid refractive index chromatograms and corresponding Mw-time calibration lines. The injected mass was 250 µg for all samples. Abbreviations: A, amide; E, ester.
**Figure S2.** SEC-MALS analysis of OligoG in the absence and presence of 10% w/w colistin, showing that they do not form strong complexes since the elution profile is identical.

**Figure S3.** Diffusion-ordered spectroscopy (DOSY) of (a-c) three different batches of OligoG-A-colistin conjugates and (d) one batch of OligoG-E-colistin conjugate. The assignment of the unique signals for OligoG and colistin is indicated at the top of each panel and the red lines indicate the average diffusion coefficients of the molecules.
Figure S4. Drug release of OligoG-polymyxin conjugates in phosphate buffered saline (PBS) at pH 5, pH 7 or pH 7 containing alginate lyase (AlgL). (a) Content of free polymyxin and (b) change in molecular weight were determined by fast protein liquid chromatography (FPLC) and size exclusion chromatography with refractive index detection (SEC-RI), respectively, over 48 h incubation. Abbreviations: A, amide; E, ester.
### Table S1. Gram-negative bacterial isolates used for characterisation of OligoG-polymyxin conjugates.

<table>
<thead>
<tr>
<th>Bacterial Isolate</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> R22</td>
<td>VIM-2</td>
<td>China</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> MDR 301</td>
<td>VIM-2</td>
<td>Poland</td>
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<td><em>P. aeruginosa</em> NH57388A</td>
<td>VIM-2</td>
<td>Denmark</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NCTC 10662</td>
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<td>Reference strain</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> KP05 506</td>
<td>NDM-1</td>
<td>India</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> IR25</td>
<td>NDM-1</td>
<td>India</td>
</tr>
<tr>
<td><em>A. baumannii</em> MDR ACB</td>
<td>MDR</td>
<td>Libya</td>
</tr>
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<td><em>A. baumannii</em> 7789</td>
<td>MDR</td>
<td>United Kingdom</td>
</tr>
<tr>
<td><em>E. coli</em> AIM-1</td>
<td>AIM-1</td>
<td>Australia</td>
</tr>
<tr>
<td><em>E. coli</em> IR57</td>
<td>NDM-1</td>
<td>India</td>
</tr>
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<td><em>E. coli</em> 5702</td>
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<td>United Kingdom</td>
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<td><em>E. coli</em> NCTC 10418</td>
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<td>Reference strain</td>
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<tr>
<td><em>E. coli</em> PN21</td>
<td><em>mcr-1</em></td>
<td>Thailand</td>
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<tr>
<td><em>E. coli</em> PN25</td>
<td><em>mcr-1</em></td>
<td>Thailand</td>
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<tr>
<td><em>E. coli</em> PN26</td>
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<td>Thailand</td>
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<tr>
<td><em>E. coli</em> ATCC 25922</td>
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<td>Reference strain</td>
</tr>
</tbody>
</table>

VIM-2, carbapenem-hydrolyzing metallo-β-lactamase; NDM-1, New Delhi metallo-β-lactamase; AIM-1, metallo-β-lactamase; MDR, multidrug resistant; *mcr-1*, plasmid-encoded colistin resistance.
Table S2. Physicochemical characteristics and batch details of OligoG-polymyxin conjugates used in this study.

<table>
<thead>
<tr>
<th>Tested Compound</th>
<th>Mw (g/mol) (PDI) by SEC-RI*</th>
<th>Mw (g/mol) (PDI) by SEC-MALS</th>
<th>Drug Content (% w/w)</th>
<th>Molar Ratio (per Colistin)</th>
<th>Conjugated NH2 per Molecule</th>
<th>Free Drug (%)</th>
<th>Experiments Performed</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Cytotoxicity</td>
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<td></td>
<td></td>
<td></td>
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<td>Antimicrobial Activity</td>
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<tr>
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<td>Growth Curves</td>
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<td></td>
<td>Biofilm Formation</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PK-PD</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>25,500 (2.6)</td>
<td>12,300 (1.4)</td>
<td>8.7</td>
<td>4.6</td>
<td>2.8</td>
<td>5.7</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>22,500 (2.3)</td>
<td>9100 (1.3)</td>
<td>8.8</td>
<td>4.6</td>
<td>2.7</td>
<td>1.5</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>24,500 (2.3)</td>
<td>8300 (1.3)</td>
<td>9.1</td>
<td>4.4</td>
<td>3.6</td>
<td>2.5</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>27,000 (2.5)</td>
<td>8200 (1.2)</td>
<td>12.5</td>
<td>3.1</td>
<td>3.3</td>
<td>2.4</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>24,500 (2.2)</td>
<td>8200 (1.3)</td>
<td>8.1</td>
<td>5.0</td>
<td>4.6</td>
<td>4.0</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-E-colistin</td>
<td>14,500 (2.3)</td>
<td>5900 (1.2)</td>
<td>12.9</td>
<td>3.0</td>
<td>N/A</td>
<td>2.0</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-E-colistin</td>
<td>16,500 (2.2)</td>
<td>5200 (1.3)</td>
<td>11.5</td>
<td>3.4</td>
<td>N/A</td>
<td>3.5</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-E-colistin</td>
<td>20,000 (3.1)</td>
<td>ND</td>
<td>8.3</td>
<td>4.9</td>
<td>N/A</td>
<td>2.7</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-A-polymyxin B</td>
<td>23,000 (2.7)</td>
<td>12,800 (1.5)</td>
<td>8.0</td>
<td>5.1</td>
<td>1.9</td>
<td>1.6</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-A-polymyxin B</td>
<td>23,500 (2.2)</td>
<td>9100 (1.3)</td>
<td>6.1</td>
<td>6.8</td>
<td>2.0</td>
<td>1.6</td>
<td>x</td>
</tr>
<tr>
<td>OligoG-E-polymyxin B</td>
<td>15,500 (2.1)</td>
<td>6200 (1.2)</td>
<td>7.0</td>
<td>5.9</td>
<td>N/A</td>
<td>2.7</td>
<td>x</td>
</tr>
</tbody>
</table>

Abbreviations: A, amide; E, ester; SEC-MALS, size exclusion chromatography with multi-angle light scattering detection; SEC-RI, size exclusion chromatography with refractive index detection; PDI, polydispersity index; ND, not determined; N/A, not applicable. *Molecular weight was estimated relative to pullulan standards.
### Table S3. Weight and number average molecular weights of OligoG and OligoG-colistin conjugates.

<table>
<thead>
<tr>
<th>Tested Compound</th>
<th>Drug Content (% w/w)</th>
<th>Mn (kDa)</th>
<th>Mw (kDa)</th>
<th>Molar Ratio (per Colistin)*</th>
<th>Mn (kDa)*</th>
<th>Mw (kDa)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OligoG</td>
<td>N/A</td>
<td>3.9</td>
<td>5.5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>9.1</td>
<td>6.6</td>
<td>8.3</td>
<td>2</td>
<td>4.3</td>
<td>8.3</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>12.5</td>
<td>6.6</td>
<td>8.2</td>
<td>1.4</td>
<td>4.4</td>
<td>9.4</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>8.1</td>
<td>6.4</td>
<td>8.2</td>
<td>2.5</td>
<td>4.2</td>
<td>8.0</td>
</tr>
<tr>
<td>OligoG-E-colistin</td>
<td>11.5</td>
<td>4.0</td>
<td>5.2</td>
<td>1.7</td>
<td>4.3</td>
<td>9.1</td>
</tr>
</tbody>
</table>

*Expected values assuming 100% conjugation calculated from the Mn and Mw values of OligoG and the % w/w of each sample.

Abbreviations: A, amide; E, ester.

### Table S4. Selectivity index (SI) values of OligoG-polymyxin conjugates against a range of Gram-negative bacterial pathogens.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>SI Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa R22</em></td>
<td>52</td>
</tr>
<tr>
<td><em>P. aeruginosa MDR 301</em></td>
<td>52</td>
</tr>
<tr>
<td><em>P. aeruginosa NH57388A</em></td>
<td>104</td>
</tr>
<tr>
<td><em>P. aeruginosa NCTC 10662</em></td>
<td>208</td>
</tr>
<tr>
<td><em>K. pneumoniae KP05 506</em></td>
<td>208</td>
</tr>
<tr>
<td><em>K. pneumoniae IR25</em></td>
<td>413</td>
</tr>
<tr>
<td><em>A. baumannii MDR ACB</em></td>
<td>52</td>
</tr>
<tr>
<td><em>A. baumannii 7789</em></td>
<td>104</td>
</tr>
<tr>
<td><em>E. coli AIM-1</em></td>
<td>208</td>
</tr>
<tr>
<td><em>E. coli IR57</em></td>
<td>839</td>
</tr>
<tr>
<td><em>E. coli 5702</em></td>
<td>208</td>
</tr>
<tr>
<td><em>E. coli NCTC 10418</em></td>
<td>3</td>
</tr>
<tr>
<td><em>E. coli PN21</em></td>
<td>3</td>
</tr>
<tr>
<td><em>E. coli PN25</em></td>
<td>208</td>
</tr>
<tr>
<td><em>E. coli PN26</em></td>
<td>104</td>
</tr>
<tr>
<td><em>E. coli ATCC 25922</em></td>
<td>52</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>184</strong></td>
</tr>
</tbody>
</table>

Selectivity index (SI) = IC_{50} (μg/mL)/MIC (μg/mL). Mean SI is shown in **bold**. Abbreviations: A, amide; E, ester.
Table S5. Microbiological efficacy (MICs) of OligoG-colistin conjugates in the presence of alginate lyase in Mueller–Hinton (MH) broth or after pre-incubation with alginate lyase against Gram-negative bacterial pathogens.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Tested Compound</th>
<th>MIC (µg/mL Drug Base) at Indicated Alginate Lyase Concentration (U/mL)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>0</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginate Lyase in MH Broth</td>
<td>0.25</td>
<td>0.125</td>
<td>0.063</td>
<td>0.125</td>
<td>0.063</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre-incubation with Alginate Lyase</td>
<td>0.25</td>
<td>0.125</td>
<td>0.063</td>
<td>0.125</td>
<td>0.063</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
</tr>
<tr>
<td>P. aeruginosa MDR 301</td>
<td>OligoG-A-Colistin</td>
<td>0.25</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae KP05 506</td>
<td>OligoG-A-Colistin</td>
<td>0.063</td>
<td>0.125</td>
<td>0.063</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli AIM-1</td>
<td>OligoG-A-Colistin</td>
<td>0.00002</td>
<td>0.0001</td>
<td>0.0002</td>
<td>0.0000008</td>
<td>0.0002</td>
<td>0.0002</td>
<td>0.00006</td>
<td>0.00006</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>A. baumannii 7789</td>
<td>OligoG-A-Colistin</td>
<td>0.063</td>
<td>0.063</td>
<td>0.008</td>
<td>0.125</td>
<td>0.125</td>
<td>0.002</td>
<td>0.004</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa MDR 301</td>
<td>OligoG-E-Colistin</td>
<td>0.25</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae KP05 506</td>
<td>OligoG-E-Colistin</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
<td>0.004</td>
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</tr>
<tr>
<td>E. coli AIM-1</td>
<td>OligoG-E-Colistin</td>
<td>0.00002</td>
<td>0.001</td>
<td>0.0002</td>
<td>0.0000008</td>
<td>0.001</td>
<td>0.001</td>
<td>0.00006</td>
<td>0.00006</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>A. baumannii 7789</td>
<td>OligoG-E-Colistin</td>
<td>0.063</td>
<td>0.016</td>
<td>0.004</td>
<td>0.125</td>
<td>0.063</td>
<td>0.002</td>
<td>0.002</td>
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<tr>
<td>Polymyxin B</td>
<td>Colistin Sulphate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
<td>0.004</td>
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</tr>
<tr>
<td>Polymyxin B</td>
<td>Colistin Sulphate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
<td>0.063</td>
<td>0.008</td>
<td>0.004</td>
<td>0.004</td>
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<td></td>
</tr>
</tbody>
</table>

Abbreviations: A, amide; E, ester.

Table S6. Microbiological efficacy (MICs) of polymyxins and antibiotic conjugates in the absence and presence of mucin against Gram-negative bacterial pathogens.

<table>
<thead>
<tr>
<th>Tested Compound</th>
<th>MIC (µg/mL Drug Base)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td></td>
<td>R22 MDR ACB IR25</td>
</tr>
<tr>
<td>OligoG-A-colistin</td>
<td>4  4  64</td>
</tr>
<tr>
<td>OligoG-E-colistin</td>
<td>1  16  64</td>
</tr>
<tr>
<td>0.2% OligoG + colistin</td>
<td>0.25  2  32</td>
</tr>
<tr>
<td>2% OligoG + colistin</td>
<td>1  4  32</td>
</tr>
<tr>
<td>Colistin sulphate</td>
<td>0.5  2  16</td>
</tr>
<tr>
<td>OligoG-E-polymyxin B</td>
<td>0.5  2  32</td>
</tr>
<tr>
<td>0.2% OligoG + polymyxin B</td>
<td>0.5  4  64</td>
</tr>
<tr>
<td>2% OligoG + polymyxin B</td>
<td>1  4  32</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0.25  4  64</td>
</tr>
</tbody>
</table>

Abbreviations: A, amide; E, ester; ND, not determined.
### Table S7. Comparison of the effect of growth medium (AS medium and MH broth) on antimicrobial activity (MIC determinations) of polymyxins and antibiotic conjugates.

<table>
<thead>
<tr>
<th>Tested compound ↓</th>
<th>Medium →</th>
<th>P. aeruginosa MDR 301 AS</th>
<th>P. aeruginosa NH57388A AS</th>
<th>K. pneumoniae KP05 506 AS</th>
<th>E. coli IR57 AS</th>
<th>E. coli 5702 AS</th>
<th>A. baumannii 7789 AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>OligoG-A-colistin</td>
<td>MH</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1.25</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>OligoG-E-colistin</td>
<td>MH</td>
<td>4</td>
<td>0.5</td>
<td>1</td>
<td>1.25</td>
<td>0.063</td>
<td>0.25</td>
</tr>
<tr>
<td>Colistin sulphate</td>
<td>MH</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>OligoG-A-polymyxin B</td>
<td>MH</td>
<td>16</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>OligoG-E-polymyxin B</td>
<td>MH</td>
<td>8</td>
<td>0.5</td>
<td>2</td>
<td>0.25</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>MH</td>
<td>4</td>
<td>0.5</td>
<td>1</td>
<td>0.25</td>
<td>0.125</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Abbreviations: A, amide; E, ester.

### Table S8. Fractional inhibitory concentration index (FICI) values of OligoG-colistin conjugates or colistin in combination with azithromycin dihydrate.

<table>
<thead>
<tr>
<th>Tested Compound</th>
<th>P. aeruginosa MDR 301</th>
<th>K. pneumoniae KP05 506</th>
<th>A. baumannii 7789</th>
<th>E. coli NCTC 10418</th>
</tr>
</thead>
<tbody>
<tr>
<td>OligoG-A-colistin + Azithromycin</td>
<td>1.35 (Additive)</td>
<td>3.15 (Indifferent)</td>
<td>3.40 (Indifferent)</td>
<td>0.64 (Additive)</td>
</tr>
<tr>
<td>OligoG-E-colistin + Azithromycin</td>
<td>1.45 (Additive)</td>
<td>2.43 (Indifferent)</td>
<td>2.53 (Indifferent)</td>
<td>0.46 (Synergy)</td>
</tr>
<tr>
<td>Colistin sulphate + Azithromycin</td>
<td>1.51 (Additive)</td>
<td>2.92 (Indifferent)</td>
<td>1.14 (Additive)</td>
<td>0.83 (Additive)</td>
</tr>
</tbody>
</table>