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Wound Biology Group, Tissue Engineering and Reparative Dentistry, Cardiff University School of Dentistry, Cardiff, United Kingdom; SINTEF Materials and Chemistry, Trondheim, Norway; AlgiPharma AS, Sandvika, Norway; Microbiology Cardiff (Velindre NHS Trust), UHW, Heath Park, Cardiff, United Kingdom; and Department of Medical Microbiology, School of Medicine, Cardiff University, Heath Park, Cardiff, United Kingdom

The uncontrolled, often inappropriate use of antibiotics has resulted in the increasing prevalence of antibiotic-resistant pathogens, with major cost implications for both United States and European health care systems. We describe the utilization of a low-molecular-weight oligosaccharide nanomedicine (OligoG), based on the biopolymer alginate, which is able to perturb multidrug-resistant (MDR) bacteria by modulating biofilm formation and persistence and reducing resistance to antibiotic treatment, as evident using conventional and robotic MIC screening and microscopic analyses of biofilm structure. OligoG increased (up to 512-fold) the efficacy of conventional antibiotics against important MDR pathogens, including *Pseudomonas*, *Acinetobacter*, and *Burkholderia* spp., appearing to be effective with several classes of antibiotic (i.e., macrolides, β-lactams, and tetracyclines). Using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM), increasing concentrations (2%, 6%, and 10%) of alginate oligomer were shown to have a direct effect on the quality of the biofilms produced and on the health of the cells within that biofilm. Biofilm growth was visibly weakened in the presence of 10% OligoG, as seen by decreased biomass and increased intercellular spaces, with the bacterial cells themselves becoming distorted and uneven due to apparently damaged cell membranes. This report demonstrates the feasibility of reducing the tolerance of wound biofilms to antibiotics with the use of specific alginate preparations.

The evolution of multidrug resistance (MDR) represents an increasingly formidable challenge in the developed and developing worlds, having a colossal clinical and economic impact (26). This escalating problem is a major world-health issue, compounded by the lack of new antibiotics in development, particularly for Gram-negative MDR bacteria. The need for new strategies to improve the diagnosis and treatment of these infections is, therefore, acute (15, 16).

*In vivo*, the inability to eradicate bacteria from a diverse number of clinical situations ranging from the periodontal pocket to the cystic fibrosis (CF) lung has been attributed to the acquisition of a biofilm state (12, 13). Antimicrobial resistance can also arise by a plethora of mechanisms involving chromosomal mutations or horizontal gene transfer. Resistance may result from destruction of the antibiotic by, e.g., β-lactamases, typified by the recently described New-Delhi metallo-β-lactamase-1 (NDM-1) (21); mutations in specific antibiotic target sites, e.g., RNA polymerase and DNA gyrase (22); limitation of local antibiotic concentrations due to outer membrane lipid bilayer modifications or porin-mediated permeability (5); the action of specific multidrug efflux pump mechanisms, e.g., MexAB-OprM (28); acquisition of resistance genes such as meca in methicillin-resistant *Staphylococcus aureus* (MRSA) (22); or the sequestration of antibiotics within the bacterial biofilm (25).

Attempts to develop (and screen) alternative antibacterial strategies to potentiate the activity of conventional antibiotics are becoming increasingly important. Antibacterial strategies have included use of antimicrobial cationic peptides (AMPs) (7); cell membrane permeabilization (7); molecular chaperones (e.g., heat shock proteins) (4); inhibitors of RecA-mediated strand exchange (34); inhibitors of DNA synthesis (6); inhibitors of penicillin-binding protein production (18); and use of efflux pump inhibitors or inhibitors of beta-lactamase expression (27). Despite almost 20 years of intense research, none of these agents deemed useful *in vitro* have progressed to clinical use.

Alginate is a biopolymer which is found in brown algae and bacterial extracellular polysaccharide (EPS) and is a linear polymer composed of (1→4)-linked α-L-guluronate (G) and β-D-mannurionate (M) residues. It is extensively utilized in foods and medicines (e.g., wound dressings) due to its physical cross-linking and gel-forming properties (30). Sodium alginate is recognized as safe by the U.S. Food and Drug Administration (reference no. 21CFR184.1724). While naturally occurring alginate is polydispersed in relation to molecular weight and G/M composition, it is possible to generate polymers of defined molecular weight and also G/M composition (11), which have been employed to modulate immunological responses *in vivo* (35). Previous workers have highlighted the potential of nanoscale biosensor-based diagnostics for the detection of life-threatening bacterial infections (16). We describe here the development of the first polymer therapeutics (based on alginate oligosaccharides) designed to specifically modulate bacterial responses and treat infections of MDR bacteria, with phase IIA human clinical studies under way.
V1  Pseudomonas aeruginosa (R22)  VIM-2, aac(6′)Ib, aadA256  China
V2  Pseudomonas aeruginosa (301)  VIM-2b,c  Poland
V13 Pseudomonas aeruginosa (PAO1, ATCC 15692)  VIM-2b,c  Australia
V79 Pseudomonas aeruginosa (ATCC 39324)  —  United States
V33 Burkholderia cepacia (ATCC 25416)  —  United States
V51 Burkholderia contaminans (LMG 23255)  —  Czech Republic
V92 Burkholderia dolosa (ATCC 10154)  —  United States
V19 Acinetobacter baumannii  —b,c  United Kingdom
V9 Acinetobacter baumannii  VIM-2b,c  Egypt
V4 Acinetobacter baumannii  —b,c  Libya
V22 Acinetobacter h腹fibii  NDM-1b,c  United Kingdom
V10 Acinetobacter h腹fibii  VIM-2b,c  Tunisia
V3 Klebsiella pneumoniae  NDM-1b,c  India
V6 Klebsiella pneumoniae  NDM-1b,c  India
V5 Escherichia coli  AIM-1b,c  Australia
V7 Escherichia coli  NDM-1b,c  India
U204 Staphylococcus aureus NCIMB 9518  —  United States
E68 Staphylococcus aureus NCTC 6571  —  United Kingdom
U50 Staphylococcus aureus 1040 (MRSA)  mecA  United Kingdom
V17 Streptococcus oralis 5610  —  United Kingdom

a AIM-1, metallo-β-lactamase; NDM-1, New Delhi metallo-β-lactamase; MRSA, methicillin-resistant Staphylococcus aureus.
b Sensitive to colistin only.
c MDR, multidrug resistant (resistant to 3 or more classes of antibiotic).

Alginic oligosaccharides of similar molecular weights but altered M content were generated via epimerisation technology and also tested. M-rich alginates (pure mannuronan) from a nonpathogenic high-expressing mutant (P. fluorescens NCIMB10525) were used as a substrate for mannuronan C-5-epimerase AlgE4, converting alternating M residues on the alginic backbone into G residues. These were developed with similar molecular-weight profiles and defined, based on G:M composition, as OligoM (100% M; 3,000 to 3,500 Mw) (11) and OligoMG (46% G; 4,000 to 4,500 Mw) (3).

Effects of OligoG on bacterial growth in liquid culture. The effects of alginic oligosaccharide OligoG on a collection of clinically relevant human Gram-negative and Gram-positive MDR and non-MDR pathogens (Table 1) were studied using robotic high-throughput screening (HTS; Beckman-Coulter) and conventional screening and a range (0% to 10%) of OligoG concentrations. Overnight (O/N) bacterial cultures grown in tryptone soya broth (TSB) were inoculated into Mueller-Hinton (MH) broth with increasing concentrations (0%, 2%, 6%, and 10%) of OligoG. After cultivation for 19 h, cell densities were determined by measuring optical density at 600 nm (OD600). The OligoG-induced reduction in optical density observed in these assays correlates directly with reduced bacterial dry weight/biomass (data not shown).

Growth of biofilms for SEM imaging or LIVE/DEAD staining. The ability of OligoG to modulate the development of biofilms as well as their effect on 24-h biofilms in vitro was investigated. Overnight cultures of P. aeruginosa PAO1 were grown in TSB. After dilution of the bacterial cultures to 0.5 McFarland in MH broth with mucin (2.5 g/liter), 1 ml was transferred to the wells of a flat-bottom 12-well plate containing sterile plastic Thermaxov coverslips (Agraf Scientific, Essex, United Kingdom) for scanning electron microscopy (SEM) or glass coverslips (Fisher Scientific) for confocal laser scanning microscopy (CLSM). Plates were then wrapped in parafilm to prevent dehydration and incubated at 37°C for 6 h to allow biofilm formation. Planktonic cells and supernatant were removed and biofilms washed with 1 ml sterile PBS. Cells then were treated with a combination of OligoG (0%, 2%, 6%, and 10%) and mucin (2.5 g/liter) in 1 ml MH broth. Plates were then wrapped in parafilm and incubated at 37°C for 24 h with gentle agitation.

### MATERIALS AND METHODS

#### Bacterial strains. The strains used for susceptibility testing are shown in Table 1. They include both culture collection strains and clinical isolates: Pseudomonas aeruginosa (n = 4), Burkholderia spp. (n = 4), Acinetobacter spp. (n = 4), Enterobacteriaceae spp. (n = 4), Staphylococcus aureus (n = 3), and Streptococcus oralis (n = 1). These isolates represent the most frequently encountered resistance types (aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, monobactams, and penicillins).

#### Bacterial media and culture conditions. Bacterial colonies were grown on blood agar base no. 2 (BA; LabM) supplemented with 5% horse blood and tryptone soya broth (TSB; Oxoid) for liquid cultures. Biofilms were generated in cation-adjusted Mueller-Hinton (MH) broth (LabM). Biofilms were washed with phosphate-buffered saline (PBS). All antibiotics (Sigma-Aldrich, Gillingham, United Kingdom, or Bristol-Myers Squibb Pharmaceuticals Ltd.) used were pharmaceutical grade and included the major classes of antibiotics (β-lactams, aminoglycosides, macrolides, tetracyclines, carbapenems, and polymyxins) employed in the treatment of these organisms. Ultrapurified pig gastric mucin glycoprotein (purified by Jeff Pearson, Newcastle University) and OligoG (2,600 Mw) provided by AlgiPharma AS, Sandvika, Norway.

#### Synthesis of alginic oligosaccharides. OligoG was generated from alginate extracted from the stem of the locally sourced brown seaweed Laminaria hyperborea. Purification and fractionation of the resulting material yielded an oligomer with a high content of guluronate and a relatively narrow molecular-weight distribution. Final purification using charcoal filters was followed by spray drying. The test OligoG had 90% to 95% of the monomer residues as G residues (mean, 2,600 Mw; Fig. 1A). Purified OligoG was characterized by hydrogen-1 nuclear magnetic resonance (H-NMR) (Fig. 1B) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Fig. 1C). OligoG is anionic and highly soluble, although its solubility is limited by viscosity at higher concentrations (>15%), the rise in viscosity being offset to a degree by an increase in temperature. OligoG was tested at working concentrations of 2% to 10%, phase 1 studies having demonstrated that these concentrations are safe for clinical use (clinical phase 1 safety and toxicity studies; www.clinicaltrials.gov [identifier, NCT00970346]).
SEM of OligoG-treated Pseudomonas biofilms. Glutaraldehyde was added to OligoG-treated biofilms at a final concentration of 1.25% and fixed at room temperature for 24 h. The samples were dehydrated in a graded series of ethanol concentrations, dried in a critical point dryer (CPD 030; Balzers, Germany), mounted on aluminum stubs, coated with gold in a sputter-coater (model AE 1231; EMscope, United Kingdom), and then viewed on a scanning electron microscope (XL-20; FEI-Philips, The Netherlands).

LIVE/DEAD staining and CLSM of OligoG-treated biofilms. The effect of OligoG on cell membrane integrity (a measure of cell death) was investigated using LIVE/DEAD BacLight stain (bacterial viability kit; Invitrogen). A 2-μl volume of each stain (green fluorescent SYTO9 and red-fluorescent propidium iodide) was added to 1 ml NaCl (0.85% [wt/vol]) and mixed and 100 μl added to each test sample. The preparation was incubated in the dark for 15 min and then analyzed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany).

OligoG-treated gfp-labeled Pseudomonas aeruginosa PAO1 biofilms and staining of biofilm matrix for CLSM. A gfp (green fluorescent protein)-labeled P. aeruginosa PAO1 derivative (20) was also used for CLSM. Overnight cultures of gfp-PAO1 were grown in TSB, and 10 μl was used to inoculate 1 ml MH broth with or without 2%, 6%, or 10% OligoG. Then, 200 μl of each was transferred to individual Coverwell incubation chamber gaskets (Invitrogen) with the addition of 40 μl BODIPY 630/650-X SE stain (Invitrogen), which stains extracellular polysaccharide (EPS) matrix components. The chamber gaskets were then sealed with a Histobond (R. A. Lamb Ltd., Eastbourne, United Kingdom) positively charged slide. Each unit was inverted and incubated for 18 h at 37°C to allow biofilm formation on the Histobond slide. Coverwell incubation chamber gaskets and any supernatant and planktonic cells were removed from the Histobond slide and replaced with a glass coverslip. Undisturbed biofilms were then visualized by CLSM as described above.

Susceptibility testing by MIC assay. Potentiation of alginate oligomers was studied using standard MICs (19) and confirmed by robotic HTS using a range of international MDR (i.e., resistant to 3 or more classes of antibiotics) and non-MDR Gram-negative and Gram-positive clinical isolates (Table 1). Isolates were grown overnight in TSB and then diluted in PBS until the OD625 was between 0.08 and 0.10 (equivalent to 0.5 McFarland standard; approximately 10⁶ CFU/ml). Two-fold antibiotic serial dilutions were prepared in MH broth or MH broth with OligoG at 2%, 6%, or 10% in flat-bottom 96-well microtiter plates (100 μl in each well). The diluted bacterial cultures were diluted 10-fold in MH broth, and 5 μl was added to the microtiter plates containing the antibiotic serial dilutions to give a final concentration of 5 × 10⁸ CFU/ml. Plates were incubated at 37°C for 16 to 20 h and MICs determined as the lowest concentration at which there was no visible growth. As well as conventional culture, a robotic high-throughput screening system (see below) was used to determine MICs.

Robotic HTS. Serial dilutions for MIC determinations were performed with a Tecan Genesis RSP 200 liquid-handling workstation equipped with an 8-channel pipetting tool using sterile disposable 200-μl barrier tips. The optical density of the cultures was measured using a Beckman Coulter Robotic Core system with an integrated Beckman Coulter Biomek NX³ robotic liquid-handling unit, a Thermo Cytomat 2 450 S robotic incubator, and a Beckman Coulter Paradigm microplate reader. The 384-well microplates were incubated at 37°C for 19 h. The microplates were shaken at 1,800 rpm (2.5-mm amplitude) for 120 s prior to taking absorbance readings at 600 nm. HTS was used for determining MICs.
Effect of OligoG on bacterial motility. Two methods of motility testing were used. For the plate assay, overnight (O/N) cultures of *Proteus mirabilis* (NSM6) and *Pseudomonas aeruginosa* (PAO1) were grown in TSB at 37°C. Cultures were diluted 1:100 in MH broth supplemented with 0%, 0.2%, 0.5%, 2%, 6%, or 10% OligoG and reincubated for 18 h at 37°C. ISO-sensitest (ISO) agar plates (Oxoid) containing 0%, 0.5%, 2%, or 6% OligoG were prepared and inoculated with 10 μl of the MH cultures. (ISO plates with 10% OligoG were not used, as they did not set at that concentration.) Plates were incubated for 23 h at 37°C and values for distance of bacterial spread recorded at 2, 5, 7, 13, and 23 h.

For the stab assay, overnight cultures of *P. mirabilis* (NSM6) and *P. aeruginosa* PAO1 and control isolates of *P. mirabilis* (NCTC 11938), *Staphylococcus aureus* (NCTC 6571), *P. aeruginosa* (NCTC 10662), and *Escherichia coli* (NCTC 10418) were prepared in MH broth with or without OligoG as described above. Motility Test Agar (MTA; Mast Group Ltd., Bootle, United Kingdom) was supplemented with 0%, 0.2%, 0.5%, 2%, and 6% OligoG, with 5 ml pipetted into sterile bijou tubes. (MTA containing 10% OligoG also did not set.) The MTA was stab inoculated with the prepared MH broth cultures and incubated for 24 h at 37°C. Motility appeared as a progressive lateral diffuse pink/red color in the MTA and was scored from 0 to 4 as previously described (2). Absence of growth beyond the track of inoculation was scored as 0, and growth throughout the medium was scored as 4.

Testing development of resistance to OligoG. Daily subculturing of *P. aeruginosa* PAO1 in MH broth or MH broth with 10% OligoG was carried out over a 16-day period (>160 generations). Serial passage in escalating concentrations (0.1%, 1%, and 10%) of OligoG was also undertaken (n = 7 days for each concentration). MIC assays of the control and test cultures (on day 16 and day 21, respectively) were then performed to see if there were any apparent differences.

RESULTS
Effects of OligoG on growth of MDR bacteria. OligoG was found to reduce bacterial proliferation in liquid culture at 19 h (Fig. 2). This effect was dose dependent for the concentration range investigated (0%, 2%, 6%, and 10%), showing a general decrease in percent change (compared to the control) with increasing OligoG concentration. These effects were particularly striking for the Gram-negative pathogens *P. aeruginosa*, *A. baumannii* and *Burkholderia spp.*, and *Enterobacteriaceae*.

Effects of OligoG on biofilms generated by MDR Gram-negative bacteria examined using conventional light, scanning electron, and confocal laser scanning microscopy. SEM, LIVE/DEAD BacLight staining of PAO1 biofilms, and CLSM of gfp-labeled PAO1 biofilms generated in the presence of OligoG revealed dose-related changes (Fig. 3). Increasing concentrations (0% to 10%) of OligoG resulted in disruption of established 24-h biofilms (demonstrable by cell damage and disruption of cellular content as visualized by SEM), increased cell death (LIVE/DEAD staining), and decreased cellular density (CLSM) in the developed biofilm. CLSM showed that OligoG was able to considerably reduce gfp-labeled PAO1 biofilm formation, with clear dose-dependent alterations in the 3-dimensional structural organization of cells in the biofilm. These changes were also associated with in-
creasing numbers of nonviable bacteria seen by LIVE/DEAD staining.

The ability of alginate oligomers to potentiate the effect of existing antibiotics on Gram-negative MDR pathogens. The ability of alginate oligomers to potentiate the activity of the principal classes of antibiotics commonly employed in clinical practice in the treatment of Gram-negative infections (cephalosporins, macrolides, quinolones, tetracyclines, and carbapenems) was assessed, with selected results for three antibiotic classes shown in Table 2. OligoG treatment reduced (by up to 512-fold) the MICs of a range of antibiotics, including macrolides (azithromycin, erythromycin, clarithromycin), aztreonam, cefazidime, oxytetracycline, and ciprofloxacin. For example, *P. aeruginosa* V2 showed a ceftazidime MIC reduction from 16 to 0.5 µg/ml, representing a change from untreatable (>8 µg/ml) to treatable (<8 µg/ml) levels (0% and 10% OligoG, respectively; Table 2). This potentiation effect was particularly marked for the genus *Burkholderia*. Interestingly, while this potentiation was not seen in the control OligoMG oligomer, a similar, although smaller, effect (up to 32-fold reduction for *P. aeruginosa* and *A. baumannii*; data not shown) was evident in part for OligoM. Specific antibiotic combination effects (using two antibiotics) were also particularly evident (data not shown); for example, using *A. baumannii* and cefazidime or ciprofloxacin with azithromycin at 1 µg/ml and 10% OligoG resulted in a reduction in the MIC of these combination antibiotics to <1 µg/ml or <0.25 µg/ml, respectively (representing a >512-fold reduction). Similar potentiation, although a lesser effect, was observed with Gram-positive isolates (Table 2). OligoG-antibiotic synergy was not evident for every combination studied. Moreover, no potentiation was seen for the aminoglycoside (tobramycin and amikacin) or the polymyxin (colistin) antibiotics for any bacteria tested.

Mechanistic cellular and molecular studies to determine the mode of action of OligoG. The addition of OligoG appeared to modulate cellular structure. However, this did not appear to correlate with increased bacterial cell membrane permeability, as the effect contrasted with that observed with an EDTA control (data not shown). Longitudinal studies employing *P. aeruginosa* PAO1 MexAB-OrpM mutants also demonstrated that the effect of OligoG was not mediated via interaction with this efflux pump system, as no differences in MIC determinations with OligoG were noted using wild-type strains or knockout or hyperexpression mutants of MexAB-OrpM, showing that OligoG was not a substrate of MexAB-OrpM (data not shown). Interestingly, motility test agar stabs showed that OligoG (6%) was able to almost completely inhibit motility in *P. aeruginosa*, *E. coli*, and *P. mirabilis* (Fig. 4A). Furthermore, swarming motility in *Proteus* sp. was inhibited in a dose-dependent manner with increasing concentrations of OligoG (0% to 6%), but the effect was predominant in the presence of OligoG (Fig. 4B). Importantly, when the bacteria were exposed to OligoG only in the initial liquid growth medium (and not in the agar plate onto which they were then inoculated), the
AZM, azithromycin; ATM, aztreonam; CAZ, ceftazidime; ERY, erythromycin; oxy-TET, oxytetracycline. Shaded area represents potentiation of antibiotic with increasing OligoG concentration.

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<th>Isolate</th>
<th>AZM (μg/ml)</th>
<th>ATM (μg/ml)</th>
<th>CAZ (μg/ml)</th>
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<th>oxy-TET (μg/ml)</th>
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**DISCUSSION**

The functional properties of alginate are strongly correlated with their MIC ratios and with their ability to potentiate antibiotic activity. Alginate disruption results in a slight "carryover" effect lasting for at least 48 h after complete loss of biofilm formation. This effect of OligoG was almost but not completely lost showing drug-induced resistance. This is consistent with the observations of other studies that have shown an increase in bacterial sensitivity to antibiotics following treatment with OligoG, which may be due to the modulation of bacterial membrane constituents. Antimicrobial agents have been developed as antibiofilm therapies (23). However, unlike alginate, which is an important component of the EPS of certain bacterial biofilms and has been previously described and is a recognized mechanism by which the activity of antibiotics is enhanced, the effect of OligoG on antibiotic activity is still unclear. The exact mechanism by which OligoG exerts its effect could simply have been the result of permeabilization of the bacterial membrane, which is the mode of action of a number of antibiotics. However, OligoG treatment (both alone and in combination with antibiotics) clearly altered the bacterial membrane and in combination with antibiotics. Membrane disruption by OligoG treatment causes the disruption of the bacterial membrane and leads to an increase in bacterial sensitivity to antibiotics. This membrane disruption was characterized by an increase in membrane fluidity, as well as an increase in membrane permeability, which is consistent with previous studies showing that OligoG treatment causes the disruption of the bacterial membrane and leads to an increase in bacterial sensitivity to antibiotics. The magnitude of the effect of OligoG treatment was associated with increased cellular "clumping" and disruption of cellular contents. Hence, the mechanism by which OligoG exerts its effect could be the result of permeabilization of the bacterial membrane, which is the mode of action of a number of antibiotics. However, further studies are needed to determine the exact mechanism by which OligoG exerts its effect on bacterial membranes.
eration of lethal reactive oxygen species and nitric oxide in the bacterial membrane). The observation of the putative, but as-yet-unidentified, membrane effect seen with OligoG is supported by the observed failure to develop resistance during prolonged treatment in longitudinal experiments. Weakened swarming motility has been shown to lead to an impaired ability to form simple biofilms (29). The impairment of bacterial motility by OligoG may, at least in part, explain its mode of action in motile bacteria. The finding that OligoG exhibits activity against a number of non-motile bacterial species indicates, however, that other mechanisms are undoubtedly involved.

The potentiation of antibiotic therapy in the treatment of MDR organisms in persistent infections is a major goal of anti-infective therapy (17). Indeed, synergistic combined therapies appear to have increasing potential for future antibacterial therapies (1). In characterizing the ability of OligoG to potentiate the activity of conventional antibiotics against MDR organisms, we utilized conventional microbiological MIC assays. These experiments demonstrated that exposure to bacterial motility by OligoG may, at least in part, explain its mode of action in motile bacteria. The finding that OligoG exhibits activity against a number of non-motile bacterial species indicates, however, that other mechanisms are undoubtedly involved.

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demonstrate that the putative doses at which OligoG is effective \textit{in vitro} are safely attainable in the lung \textit{in vivo}.

OligoG represents a novel target nanomedicine for future clinical use. Its suitability for formulation in isotonic solution and safety-pharmaco kinetic profiles all favor human use. Furthermore, phase IIa human clinical studies in CF patients are now ongoing.

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**REFERENCES**


