Drugs of the Future 2010, 35(12): 1007-1014
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CCC: 0377-8282/2010
DOI: 10.1358/dof.2010.35.12.1537937

REVIEW ARTICLE

TARGETING THE *PSEUDOMONAS AERUGINOSA* BIOFILM TO COMBAT INFECTIONS IN PATIENTS WITH CYSTIC FIBROSIS

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SUMMARY

Patients with cystic fibrosis are susceptible to lung infection with the bacterium Pseudomonas aeruginosa. Chronic infection cannot be eradicated and leads to lung destruction and an increased mortality. Treatment of infections caused by P. aeruginosa is made challenging by the organism's heterogeneity, adaptability, antibiotic resistance and the significant antibiotic tolerance it achieves from existing within biofilms. Biofilms are composed of aggregates of bacteria living and communicating within an extracellular matrix, often within mucus plugs. The use of conventional antibiotics against this bacterium is often ineffective and the conventional antibiotic pipeline is running dry, and so novel strategies are required. Planktonic free-swimming bacteria are significantly more susceptible to antibiotics compared to those living within the biofilm. Candidate strategies that target the biofilm, prompting its dispersal or increasing the susceptibility of the bacteria within to antibiotics, may therefore yield new opportunities to treat these destructive infections.

BACKGROUND

Pseudomonas aeruginosa is a ubiquitous environmental organism, responsible for invasive infection in susceptible patients. Such patients include those with chronic lung conditions such as cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary disease; individuals who are immunosuppressed; those with indwelling catheters, and those receiving intensive care (1).

Once a suitable niche is colonized, this supremely adaptable organism may adjust to the local conditions and alter the immediate environment to promote its own survival (2). For those with CF, early respiratory infection with *P. aeruginosa* may be eradicated with the prompt and aggressive use of antibiotics. However, most individuals with CF will develop chronic pulmonary infection with *P. aeruginosa* (3). Chronic infection is associated with a more rapid decline in lung function, quality of life and subsequently an increased mortality (4). Inevitably, as the number of antibiotic courses to which the patient is exposed increases, the bacteria respond to selective pressure and develop antibiotic resistance (5).

CHALLENGES POSED BY *P. AERUGINOSA* AND ITS BEHAVIOR

The ability of *P. aeruginosa* to survive in the susceptible host is enhanced by its hypermutability (6), heterogeneity in displaying diverse phenotypes that change over time (7) and the protection the organism gains by the biofilm mode of growth (8). There is burgeoning evidence to suggest that, where *P. aeruginosa* causes chronic pulmonary infection in CF, it can exist as a biofilm. When sputum from patients with CF has been examined using electron microscopy, clusters of *P. aeruginosa* have been observed within a densely stained matrix –structures resembling a biofilm (9). The quorum-sensing (QS) molecules, which coordinate biofilm formation, can also be detected in sputum (9). More recently, Bjarnsholt et al. described aggregates of bacteria within mucus plugs in the explanted lungs of patients undergoing lung transplantation (10). Finally, the biofilm mode of growth would explain the lack of a direct relationship between the results of in vitro antibiotic susceptibility testing and the observed clinical response (11).

However, the variability of the CF phenotype (12), the changing characteristics of the bacterial community over time (7, 13) and difficulties in obtaining a bacterial sample representative of those present in the lungs (14) add a level of complexity, such that the behavior of the bacteria in an individual patient remains uncertain.

Clinical isolates of *P. aeruginosa* from these patients grow two to three times slower than reference laboratory strains (15). Slow

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growth may be a protective mechanism against antibiotics that are active against dividing cells (16). The antibiotic susceptibility of bacteria housed within biofilms is significantly less (over 100-fold higher minimum inhibitory concentration [MIC] required [17]) than planktonic cells (8). This may result from a number of factors, including the inhibition of diffusion, or consumption of antibiotics within the biofilm (18); anaerobic conditions deep within the matrix (19); and slow growth in the biofilm. Persister cells may also contribute –instead of replicating like wild-type bacteria, persisters remain dormant and exhibit a decreased antibiotic susceptibility. Upon cessation of antibiotic administration, they are able to repopulate the biofilm (20).

Biofilm production and the biofilm mode of growth offer the bacterium an advantage over the host immune system and the administration of antibiotics. Unfortunately, the antibiotic development pipeline for agents active against *P. aeruginosa* is running dry (21). The successful management of chronic *P. aeruginosa* infection may therefore rely on antibiotic adjuvants –agents that act by rendering the organism more susceptible to attack by antibiotics or the host immune system, by rendering it less virulent or killing it by other means (22).

BIOFILM THERAPEUTIC TARGETS

Putting the diversity and heterogeneity of *P. aeruginosa* itself aside, the biofilms produced by these bacteria are complex structures. The biofilm is comprised of bacteria linked together by cell-surface lectins (23, 24), encapsulated within an extracellular matrix (25) containing DNA (26), and extracellular polysaccharides such as alginate (27), psl and pel (28). The bacteria within produce diffusible factors such as rhamnolipid, which ameliorates attack by the host immune system (29). The organisms also export toxic substances such as antibiotics from within cells via efflux pumps (30). They also serve as reservoirs of active planktonic cells that are released to the environment (31). Each component of the biofilm assembly may act as a future therapeutic target (Table I). Furthermore, resistance factors operating at the level of the individual organism may be targeted by new therapeutics (Table II).

Biofilm-specific antibiotic susceptibility testing

Traditional antibiotic susceptibility testing has assessed the effect of individual antibiotics in a broth culture of the organism isolated from the patient. This model is distant from the in vivo situation for patients with CF, who are routinely treated with a combination of two antibiotics (32) and in whom the bacteria exist in both planktonic and biofilm forms (33). A randomized controlled trial of the use of combination antibiotic susceptibility testing compared against standard susceptibility testing to direct antibiotic choice for the treatment of a pulmonary exacerbation was undertaken to determine if in vitro combination testing conferred a treatment benefit (11). There was no difference observed between the two groups. However, given the reduced antibiotic susceptibilities of bacteria grown in biofilms compared to planktonic culture, even in the presence of antibiotic combinations (8), this is not surprising.

The effect of combinations of antibiotics on the *P. aeruginosa* biofilm have been examined using combinations of antibiotics at concentrations achievable in the human lung. The most effective combination (nebulized tobramycin and meropenem) only inhibits the growth of 56% of isolates (34). These results are not unexpected, as only 76% of patients have a reduction in bacterial burden as a consequence of conventional antibiotic therapy at the end of a treatment course (35). In another study, the starkest effect of biofilm antibiotic susceptibility testing was identified with the common combination of ceftazidime and tobramycin. With conventional broth culture testing, 62.5% of isolates were susceptible compared to only 6.3% of isolates when grown in biofilms (36).

When assessing triple antibiotic combinations, the maximum effect only inhibited growth of 68% of the isolates (tobramycin, meropenem and ceftazidime). In addition, more is not always better, with the addition of azithromycin to previously susceptible dual combinations being antagonistic in 52% of cases (34). A clinical study is currently recruiting to assess the efficacy of a biofilm antimicrobial susceptibility assay for the treatment of pulmonary exacerbations for those with chronic *P. aeruginosa* infection in CF (37).

Table I. *Potential strategies to overcome Pseudomonas aeruginosa resistance at the biofilm level.*

Biofilm target	Potential strategy	Stage of development	Ref.
Antibiotic tolerance	Biofilm antibiotic susceptibility testing	Randomized, double-blind, controlled trial recruiting – due to complete May 2012	37
Increased biofilm penetration by antibiotic	Liposomal amikacin	Phase II blinded, placebo-controlled trial	92
Alginate in biofilm	Alginate lyase Alginate-derived oligomers	In vitro studies Phase I study	41-43 45
Quorum sensing	Quorum-sensing inhibitors, e.g., garlic	Pilot randomized, double-blind, controlled trial completed In vitro studies	54-56
Cell-surface lectins	Carbohydrate competitive inhibitors	Pilot safety study completed In vitro studies	59, 62
Biofilm formation	*N*-Acetylcysteine	In vitro studies regarding biofilm formation inhibition Phase IV nonrandomized, controlled trial planned	66, 68
Bacterial predation	Bacteriophages	Randomized, controlled trial completed (chronic otitis) Routine use trial – convenience sample recruiting	97, 98

Table II. *Potential strategies to overcome Pseudomonas aeruginosa resistance at the level of the individual organism.*

Cellular target	Potential strategy	Stage of development	Ref.
Enzymatic breakdown of antibiotics, e.g., β-lactamase	Antipseudomonal antibiotic combined with β-lactamase inhibitor, e.g., ceftazidime plus NXL-104	Phase II study completed (intra-abdominal infections) Phase II study ongoing (complicated urinary tract infection)	79, 99
Efflux pumps	Efflux pump inhibitors, e.g., MC-207110	Pharmacokinetic studies	73
Bacterial competition	Probiotics, e.g., *Lactobacillus casei rhamnosus*	Pilot randomized, double-blind, controlled trial completed	93

Alginate

Alginate overproduction appears to be responsible for much of the added tolerance and resistance to antibiotics that accompany the mucoid phenotype (38). However, while alginate is produced by both mucoid and nonmucoid bacteria (39), it is itself not essential for biofilm production (40).

In vitro studies have demonstrated that alginate lyase may increase cell detachment from an experimental nonbiological surface (41), promote diffusion of aminoglycosides through the extracellular matrix (42) and enhance antibiotic killing of mucoid *P. aeruginosa* (43). Clinical trials, however, have not been forthcoming. Bacteriophages may also be a delivery vector for alginate degradation, with one phage demonstrating in vitro degradation by hydrolysis, reducing the viscosity of alginate (44).

Alginate-derived oligomers are currently being investigated and a phase II trial is planned, with one product, OLigoG CF-5/20, having been granted Orphan Medicinal Product registration from the EU Commission and European Medicines Agency (45). It is reported that such oligomers "may interact with the extracellular polymers of the biofilm, and thereby weaken it, enabling or facilitating its removal or breakdown (or disruption), and/or facilitating the access of antimicrobial agents to the biofilm, thereby enhancing their efficacy against the biofilm" (46).

Quorum sensing

Biofilm formation is under QS regulatory control (47). Signal molecules are produced by each bacterial cell within a colony, the amount of signal in the environment being directly proportional to the number of cells producing the signal. Once the signal levels around and within the cells reach a threshold, virulence gene expression is upregulated. QS regulation of gene products is integral to the virulence of *P. aeruginosa* and bacteria within biofilms express a typical QS signal pattern (9). The production of alginate (48), rhamnolipid (29), elastase (49) and lectins (50), among many others, is regulated by QS. QS-proficient isolates are significantly more virulent and are also more likely to induce ventilator-associated pneumonia in those not affected by CF (51). As the infection progresses with age, QS-negative mutants may become more prominent, reducing their virulence and increasing the diversity (13), and reducing the virulence of the infecting bacterial communities (52).

QS inhibitors have been demonstrated to alter the architecture of the biofilm (53), with one such candidate, garlic, increasing the susceptibility of biofilms to antibiotics. In vitro biofilms grown in the presence of garlic were more susceptible to tobramycin, and survival rates of animals improved when they were pretreated with garlic in a mouse model of *P. aeruginosa* lung infection (54). The QS-inhibitory potential of garlic is conserved throughout a number of clinical isolates (55). A pilot randomized controlled trial of a garlic supplement demonstrated a nonsignificant improvement in clinical parameters, including lung function (FEV_1), clinical score and weight. The authors were also able to detect QS molecules in the plasma and sputa of these patients, indicating QS activity (56).

Lectin binding

There are two subtypes of lectin expressed on the cell surface of *P. aeruginosa*: lecA and lecB. Both are involved in bacterial aggregation, linking bacterial cells together to form a cohesive colony (23, 24). There is also evidence to suggest that lectins are involved in adherence to host cells (57) and can reduce epithelial ciliary beating (58). The lectin-inhibiting carbohydrates galactose (lecA) and fucose (lecB), and dendrimers thereof, may inhibit biofilm formation, prompt biofilm dispersal (59) and reduce lung injury and mortality in a murine model when coadministered with a liquid culture of *P. aeruginosa* (60). CdrA is an adhesin exported into the extracellular matrix by *P. aeruginosa* and acts to stabilize the biofilm by crosslinking extracellular Psl or tethering Psl to cells. The biofilm-promoting action of CdrA may be counteracted by mannose (61).

A pilot study of inhaled fucose and galactose in the treatment chronic *P. aeruginosa* infection has been completed, demonstrating that such an approach was well tolerated (62). Unfortunately, this study compared carbohydrate inhalation alone with carbohydrate inhalation in combination with antibiotics. The lack of a control group makes assessing any effect of the intervention difficult.

Biofilm niche

A popular hypothesis suggests that bacterial biofilms are suspended within mucus plugs in the respiratory tract. This has recently been supported by a histopathological and microbiological study of explanted lungs of those with CF (10), where *P. aeruginosa* aggregates were common in the sputum of the conductive zone. One might therefore expect that improving mucus clearance may confer a clinical benefit. Dornase alpha, a mucolytic, appears to confer a benefit in improving lung function (63), while hypertonic saline, improving mucociliary clearance, fails to sustain an improvement in lung function but does appear to improve quality of life and reduce pulmonary exacerbations (64). A phase III study of inhaled dry powder mannitol has recently been reported. Postulated to increase airways surface liquid and mucociliary clearance, sustained improvements in FEV_1 were observed (65).

Other biofilm inhibitors

Although the mechanism is currently not well understood, *N*-acetylcysteine (NAC) may also have biofilm-inhibitory effects (66). A systematic review of trials of NAC (and related compounds), used as mucolytics, demonstrated little evidence of efficacy (67). However, NAC has been shown in vitro in a laboratory strain to have both bactericidal and biofilm-inhibitory and dispersal effects. Using in vitro susceptibility assays, the authors were also able to demonstrate a synergistic effect when used together with ciprofloxacin in 50% of clinical isolates tested (66). A phase IV clinical trial is planned (68).

ANTIBIOTICS

Currently there are no antibiotics in clinical-stage development that exploit a novel mechanism of action. New agents of existing classes of antibiotics are in clinical trials, but few will be available to patients with CF in the near future. In some cases, clinical trials are restricted to other indications, while there is considerable attrition of candidate agents as they progress through each phase of development (69). New agents of existing classes such as doripenem (carbapenem), tigecycline (glycylcycline), ceftobiprole (fifth-generation cephalosporin) (69) and CXA-101 (cephalosporin) (70) offer new opportunities. However, like other agents of these classes, they will not be immune from intrinsic and acquired resistance (69).

Antibiotic adjuvants

Antibiotic adjuvants are a diverse group of agents that act by rendering the organism more susceptible to attack by antibiotics or the host immune system, by rendering it less virulent or killing it by other means. An ideal adjuvant can act alongside an antibiotic, resensitizing bacteria to conventional antibiotics without selecting for resistance (22). Many of the mechanisms above (QS inhibitors, lectin inhibitors) may be considered as antibiotic adjuvants.

Efflux pump inhibitors

The expression of efflux pumps at the cell surface is regulated by QS (71) and maintains homeostasis within the cell by removing toxic substances. Efflux pump inhibitors (EPIs) are the archetypal antibiotic adjuvant, interfering with the extrusion of toxic substances and antibiotics from the bacterial cell (30). One candidate EPI (MC-207110) was demonstrated in vitro to decrease the intrinsic resistance of *P. aeruginosa* to levofloxacin without the accompanying emergence of resistant variants (72). The same group has presented data demonstrating that another candidate, MC-02595, potentiated the effect of coadministered levofloxacin in a mouse thigh infection model (73). Mpex is currently working with GlaxoSmithKline on a systemic efflux pump inhibitor.

Synergism may also be observed when assessing combinations of EPIs and other anti-*P. aeruginosa* agents. A synergistic effect upon biofilm formation has been noted when an EPI was combined with an iron chelator (74).

Iron chelation and gallium

An iron chelator in combination with gallium has been demonstrated to exert bactericidal properties in mature biofilms in vitro and reduce infection in a keratitis *P. aeruginosa* infection model (75). Also, gallium coencapsulated with gentamicin in a liposomal preparation enhances the in vitro activity of gentamicin against *P. aeruginosa* isolated from patients with CF (76). A pharmacokinetic study of gallium nitrate is under way (77).

Antibiotic resistance mediator inhibitors

Another antibiotic–adjuvant coupling is that of ceftazidime accompanied by a β-lactamase inhibitor (NXL-104) (78). The β-lactamase inhibitor is added to the commonly used antibiotic in order to maintain susceptibility of β-lactamase-producing strains. This combination reduced the in vitro MIC by up to fourfold compared to ceftazidime alone. This combination is currently subject to a phase II clinical trial in the U.S. for patients with complicated urinary tract infections (79).

Complementary antibiotic actions

Conventional antibiotics and other drugs may possess previously unrecognized effects upon *P. aeruginosa*. Trimethoprim and epinephrine appear to exert efflux pump-inhibitory actions (80) and further investigation to assess whether the combination of either of these with conventional antibiotics will be of benefit to patients is required.

In addition, azithromycin appears to exert QS-inhibitory activity (81). A Cochrane review of the use of long-term azithromycin in CF reported a modest significant improvement in lung function across all groups. However, it was not possible to clearly determine the reason for the improvement, with antistaphylococcal, antipseudomonal and immunomodulatory effects being possible (82). In three of the randomized controlled trials included in the review, a total of 82.9% of participants isolated *P. aeruginosa* from sputum at study entry (83-85). However, in the pediatric study, only 51% of participants were chronically infected (84). In the study reported by Saiman et al. (83), tobramycin solution for inhalation was "widely used" and the authors reported that azithromycin conferred clinical benefits when azithromycin was added to other therapies. Saiman et al. have recently published a report of a randomized controlled trial of azithromycin in those not infected with *P. aeruginosa*. They concluded that while azithromycin confers no improvement of pulmonary function in those free of *P. aeruginosa* (86), there was a 50% reduction in exacerbations and more weight gain in the active group. It seems likely that azithromycin may have a number of actions besides its antibiotic mechanism, including QS inhibition and anti-inflammatory properties.

Antibiotic preparations

In the absence of a novel antibacterial to treat *P. aeruginosa* infections and with biofilms requiring significantly higher antibiotic levels to exert an effect, novel mechanisms to increase the delivery to the site of infection are being explored. Encapsulation of antibiotics as microparticles, nanoparticles or within liposomes may alter the drug delivery profile and deliver the active drug in a targeted manner, delivering concentrations that would not be tolerated if administered systemically. Such delivery devices may allow for drugs to be delivered through the mucus barrier (87), the biofilm (88) and trans-

ported into the bacterial cell (89). Combinations of drugs may be coadministered in a predefined ratio (90, 91). Unfortunately, many of these technologies remain in the development phase, with few being the subject of clinical trials. One study of liposomal amikacin reports that, compared to placebo, the intervention group on the high-dose schedule reported benefits beyond the exposure period. Improvements in FEV_1, bacterial load and respiratory symptoms were observed (92). A phase III study is planned.

Probiotics

Promoting indigenous bacterial flora with "healthy bacteria" is widespread, with many products being available direct to consumers as food supplements and foodstuffs. Recently, this approach has been used to assess the efficacy of a probiotic to prevent nosocomial infections associated with intensive care (93). Patients were randomized to receive oral doses of *Lactobacillus casei rhamnosus* (Lcr35) or placebo via a nasogastric tube during their intensive care admission. Administration of Lcr35 appeared to be safe, and while there was a nonsignificant reduction of *P. aeruginosa* ventilator-associated pneumonia and gastric colonization, a statistically and clinically significant reduction in time to acquisition of respiratory colonization was described. While it is difficult to generalize the results of this small study to patients with CF, further investigation would be warranted.

Bacteriophages

Bacteriophages are naturally occurring viruses that infect bacterial cells, in many cases causing lysis of the bacterium. By definition, associated with bacterial infections, they have been used to identify epidemic strains of *P. aeruginosa* (94).

Bacteriophages have been used to treat bacterial infections for decades, albeit with a scant evidence base. Recent studies using in vitro bacterial biofilms have, however, been encouraging. In particular, bacteriophages have been demonstrated to penetrate the biofilm (95). In a murine study of intraperitoneal infection, a nonreplicating phage yielded a 70% survival rate at day 7 compared to 20% survival at day 2 in untreated mice (96).

The development of bacteriophages as a therapeutic agent is challenging; however, a recent randomized controlled trial of chronic otitis due to *P. aeruginosa* observed a significant reduction in bacterial load in the treated group (97). Various clinical trials are under way using bacteriophages, with a Polish group that routinely uses bacteriophages currently recruiting patients (98).

CONCLUSIONS

The use of conventional antibiotics is an unsustainable approach to the treatment of *P. aeruginosa* infection in patients with CF. The intrinsic resistance of the bacterium is compounded by the antibiotic tolerance that is afforded by the biofilm mode of growth. The antibiotic pipeline is running dry and novel approaches are required that can overcome antibiotic resistance and resist selection of newly resistance strains. The mechanisms of biofilm growth and virulence control may act as future therapeutic targets. A new strategy is required if we are to successfully treat this destructive infection, particularly for those with CF.

DISCLOSURES

Prof. Smyth has acted as a consultant to pharmaceutical companies for therapies in development for the treatment of pulmonary infection with *P. aeruginosa* in CF (Novartis: dry powder inhaled tobramycin; Mpex Pharmaceuticals: inhaled, nebulized levofloxacin; Biocontrol: bacteriophage therapy). Dr. Hurley is funded by the Nottingham Respiratory Biomedical Research Unit and latterly by the Wellcome Trust.

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