Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis

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ABSTRACT The aerobic Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen responsible for life-threatening acute and chronic infections in humans. As part of chronic infection *P. aeruginosa* forms biofilms, which shield the encased bacteria from host immune clearance and provide an impermeable and protective barrier against currently available antimicrobial agents.

P. aeruginosa has an absolute requirement for iron for infection success. By influencing cell–cell communication (quorum sensing) and virulence factor expression, iron is a powerful regulator of *P. aeruginosa* behaviour. Consequently, the imposed perturbation of iron acquisition systems has been proposed as a novel therapeutic approach to the treatment of *P. aeruginosa* biofilm infection.

In this review, we explore the influence of iron availability on *P. aeruginosa* infection in the lungs of the people with the autosomal recessive condition cystic fibrosis as an archetypal model of chronic *P. aeruginosa* biofilm infection. Novel therapeutics aimed at disrupting *P. aeruginosa* are discussed, with an emphasis placed on identifying the barriers that need to be overcome in order to translate these promising *in vitro* agents into effective therapies in human pulmonary infections.

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Introduction

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium which is widespread in the terrestrial environment. It is extremely robust and capable of surviving in challenging and varied environmental niches, as exemplified by its isolation from jet plane fuel and bottles of disinfectant fluid [1]. This adaptability is conferred by its large genome (approximately 6 Mb) and ability to survive as either a planktonic organism or as a member of a codependent bacterial community within the confines of a "biofilm" [2, 3].

The genetic plasticity and biofilm-forming attributes of *P. aeruginosa* make it a highly successful pathogen in multiple disease settings in eukaryotes. In humans, *P. aeruginosa* is an opportunistic pathogen, which is responsible for life-threatening acute infections in burn victims and other critically ill patients, as well as chronic infections and acute exacerbations in patients with respiratory diseases [4–6].

Iron is essential to the survival of virtually all prokaryotes and eukaryotes. The importance of iron to *P. aeruginosa* is exemplified by the fact that 6% of its transcribed genes are iron-responsive [7]. The concentration of bioavailable iron is a powerful regulator of *P. aeruginosa* behaviour, influencing intercellular communication and biofilm formation [7].

In this review we explore how iron availability within the lung influences the development of chronic *P. aeruginosa* biofilm infection in people with the autosomal recessive genetic disorder cystic fibrosis (CF), and examine current research into how the iron dependency of *P. aeruginosa* may be targeted therapeutically.

The susceptibility of the CF airway to infection

The CF airway is inherently prone to infection. In health, the luminal surface of the respiratory epithelium is coated with airway surface liquid (ASL), comprised of mucins, immune cells and antimicrobial peptides. ASL traps and kills inhaled pathogens which are then rapidly cleared by the mucociliary escalator. In CF, impaired function of the CF transmembrane conductance regulator (CFTR) on respiratory epithelial cells results in increased reabsorption of water from the airway lumen and dehydration of the ASL, with consequent slowing of mucociliary clearance [8]. In addition, defective CFTR-mediated bicarbonate export has been shown in animal models to result in a fall in ASL pH and further inhibition of ASL antimicrobial activity [9]. A similar acidic environment exists in human disease [10]. These alterations in the biophysical properties of ASL are compounded by deficits in airway innate immune defences, including defective iron sequestration and degradation of antimicrobial peptides by high concentrations of endogenous and bacterial-derived proteases, which produce an environment conducive to chronic infection [11, 12].

As CF lung disease progresses, plugging of distal airways by dehydrated, inspissated mucus creates microaerobic or frankly anaerobic pockets within the normally aerobic environment [13, 14]. This low oxygen environment drives phenotypic adaptation in incumbent bacteria and promotes the survival of bacteria capable of existing at low oxygen tensions [13]. Bacterial respiration may further lower oxygen tensions and potentially contribute to alteration in the pH of ASL, which will further impair the bactericidal effects of several antibiotics (especially aminoglycosides) commonly used in CF [13, 15].

Respiratory tract infections in CF begin very early in life [16]. Initial intermittent infections are typically caused by the common respiratory pathogens *Staphylococcus aureus* and nontypeable *Haemophilus influenzae* [17]. By adulthood, a chronic polymicrobial airway infection develops, with *P. aeruginosa* becoming the dominant pathogen in 80% of cases [17, 18]. Chronic *P. aeruginosa* infection leads to an increased rate of lung function decline, morbidity and mortality [19]. Recent culture-independent (metagenomic) microbiological techniques suggest that a wide range of additional bacterial species may also infect the CF airway (including anaerobes), although little is currently known about the pathological significance of these microbes [20, 21]. A key factor in the interplay between host tissues and bacterial pathogens is the management of iron metabolism. The lung is exposed daily to a high oxygen concentration, and unbound iron in atmospheric particulate matter can potentially catalyse the formation of reactive oxygen species, as can ferrous and ferric iron in ASL. This provides the lung with unique challenges with regards to iron homeostasis [22]. Airway cells rapidly sequester iron to prevent the generation of damaging free radicals, and to withhold this key nutrient from inhaled pathogens. This is achieved through uptake of nonprotein-bound iron by divalent metal-ion transporter 1 on the apical surface of bronchial epithelial cells and by the secretion of the iron chelating proteins lactoferrin and transferrin into ASL [23].

The lung is highly adept at iron detoxification and iron is barely detectable in normal airway secretions. The resulting lack of accessible iron inhibits the growth of infectious bacteria. However, respiratory secretions and sputum from patients with CF contain micromolar concentrations of iron, making this micronutrient more readily available to inhaled pathogens (airway iron indices from existing studies are presented in

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TABLE 1 Studies reporting iron concentration in respiratory secretions from patients with cystic fibrosis (CF)

First author [ref.]	Year	Population	Controls	Substrate	Assay	Iron concentration	
						Controls	CF
Gifford [27]	2011	Adults	None	Expectorated sputum	Inductively coupled plasma mass spectrometry	Not available	Stable 1.11 (0.09–4.01) mg·mL^{-1} Exacerbation 2.22 (0.77–7.04) mg·mL^{-1}
Reid [28]	2007	Adult and paediatric	Healthy	Expectorated sputum	Colorimetric	0 (0–15.8) μmol·L^{-1}	*Pseudomonas aeruginosa* infection 34 (2.4–78) μmol·L^{-1} No *P. aeruginosa* infection 18 (8–118) μmol·L^{-1}
Reid [25]	2004	Adult	Healthy	Expectorated sputum	Colorimetric	0 (0–13.2) μmol·L^{-1}	Stable 33.3 (0–111.2) μmol·L^{-1} Exacerbation 44.4 (17.0–128.7) μmol·L^{-1}
Stites [24]	1999	Adult	Healthy nonsmokers	BALF	Colorimetric	0±0 μg·dL^{-1}	42±11.6 μg·dL^{-1}
Stites [26]	1998	Adult	Nonsmokers recent URTI	Expectorated sputum	Coulometry	0±0 ng·mg^{-1}	242±47 ng·mg^{-1}

Data are presented as median (range) or mean±SD, unless otherwise stated. BALF: bronchoalveolar lavage fluid; URTI: upper respiratory tract infection.

table 1) [24–28]. *In vitro* data suggest that this increase in lung iron may partly be due to defective iron handling by CF bronchial epithelial (CFBE) cells [12].

Neutrophils represent the first line of cellular defence against bacterial pathogens and also participate in iron-withholding by secreting lactoferrin and lipocalins. Lipocalin 2 binds and inactivates bacterial-derived iron scavenging molecules (siderophores), although it is not thought to bind to the *P. aeruginosa*-derived siderophores [29, 30]. The role of lipocalin 2 in the setting of polymicrobial infection has not been explored in CF, although serum levels increase when patients develop an increased infective burden [31].

The development of *P. aeruginosa* biofilms in CF airways

Following initial airway infection, planktonic *P. aeruginosa* undergoes rapid phenotypic and genotypic adaptation to prevent immune recognition. This is achieved by the formation of a biofilm, which offers physical protection and downregulation of virulence factors [2, 32].

Biofilms comprise an extracellular matrix (ECM) of exopolysaccharides, extracellular DNA (eDNA) and proteins produced by the resident bacteria. By trapping essential nutrients and providing a physical barrier to host immune attack, biofilms offer a survival advantage to embedded bacteria. In the CF lung it is proposed that *P. aeruginosa* binds abnormal mucins present in ASL to form biofilm "rafts" which float on the respiratory epithelium [32]. Established biofilm infections cannot be eradicated with currently available antibiotics or by the host's neutrophilic inflammatory response [33].

Biofilm development is largely determined by its environment and available nutrients [34]. *In vitro*, biofilms develop complex three-dimensional structures containing phenotypically distinct subpopulations of bacteria connected by water channels formed within the ECM [35]. Iron is essential as a bacterial nutrient, and lack of iron interferes with biofilm development [36]. Iron also contributes to the structural integrity of the biofilm by cross-linking exopolysaccharide strands [37].

P. aeruginosa biofilm development is dependent on cell–cell communication. Quorum sensing is a population density dependent form of communication employed by bacteria to control the synthesis of key regulatory proteins. Quorum sensing is integral to all activities of the bacterial community, including biofilm formation. *P. aeruginosa* employs three quorum sensing systems (Las, Rhl and *Pseudomonas* quinolone signal (PQS)), each of which is iron responsive [38–41].

Under conditions of limited iron availability, both the Las and Rhl systems are activated [38, 39]. The relationship between the PQS system and iron is complex. PQS is able to operate as an iron chelator, thereby controlling the activation of the Las and Rhl systems through iron limitation [42]. Conversely, PQS synthesis is increased under conditions of both iron limitation and excess [40].

An important gene cluster under the control of Rhl is the *rhlAB* operon that regulates production of the biosurfactant rhamnolipid. Rhamnolipid acts as a "wetting agent", reducing surface tension and promoting surface-associated movement (twitching motility). Correctly timed production of small quantities of rhamnolipid is critical for the production of water channels within the core of mature *P. aeruginosa* biofilms, through which motile bacteria are able to travel. *In vitro*, inhibition of rhamnolipid production leads to the formation of flat, thick, immature biofilms [43]. In contrast, excessive rhamnolipid promotes the dispersal of mature biofilms and causes newly formed biofilms to be thin and flat [44, 45]. When iron availability is limited, increased rhamnolipid production and twitching motility prevents biofilm development, or triggers biofilm dispersal, depending on the stage of biofilm maturation [44, 46].

Paradoxically, supraphysiological iron concentrations appear to be detrimental to biofilm development. Normal human plasma contains 20–25 μM of iron, <1 μM of which is protein-bound. Biofilms grown in medium containing 100 μM of iron contain less eDNA, fail to develop complex macrocolonies and are more susceptible to antimicrobials compared with biofilms grown in an equivalent 1 μM iron medium [47]. Similarly, exposing established biofilms to medium containing 200 μM of ferric ammonium citrate triggers dispersal events and facilitates antibiotic killing [48].

Iron acquisition by *P. aeruginosa*

P. aeruginosa may take up iron from either haem or nonhaem iron sources (fig. 1). Two haem uptake systems have been described in *P. aeruginosa* (Phu and Has) [49]. The Phu system relies on direct binding of haem or haem-containing proteins to a membrane-bound receptor, whereas the Has system secretes a haem-binding protein (HasAp) which is reabsorbed through the Has receptor (HasR) when bound to haem [50, 51]. The *P. aeruginosa* genome contains a third haem receptor-encoding gene (*hxuC*); however, its functional regulation has yet to be characterised [52]. It is unknown whether haem uptake systems are employed by *P. aeruginosa* within the CF lung; however, patients frequently have frank blood in their sputum and subclinical bleeding into the airway is probably common.

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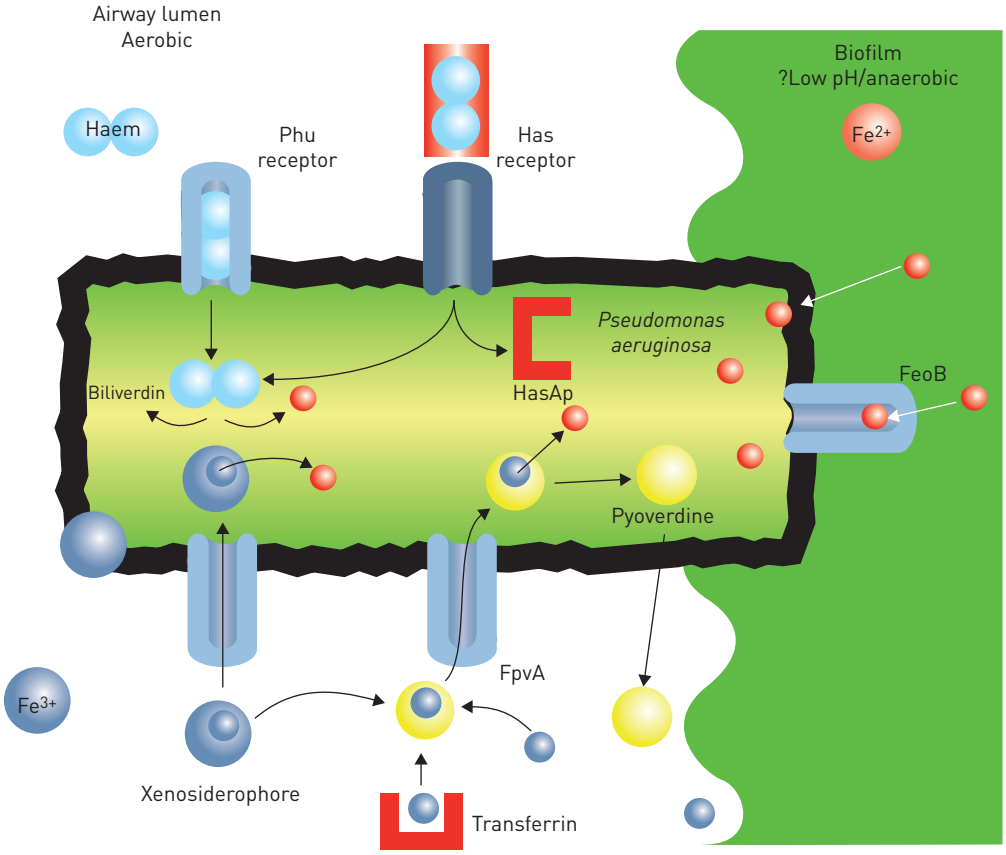

FIGURE 1 *Pseudomonas aeruginosa* iron acquisition pathways.

Haem is an uncommon iron source in the natural environment and *P. aeruginosa* must also be capable of scavenging nonhaem iron, which under aerobic conditions is most probably present in the poorly soluble ferric (Fe^{3+}) form. *P. aeruginosa* (and other bacteria and fungi) therefore produces high-affinity iron chelating siderophores [53]. Siderophores are secreted by *P. aeruginosa* into the local environment to chelate free iron and "strip" iron from host iron-binding proteins.

Two distinct siderophores have been characterised in *P. aeruginosa*: pyoverdine and pyochelin. >50 distinct pyoverdine subtypes have been characterised and are responsible for the distinctive yellow-green fluorescence of certain pseudomonads [54]. Pyoverdines are the primary siderophore produced by *P. aeruginosa*, with one of three distinct subclasses being produced by individual strains [55].

Pyochelin is considered a secondary siderophore in *P. aeruginosa*, having a much lower iron binding affinity than pyoverdine [52, 53]. Pyochelin appears to have less influence on the biofilm forming capacity of *P. aeruginosa* than pyoverdine, and its importance for iron acquisition during clinical airway infections is unclear [36, 53]. In addition to acquiring iron using autologous siderophores, *P. aeruginosa* has a high capacity to take up iron-laden siderophores produced by other bacteria and fungi [52].

P. aeruginosa, while naturally an aerobic bacterium, is capable of adapting to low oxygen environments such as those encountered within plugged CF airways. Within these regions of low oxygen tension and low pH there is potential for the redox status of iron to change to the more "soluble" ferrous (Fe^{2+}) form, but there are currently no data on this scenario in CF lung disease. Ferrous iron may be acquired by *P. aeruginosa* by passive diffusion or uptake through the FeoB receptor, although the role of these mechanisms in the clinical setting is at present unclear [56].

P. aeruginosa iron acquisition systems are tightly controlled by the ferric uptake regulator (Fur). Fur acts both directly and indirectly, through extracytoplasmic sigma factors (including PvdS), to limit iron absorption [57]. Under iron-replete conditions, Fur binds ferrous iron and attaches to a consensus sequence (Fur-box) in the promoter region of genes instrumental in iron acquisition, thus suppressing their transcription [58]. In the presence of iron, Fur inhibits iron conservation strategies by suppressing the production of two small RNAs (PrrF1 and PrrF2) [59]. In the absence of iron these small RNAs are

synthesised and facilitate inhibition of genes that encode ''nonessential'' iron-containing proteins, thereby maintaining the cytoplasmic iron pool for essential use [60]. Under low iron environments siderophore synthesis increases and nonessential iron-consuming processes are downregulated. Several excellent comprehensive reviews of the iron acquisition systems employed by *P. aeruginosa* have recently been published [36, 52, 53, 57, 60], but the above overview highlights the central role of iron in *P. aeruginosa* biofilm development.

Targeting bacterial iron acquisition as a therapeutic strategy

The critical role of iron in *P. aeruginosa* survival and biofilm formation may represent a potential ''Achilles' heel'' in the defensive armamentarium of this fastidious pathogen. Thus considerable research endeavours on a variety of fronts are being undertaken to develop novel therapeutic strategies based on disruption of bacterial iron homeostasis. These therapeutic strategies may be particularly important in CF where host iron homeostatic mechanisms appear to be abnormal.

Delivering toxic amounts of iron to P. aeruginosa

In vitro studies have suggested that iron-laden synthetic chelators can be utilised to deliver high concentrations of iron to biofilm-dwelling *P. aeruginosa* with resultant biofilm disruption [61]. While this approach demonstrates promise *in vitro*, the high redox activity of iron and potential for harmful reactive oxygen species generation within the human airway must be considered. Animal studies suggest that iron loading can potentiate proinflammatory cytokine responses to *P. aeruginosa* lipopolysaccharide and increase lung injury, highlighting the potential danger of iron therapy [62]. Furthermore, detrimental effects of iron in the lung are well described [63], and this may potentially be accentuated in the CF lung where iron handling appears to be defective [12].

Iron mimetics

Gallium (Ga^{3+}) has a similar ionic radius to Fe^{3+} and is mistaken for Fe^{3+} by many biological systems. However, Ga^{3+} lacks the redox activity of iron and consequently competitively inhibits iron-dependent processes [64]. *In vitro* studies have shown that Ga^{3+} can prevent the growth of planktonic and biofilm-dwelling *P. aeruginosa* and disperse established biofilms, with transcriptomic analysis suggesting that this effect is mediated through inhibition of iron acquisition systems including repression of *pvdS* gene [65]. Mouse infection models have demonstrated ''cure'' of *P. aeruginosa*-induced pneumonia and wound infections by local application of Ga^{3+} [65, 66]. A preparation of gallium conjugated to the siderophore desferrioxamine is undergoing *in vitro* and animal studies. This preparation aims to utilise the siderophore to improve delivery of gallium to biofilm-dwelling bacteria. Initial studies indicate that this agent has powerful anti-*P. aeruginosa* biofilm actions, in particular when combined with the aminoglycoside antibiotic gentamicin [67].

Gallium salts have established medical applications in the systemic treatment of malignant hypercalcaemia and in the diagnostic imaging of haematological malignancies [68]. Currently licensed preparations have poor oral bioavailability and are associated with a risk of nephrotoxicity, diarrhoea, hypocalcaemia, microcytic anaemia and immunosuppression when administered systemically [68]. Although the risk of toxicity is acceptably low when Ga^{3+} is used in short courses for currently licensed indications, little is known about its cumulative toxicity when used in long-term maintenance regimens as would probably be required to prevent *P. aeruginosa* infection in the CF airway. A safety study of intravenous gallium nitrate (Ganite; Genta Inc., Berkeley Heights, NJ, USA) (dose regimen 100 or 200 $mg \cdot m^{-2} \cdot day^{-1}$ for 5 days) in patients with CF was commenced in April 2010 and the results of this study are awaited (clinicaltrials.gov/ct2/show/NCT01093521).

An inhalational preparation of gallium would potentially overcome the obstacle of poor bioavailability and deliver high concentrations to biofilms while limiting systemic toxicity, but there are limited data about the safety of this approach. Gallium arsenide is utilised in the microelectronics industry and has undergone toxicological studies to assess the risk to workers from inhalation exposure [68]. Reported changes induced by gallium arsenide inhalation or tracheal instillation in animal models include epithelial hyperplasia, squamous metaplasia, benign and malignant lung tumours, and haematological malignancy [68]. Although these side-effects may be attributed to arsenide, a potentially toxic effect of gallium must also be considered. To the best of our knowledge the safety of gallium nitrate by inhalation in animal models has only been reported in abstract form [69]. In this single study, no excess toxicity was demonstrated; however, dosing was limited to a single 6-h exposure.

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Iron chelators

Exogenously administered, high-affinity iron chelators may be utilised to out-compete *P. aeruginosa* siderophores for available iron. Two such approaches have been proposed, first through the use of naturally occurring biological chelators such as lactoferrin, and secondly through the administration of entirely synthetic compounds.

Biological iron chelators

Lactoferrin

Lactoferrin is an antimicrobial glycoprotein with iron chelating properties. Lactoferrin represents a major endogenous antimicrobial constituent of airway secretions [70]. In addition to iron chelation, lactoferrin may induce bacterial cell lysis through interactions with lipopolysaccarhide and it may also prevent bacterial invasion of epithelial cells through competitive binding and proteolytic degradation of surface associated adhesion proteins [71].

In the presence of intense neutrophilic inflammation, as seen in CF airway infection, lactoferrin concentrations would be expected to be greatly elevated in respiratory secretions. However, the CF lung displays relatively low levels of lactoferrin, which are most depleted in the presence of *P. aeruginosa* [72]. This reduction is due partly to proteolytic degradation by high concentrations of proteases present in the CF airways, which serves to increase susceptibility to *P. aeruginosa* infection and promote biofilm growth [72].

In vitro, lactoferrin is capable of inhibiting *P. aeruginosa* biofilm development; however, there is conflicting evidence over whether or not this is mediated through iron chelation [73–75]. In pivotal studies conducted by SINGH [75] and others, lactoferrin induced twitching motility and repressed biofilm formation in a manner similar to that seen with iron limitation. Similarly, the biofilm-disrupting effects of apo-lactoferrin were neutralised by pre-loading lactoferrin with iron, suggesting that at least some of the effect was mediated by iron chelation [74, 75]. However, O'MAY *et al.* [73] demonstrated that the efficacy of lactoferrin in biofilm disruption was augmented at higher iron concentrations (250–500 μM), suggesting an iron chelation-independent method of biofilm disruption.

The efficacy of lactoferrin supplementation *in vivo* is beginning to be investigated. However, the potential for proteolytic degradation may impact on the clinical efficacy of this therapeutic approach *in vivo*.

Lactoferrin combined with hypothiocyanate

Production of hypothiocyanate in ASL is another important innate immune defence strategy that appears to be defective in CF lung [11]. Hypothiocyanate is normally formed by the oxidation of thiocyanate, but CF epithelial cells do not secrete thiocyanate [11]. A combination preparation of lactoferrin and hypothiocyanate (Meveol; Alaxia, Lyon, France) delivered by inhalation is undergoing development (www.alaxia-pharma.eu/meveol), and has been granted orphan drug status to promote clinical trials. To date, *in vitro* and animal data demonstrating its antimicrobial actions have only been presented in abstract form.

Synthetic iron chelators

Synthetic iron chelators developed primarily for the treatment of conditions associated with systemic iron overload display much higher iron binding affinities than biological iron-carrying proteins and therefore potentially offer greater competition to bacterial siderophores. A number of authors have reported on the ability of these agents to disrupt *P. aeruginosa* biofilms; however, the bacterial strains studied, chelators employed and culture models utilised have varied between studies (table 2).

MOREAU-MARQUIS *et al.* [76] investigated the effects of the currently licensed iron chelators deferasirox and deferoxamine on *P. aeruginosa* biofilms grown on CF epithelial cells. These studies indicated that both agents were able to prevent biofilm growth as well as disrupt established biofilms. Their efficacy was further enhanced when they were co-administered with the antipseudomonal antibiotic tobramycin.

In addition to demonstrating the antibiofilm properties of a number of synthetic chelators, O'MAY *et al.* [73] showed an increased efficacy of these agents against anaerobically grown biofilms, highlighting the important role that local environmental conditions may play when these interventions are deployed *in vivo*. In similar experiments, BANIN *et al.* [30] demonstrated disruption of *P. aeruginosa* PAO1 biofilms by EDTA, which was augmented by the aminoglycoside gentamicin. However, in contrast, LIU *et al.* [77] suggested that EDTA administered alone could potentiate PAO1 biofilm formation, yet it inhibited biofilm growth when co-administered with the efflux pump inhibitor phenyl-arginine-β-naphthylamide. Possible explanations for the different findings in these two studies include differences in biofilm model, the ability of EDTA to chelate multiple divalent cations in addition to Fe^{2+} and the chelator concentrations used (14.6 $\mu g \cdot mL^{-1}$ *versus* 5 $\mu g \cdot mL^{-1}$) [30, 77].

TABLE 2 *In vitro* studies employing synthetic iron chelators in the treatment of *Pseudomonas aeruginosa* biofilms

First author [ref.]	Year	Iron chelators	Adjuvant treatment	Biofilm model employed	Outcomes
Liu [77]	2010	2DP Acetohydroxamic acid EDTA	PaβN	Coverslip	EDTA, 2DP and acetohydroxamic acid each worked synergistically to reduce biofilm growth EDTA alone increased biofilm growth
Moreau-Marquis [76]	2009	Deferasirox Deferoxamine	Tobramycin	CFBE cell-lined flow cells Static CFBE cells Abiotic static culture	Deferasirox and deferoxamine reduced biofilm growth on CFBE cells and potentiated the effects of tobramycin Deferasirox and deferoxamine acted synergistically with tobramycin to disrupt biofilms grown on CFBE cells Neither deferasirox nor deferoxamine disrupted biofilms on abiotic static surfaces
O'May [73]	2009	DPTA Deferoxamine 2DP EDDA EDTA		Borosilicate glass tubes (aerobic and anaerobic) Flow cells	2DP, DPTA and EDTA impaired biofilm growth 2DP disrupted established biofilms Antibiofilm effects of all iron chelators were greatest against anaerobically cultured biofilms
Banin [30]	2006	EDTA	Gentamicin	Disk reactor Flow cells	EDTA reduced biofilm-associated cells by >99% EDTA increased biofilm dispersal events Coadministration of gentamicin increased bacterial killing Antibiofilm effects were overcome by divalent cationic Mg^{2+}, Ca^{2+} and Fe^{2+}

2DP: 2,2,dipyridyl; PaβN: phenyl-arginine-β-naphthylamide; CFBE: cystic fibrosis bronchial epithelial; DTPA: diethylenetriaminepentacetic acid; EDDA: ethylenediamine-N,N9-diacetic acid.

Siderophore–antibiotic conjugates and the "Trojan horse" approach

Reduced membrane permeability, antibiotic efflux pumps and antimicrobial inactivating enzymes (*e.g.* β-lactamases) are defence strategies employed by biofilm-dwelling bacteria which augment the physical protection offered by the ECM. The essential requirement for iron trafficking mediated by siderophores in biofilm-dwelling pseudomonads has driven the concept of "hijacking" this system to circumvent the protection offered by the ECM and cell membrane impermeability. As a result, siderophore–antibiotic conjugates (SACs) have been developed which may function as "Trojan horses" [78–80].

Naturally occurring SACs termed sideromycins were discovered many years prior to the description of siderophore trafficking [80]. Sideromycins are produced by *Actinomyces* and *Streptomyces* species as antimicrobials against competing micro-organisms. These agents rely heavily upon their recognition by the iron uptake system of the target species and, disappointingly, they display limited activity against *P. aeruginosa* [81, 82].

Penicillin–siderophore conjugates have been proposed as leading candidates for synthetic SACs. These compounds have the advantage of having a distinct antibiotic active site and siderophore conjugation site, which means that there is no need for the antibiotic to dissociate from the siderophore to exert its effect. Furthermore, the antibacterial action of penicillin is exerted through attachment to penicillin binding proteins located in the periplasm. Thus, the conjugated molecule needs only traverse the bacterial outer membrane to be effective. Recent *in vitro* and mouse model data have demonstrated that an ampicillin-based SAC has superior antibacterial actions against a range of laboratory and clinical strains of *P. aeruginosa* (and other Gram-negative bacteria) compared to the commonly prescribed antipseudomonal antibiotics meropenem, imipenem and ciprofloxacin [83]. Similar *in vitro* experiments performed with β-lactam antibiotics conjugates have yielded mixed results [78, 84]. A sulfactam-containing SAC has demonstrated potent activity against multi-antibiotic resistant *P. aeruginosa* strains (minimum inhibitory concentration required to produce 90% inhibition 8 $\mu g \cdot mL^{-1}$), whereas a monobactam SAC demonstrated only modest improvements in minimum inhibitory concentrations against "epidemic" CF *P. aeruginosa* strains when compared to established antipseudomonal antibiotics [78, 84].

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Other potential targets based on iron homeostasis

Additional potential strategies to disrupt *P. aeruginosa* iron homeostasis include competitive inhibition of siderophore uptake through the use of siderophore mimetics or monoclonal antibodies, which bind to bacterial siderophore receptors but do not deliver bioavailable iron [85, 86]. These techniques are in their infancy and there is little published work on the effect of these strategies with regards to *P. aeruginosa*. Such therapies are likely to be very expensive.

Advances in crystallography are defining the structural composition of enzymes involved in bacterial siderophore synthesis, which may lead to targeted inhibitors of these pathways. Characterisation of the structure of salicylation enzymes involved in the synthesis of siderophores by *Mycobacterium tuberculosis* and *Yersinia pestis* have resulted in the development of the synthetic compound 5-O-(N-salicylsulfamoyl)adenosine (salicyl-AMS), which has been shown to inhibit the growth of both *M. tuberculosis* and *Y. pestis* under iron-limiting conditions [87]. The design of similar agents that are active against *P. aeruginosa* has yet to be described, although they are likely to be developed in time.

Finally, iron acquisition pathways may be targeted in vaccine development. Attempts to develop clinically efficacious vaccines against *P. aeruginosa* have, to date, been unsuccessful [88]. Obstacles include *P. aeruginosa*'s multiple antigenic determinants, multiple serotypes of these determinants between clinical strains and the different expression of determinants under different conditions (*e.g.* planktonic and biofilm growth) [89]. Application of proteomic and bioinformatics techniques to the study of uropathogenic *Escherichia coli* identified six highly conserved iron uptake surface membrane receptors [90]. Deployment of a polyvalent vaccine against three of these receptors in a murine model resulted in effective protection against urinary tract infection [90]. *P. aeruginosa* iron-regulated outer membrane proteins are also immunogenic, but their potential as vaccine targets has not been explored [91].

Strategies to limit iron in the setting of a polymicrobial infection

Any new intervention directed against *P. aeruginosa* must consider the potential impact on copathogens, as suppression of the dominant pathogen may allow the emergence of other, potentially more harmful, infections.

In common with *P. aeruginosa*, other commonly isolated CF airway pathogens, including *S. aureus*, *H. influenzae* and *Burkholderia cepacia* complex (BCC), are capable of biofilm development and each have an absolute requirement for iron [92–96].

In a single published study on the effect of gallium on planktonic and biofilm grown BCC, strains were exposed to gallium nitrate at concentrations of up to 64 $mg \cdot L^{-1}$ (~250 μM Ga^{3+}) [97]. Disappointingly, there was little effect seen on either planktonic or biofilm growth. These results have been challenged on the basis that the concentration of gallium used was lower than could be safely administered therapeutically [98]. However, in a similar study examining the effects of gallium maltolate on the growth of *S. aureus* and *S. epidermidis* biofilms, equally disappointing results were reported, and minimal inhibitory concentrations far in excess of those that could be safely administered systemically (>3000 $mg \cdot L^{-1}$) were needed to achieve biofilm inhibition [99].

There are few studies of iron chelator effects on CF bacterial pathogens other than *P. aeruginosa* (table 3) [100–103]. The effect of the synthetic chelators deferiprone and deferoxamine against a number of staphylococcal species grown in broth cultures has been examined [103]. Deferiprone inhibited growth of all species studied, but desferrioxamine promoted growth in a number of staphylococcal species [103]. Similarly, it has been demonstrated that *S. aureus* can take up iron hydroxamates such as desferrioxamine and utilise them as an iron source to promote biofilm growth [101, 104].

Translational research and the challenges of targeting *P. aeruginosa* iron homeostasis in the human lung

Despite the early promise of a number of the agents discussed above *in vitro*, important questions remain to be answered about their safety and efficacy before advancing to human trials.

The majority of the work presented above has been performed using common laboratory-adapted strains of *P. aeruginosa*, which vary both genetically and phenotypically from clinical strains isolated from the CF lung. Additionally, studies have considered only a limited number of environmental variables and often use conditions that are distinct from those within the CF lung, where there is reduced oxygen tension, significant amounts of extracellular iron, low pH and a hostile milieu replete with proteases and free radicals [2, 10, 13]. In the very limited work performed with clinical isolates, different responses to iron-targeted therapies have been reported, both between clinical and laboratory strains, and between clinical isolates from different patients [73].

TABLE 3 The effect of iron chelators on common cystic fibrosis airway pathogens

First author [ref.]	Year	Bacteria tested	Iron chelators	Culture model employed	Outcomes
Aguila [102]	2001	*S. aureus* (clinical and laboratory isolates); MRSA	Lactoferrin	Broth cultures in iron depleted minimal media or normal human serum	Lactoferrin was bacteriostatic against most clinical and laboratory strains, including many antibiotic-resistant strains
Percival [100]	2005	*S. aureus*; *S. epidermidis*; *P. aeruginosa*; MRSA; *E. coli*; *K. pneumoniae*	EDTA	Silicon tubing, central venous catheter model	Exposure of catheter-related biofilm to EDTA for 25 h inhibited biofilm growth of all bacterial species
Kim [103]	2009	*S. aureus*; *S. epidermidis*; *S. saprophyticus*	DFO Deferiprone	Broth culture in minimal media	DFO promoted the growth of some species (especially *S. aureus*) Deferiprone inihibited the growth of all species tested
Al-Azemi [101]	2011	*S. aureus*[#]	EDTA DFO	Coverslip static biofilm	EDTA impaired biofilm growth DFO at low concentration (100 μM) stimulated biofilm growth DFO concentrations >1 mM inhibited growth EDTA and DFO displayed synergistic antibiofilm effects

S. aureus: *Staphylococcus aureus*; MRSA: methicillin-resistant *S. aureus*; *S. epidermidis*: *Staphylococcus epidermidis*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*; *S. saprophyticus*: *Staphylococcus saprophyticus*; DFO: desferrioxamine B. [#]: Panton–Valentine leukocidin-positive community-acquired methicillin-sensitive *S. aureus*.

Although there have been no studies of treatments targeting bacterial iron homeostasis under "CF lung conditions", factors including pH, glucose source and oxygen availability have been shown to affect the biofilm-forming capacity of airway pathogens [32, 73, 101]. Consequently, if new agents are to be successful they must remain active over a wide pH range, and compete with both ferrous and ferric iron acquisition systems.

Iron limitation *in vitro* triggers the dispersal of motile planktonic bacteria with increased virulence compared to their biofilm-dwelling counterparts, and thus the potential for biofilm disruption to trigger an acute host inflammatory response [105]. To better understand the inflammatory potential of these agents testing in an animal model is desirable; however, representative models of CF airway infection are limited. Mice containing the major *CFTR* gene mutations (*e.g.* DeltaF508, G551D) do not develop spontaneous airway infections and *P. aeruginosa* has to be introduced directly into the mouse lung where it is either spontaneously cleared or results in overwhelming infection [106, 107]. Successful chronic mouse airway infection has been achieved by introducing *P. aeruginosa* bound to agar beads into the trachea and by contaminating drinking water with *P. aeruginosa* [108], but how closely this reflects human disease is debated. More recently, pig and ferret models of CF have been developed, which may more closely mimic human respiratory disease [109, 110].

Finally, the route of administration must be considered. The concentrations of gallium required for activity against *S. aureus* and BCC biofilms are well above those considered safe for systemic delivery in humans, suggesting that inhalation may be the only viable option to safely administer the required dose. Similarly, *in vitro* studies suggest iron chelators delivered directly to biofilms grown on the apical membrane of CFBE cells inhibit growth more effectively than when they are applied to the basal membrane, suggesting that direct delivery to the airway may also be the preferred mode of delivery for these compounds [76]. The possibility of localised delivery of chelators is supported by *in vitro* modelling, which has suggested that chelated iron may be effectively aerosolised to a particle size suitable for lung delivery [61].

Conclusions

As our understanding of the biology of bacterial biofilms expands, new therapeutic possibilities present themselves. Given the absolute requirement for iron of *P. aeruginosa* and other CF airway pathogens, disrupting iron utilisation is an exciting avenue for further research. The results of the safety trial of intravenous gallium are eagerly awaited. Future studies of iron chelation therapy will need to test the efficacy of these agents against clinically relevant *P. aeruginosa* strains and establish their safety within animal models, before proceeding to human trials.

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