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In Situ Identification of Gram-negative Bacteria in Human Lungs Using a Topical Fluorescent Peptide Targeting Lipid A

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One Sentence Summary: A topically administered fluorescently labelled peptide targeting Lipid A permits rapid, real-time visualization of bacteria in the distal human lung.

Abstract: Respiratory infections in mechanically ventilated patients caused by gram-negative bacteria are a significant cause of morbidity and antibiotic administration. Rapid and unequivocal determination of the presence, localization and abundance of bacteria in the distal lung would be helpful for the diagnosis of bacterial infection and for patient stratification and could be used for monitoring treatment efficacy. Thus, we developed an in situ approach to simultaneously visualize gram-negative bacterial species and cellular infiltrates in distal human lungs. We used optical endomicroscopy to visualize a water-soluble optical imaging probe based on the antimicrobial peptide polymyxin conjugated to an environmentally sensitive fluorophore. The probe was chemically stable, non-toxic and, after in-human intrapulmonary microdosing, enabled the specific detection of gram-negative bacteria in distal human airways and alveoli within minutes. The results suggest that pulmonary molecular imaging using a topically administered fluorescent probe specific for Lipid A is safe and practical, enabling rapid in situ identification of gram-negative bacteria in humans.

Introduction

Gram-negative pulmonary infection (GNPI) is a frequent and severe consequence of hospitalization, immunosuppression and mechanical ventilation[1]. Unfortunately, the accurate and rapid diagnosis of gram-negative pneumonia is challenging. The gold standard diagnostic in situ procedure for pneumonia (defined as infection in the gas-exchanging regions of the human lung alongside a host cellular response), remains biopsy and culture[2, 3]. However, biopsy is rarely performed as a diagnostic tool for pneumonia, and would have safety concerns if performed in mechanically ventilated patients.

The accurate determination of the aetiology of new pulmonary infiltrates on the chest x-ray, represent one of the major diagnostic challenges in mechanically ventilated patients in the Intensive Care Unit (ICU). The clinical suspicion of nosocomial pneumonia is overly sensitive[4], often leading to inappropriate and/or overtreatment with broad-spectrum antimicrobial therapy. Current approaches to guide antimicrobial therapy for suspected pneumonia rely on the growth of bacteria from aspirated fluids or expectorated samples alongwith antimicrobial susceptibility testing[4-6]. In mechanically ventilated patients and immunosuppressed lung transplant patients, bronchoalveolar lavage (BAL) is a routinely used sampling methodology. However, BAL has suboptimal specificity and sensitivity[7] and is associated with significant delays as culture results take up to 72 hours[8]. Furthermore, although BAL aims to sample the alveolar space, it is invariably contaminated by tracheobronchial organisms resulting in low specificity[6, 9] and subsequent over-treatment. Similarly, negative culture results from distal airways sampling may lack validity as a result of concurrent antimicrobial therapy[10] or poor sampling[4, 11, 12]. In addition, molecular techniques such as polymerase chain reaction (PCR) that employ amplification and sequencing of potential microbial species in expectorated or sampled fluids are inherently over sensitive[13].

Given these limitations of existing approaches, an in situ methodology for the rapid and accurate identification of GNPI has potential benefits. Therefore, we developed and tested the feasibility of an optical molecular imaging (OMI) approach combining intrapulmonary delivery of a fluorescent probe (SmartProbe) specific for the presence of Gram-negative bacteria with optical endomicroscopy (OEM) of the distal lung.

We investigated the utility of Polymyxins (PMXs), which are cationic, amphipathic, cyclic antimicrobial peptides, naturally formed by the bacterium *Paenibacillus polymyxa*(*14*), as a selective binding agent for Lipid A of lipopolysaccharide (LPS) on the outer membrane of gram-negative bacteria(*15, 16*). We conjugated PMX to an environmentally sensitive fluorophore, 7-nitrobenz-2-oxa-1,3-diazole (NBD), generating NBD-PMX which had excellent signal-to-noise ratios with fluorescent amplification only upon entry into the hydrophobic environment of the bacterial membrane. Following in vitro and ex vivo validation in a ventilated ovine lung model, the compound underwent preclinical toxicology and was manufactured to good manufacturing practice (GMP) for a first-in-human study. Here, we used NBD-PMX with OEM in patients with bronchiectasis and in a cohort of critically ill mechanically ventilated patients in the ICU who had suspected pneumonia, to rapidly and specifically visualize gram-negative bacteria alongside autofluorescent cellular infiltrates in the distal lung.

This method showed the potential to provide in situ molecular imaging of specific bacteria in humans that might be used as a diagnostic and monitoring tool and also as a platform to increase understanding of the pathophysiology of microbial infection.

Results

Structure-activity relationship of NBD-PMX constructs demonstrate an optimal SmartProbe to selectively label gram-negative bacteria with high signal-to-noise in alveolar tissue

A panel of modified PMX constructs was chemically synthesized and evaluated for specificity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. PMX was modified by removal of the hydrophobic tail and two amino acid residues and replaced with various linkers attached to the fluorophore NBD (without alteration of cyclic ring component). Progressive lengthening of linker led to loss of gram selectivity (fig. S1). The lead compound (NBD-PMX, Fig. 1A) with an amino-PEG2-carboxylate spacer showed fluorescence amplification in increasingly polar environments generated by increasing dimethylsulphoxide (DMSO) concentration, demonstrating the required environmental reporting for fluorescence amplification upon bacterial membrane insertion in vivo, where wash steps are not possible (Fig. 1B). Moreover, the lead compound labelled *P. aeruginosa,* in a concentration dependent manner with high signal-to-noise ratio (Fig. 1C).

Gram selectivity was assessed against a broad and clinically relevant panel of organisms and demonstrated labelling selectivity for gram-negative bacterial species; *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Escherichia. coli, Haemophilus influenzae, Acinetobacter baumannii* and *Stenotrophomonas maltophilia* but not for the gram-positive bacterial species Methicillin sensitive *S. aureus* (MSSA), Methicillin resistant *S. aureus* (MRSA) and *Streptococcus pneumoniae* (Fig. 1D-F)*.* To demonstrate selectivity over mammalian cells, NBD-PMX was imaged in co-culture with freshly isolated human peripheral blood granulocytes and mononuclear cells and also with alveolar macrophages retrieved from BAL (Fig. 2A-C). Furthermore, there was no labelling of human lung epithelial cells demonstrated in a bacterial-human lung co-culture experiment (Fig. 2D), and low concentrations of NBD-PMX preferentially labelled bacteria in the presence of surfactant (fig. S2).

To determine the mode of action of NBD-PMX, the *Burkholderia cenocepacia* strain K56-2, which is highly resistant to PMX through mutations in its Lipid A component([17, 18](cite)) was treated with the NBD-PMX probe. This showed a lack of binding, suggesting that NBD-PMX labelling of gram-negative bacteria is through Lipid A binding (fig. S3).

NBD-PMX demonstrated no toxicity and chemical stability

NBD-PMX demonstrated no membrane toxicity in an in vitro haemolysis assay and no toxicity or pulmonary inflammatory cell recruitment after intratracheal instillation in mice (fig. S4). Furthermore, no adverse histopathological observations were noted between the two groups at 48 hours or day 14 post administration in lung, liver or kidney (fig. S4). This was confirmed by a contract research organization good laboratory practice (GLP) rat single-dose intratracheal toxicology study (with over 600 times the human dose based on lung weight) where no changes in weight, haematological, coagulation and clinical chemistry parameters were observed (table S1). NBD-PMX was manufactured to GMP and stability studies (24 months) demonstrated chemical stability of the aqueous drug product under frozen (-20^0C) storage conditions. (table S2).

NBD-PMX visualizes gram-negative bacterial bioburden in the distal lung with topical microdosed endobronchial delivery and OEM in a large animal ex vivo lung model

An important technical prerequisite of the approach required evidence that bacteria could be rapidly detected in the distal alveolar regions with a clinically-relevant limit of detection (LoD) and sampling methodology. We developed an ovine ex vivo whole lung ventilated model of distal lung bacterial burden to evaluate NBD-PMX against a diverse panel of clinically relevant pathogenic bacteria in a human-size-relevant lung model (Fig. 3A). This model allowed the use of clinical equipment and the thorough testing of the technical feasibility of delivering microdoses (<100 mcgs) of NBD-PMX and detection of labelled bacteria in distinct bronchopulmonary segments after regional OEM. OEM enables the delineation between bronchiolar imaging and alveolar imaging through the very distinct autofluorescence patterns of elastin. The presence and distribution of bacteria were initially modelled *in situ* with the instillation of Green Fluorescent Protein (GFP) *S. aureus,* which demonstrated a characteristic imaging pattern of punctate twinkling bacteria (movie S1). Anatomically distinct bronchopulmonary segments were instilled with bacteria or vehicle control and subsequently microdoses of NBD-PMX were instilled and lung segments immediately imaged using OEM. This was achieved by passing an OEM fibre into disparate bronchopulmonary segments (up to 5 passes per

bronchopulmonary segment to capture a regional representation of fluorescence), recording images at 12 frames per second and imaging for up to 5 minutes. The regional exploration of the bronchopulmonary segments was intended to mimic the clinical scenario of performing sampling in bronchopulmonary segments guided by chest x-ray infiltrates. Using this approach we could detect gram-negative bacteria, such as *P. aeruginosa, K. pneumoniae* and *E.coli* but not the gram-positive bacteria MSSA, MRSA and *S. pneumoniae* (Fig. 3B-C). An image analysis algorithm was developed to objectively detect bacteria and analyze and quantify the videos on a frame-by-frame basis (file S1). The analysis confirmed the ability of NBD-PMX to discriminate gram-negative bacteria from gram-positive and buffer control segments (Fig. 3D-F) with bacteria visualized within minutes of NBD-PMX instillation. The imaging algorithm was developed to discriminate a clinically relevant LoD of $1x10^5$ colony forming units/ml in BAL. To test whether the algorithm was able to distinguish various bioburdens of bacteria, different concentrations of *E. coli* were instilled and imaging performed as described above. Using the developed algorithm, ranges of $1x10^5$ to $1x 10^9$ bacterial bioburden in the distal alveoli were distinguishable (fig. S5).

NBD-PMX enables immediate in situ visualization of gram-negative bacteria in the distal lung of bronchiectasis patients

Bronchiectasis is a chronic suppurative pulmonary disease characterized by distal lung bacterial colonization and repeated infective exacerbations(*19*) with pathogens, often gram-negative, that are commonly found in mechanically ventilated patients and patients who receive lung allografts. To test the clinical potential of this imaging method, GMP manufactured NBD-PMX was topically administered as a microdose via an intrapulmonary catheter during a bronchoscopy procedure to six patients with bronchiectasis and imaging of the distal airways and alveoli initiated immediately after NBD-PMX administration. Four females and two males with a mean age of 65 comprised the study population and no patient had a serious adverse event (table S3). Minor adverse events such as cough attributable to the bronchoscopy procedure were observed in three patients. BAL and sputum culture was performed after the imaging procedure and bacterial species identified. The imaging videos

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obtained following NBD-PMX demonstrated that the method was able to clearly detect the presence (or absence) of gram-negative bacteria (movies S2-7).

Furthermore, using an image analysis algorithm analogous to that used in the ovine ex vivo model, and by deploying an arbitrary cut off value determined by the ex vivo model but adjusted for the larger field of view in the clinical system (file S2), we clearly distinguished positive from negative frames (Fig. 4). Negative bacterial signal was seen in three patients: D1, D3 and D4. Upon culture, patients D1 and D3 grew only a gram-positive organism (*S. aureus* and *S. pneumoniae* respectively) and D4 grew a PMX resistant gram-negative organism (*Proteus miribalis*) (fig. 4 and movies S2, S4 and S5). Positive bacterial signals were seen in D2, D5 and D6. D2 and D5 grew *P. aeruginosa*, (Fig. 4 and movies S3 and S6), while D6 grew two different species; *S. pneumoniae* and *H. influenzae.* Therefore, this positive signal was attributed to *H. influenzae* presence. Bacterial signals were clearly observed in the distal alveolar regions, which conventionally are considered to be low in bacterial bioburden in chronic suppurative lung disease at post-mortem(*20*). Bacterial aggregates were also observed in the distal bronchioles of these patients (fig. S6).

NBD-PMX and OEM enable immediate in situ visualization of gram-negative bacteria and cellular infiltrates in the distal lung of ICU patients

We then performed the procedure in seven ICU patients with pulmonary infiltrates and suspected pneumonia to demonstrate early feasibility, assess safety and to discern in an unselected cohort if a qualitative signature of infection in the distal lung could be observed. One patient was excluded from the analysis as no videos could be analyzed and no BAL was retrieved. In six patients, imaging and BAL analysis was performed to provide initial proof-of-concept (Fig 5, table S4 and movies S8-S13). No study related serious adverse events were noted and the average procedure time (including BAL) was 14.7 minutes with 4.8 passes in the endobronchial segments imaged. Two patients, D7 and D11 (Fig 5 and movies S8 and S11), demonstrated a characteristic bacterial signal and an autofluorescent cellular infiltrate. BAL yielded no significant bacterial growth for patient D7, which was unsurprising as the patient was on multiple antimicrobials including metronidazole, ciprofloxacin, vancomycin and

the anti-fungal fluconazole for a perforated abdominal viscus. BAL from patient D11 demonstrated growth of the gram-negative bacteria *Klebsiella oxytoca* at low levels (table S4) (BAL microbial culture was likely suppressed by the prior administration of intravenous temocillin) and the fungus *Candida albicans*. Ex vivo labelling of BAL from D11 with NBD-PMX demonstrated labelling of *Klebsiella oxytoca* but not *Candida albicans* (fig S7). Patients D8 and D13 demonstrated no bacterial labelling and no inflammatory cellular infiltrate in the alveoli, but did demonstrate the absence of well-aerated alveoli, as there were condensed/compressed elastin structures (Fig 5. and movie S9 and S13) consistent with atelectasis of the segment. Growth of *S. maltophilia* was demonstrated for D8 in both tracheal aspirates prior to procedure, and on BAL at 1×10^4 CFU/ml. Given the absence of distal alveolar bacterial signal and the absence of a cellular infiltrate, this was attributed to airway colonisation. Patient D9 and D12 both demonstrated no distal alveolar bacteria, no cellular infiltrate and BAL grew bacteria below the threshold for VAP, consistent with absence of VAP. Patient D12 developed a pulmonary abscess prior to imaging, which was additionally identified on OEM by the absence of elastin autofluorescence. Whilst these are preliminary studies in an unselected cohort of mechanically ventilated critically ill patients with new pulmonary infiltrates on CXR, they demonstrate the feasibility of this point-of-care technology platform and the utility of a qualitative imaging signature of bacterial presence and alveolar cellular infiltrate.

Discussion

The inexorable rise of antimicrobial resistance (AMR) has in part been driven by the indiscriminate administration of antibiotics to individuals suspected of having infections(*21*). Upper airway and bronchoscopic sampling and subsequent microbiological culture are routinely used in mechanically ventilated patients and in patients who have received lung allografts. However, these diagnostic approaches have limited sensitivity and specificity and significant delays in reporting(*7, 8*), such that empirical antibiotic therapy is frequently initiated leading to inappropriate and/or overtreatment with broad-spectrum antibiotics, which leads to increased morbidity, exposes patients to potential drug toxicity, promotes AMR, increases the risk of antibiotic-associated infections and wastes health service resource(*21-24*). Moreover, there are no internationally agreed refinement/de-escalation protocols for antimicrobial therapy within ICU (*21*). Therefore, technologies that enable bacterial detection, bacterial bioburden and bedside qualitative measure of pulmonary infection in the distal lung may have significant utility. Molecular imaging of infection in humans has been dominated by nuclear medicine approaches and whilst these do offer whole-body imaging, they are expensive, cumbersome, limited to large centers, are not point-of-care, offer poor specificity and variable sensitivity(*25*) and have not been widely adopted. Indeed, using whole-body approaches, it is impossible to delineate true distal lung alveolar infection from tracheobronchial colonization and in the specific case of mechanically ventilated patients, adds time, safety and often transport challenges as critically-ill patients need to be moved to the scanners.

Although there have been significant technical developments in developing bacterial OMI for small animals or tissue explants(*26-29*), clinical translation is mostly lacking. Partnering intrapulmonary microdosed fluorescent probes with OEM is a platform technology enabling molecular imaging of the distal lung at high resolution, with the potential for serial exploration of pulmonary segments. The distal airway and alveolar elastin networks allow clear visualization of the spatial location within the lung through autofluorescence imaging at 488nm excitation(*30*). Importantly, the OEM fibre is only extended through the working channel of the bronchoscope once it is wedged in the distal lung. This simple method avoids proximal tracheobronchial contamination which is a major issue with existing

methods (*6*). The targeting nature of the NBD-PMX coupled with selected distal lung optical imaging ensures potentially high specificity and coupling the bacterial imaging with the imaging signature of a cellular infiltrate has the added potential to be more predictive of alveolar infection vs colonization. The true signature of infection vs colonization requires larger studies with further evaluation compared to accepted reference standards.

In clinical practice, PMX isomers are used as antimicrobial agents(*31, 32*), hence we chose to use PMX as a binding ligand for OMI. PMX was linked to an environmentally sensitive fluorophore to allow signal generation only upon bacterial membrane engagement and generation of high signal-to-noise when administered topically into the human lung. Salient chemical modifications of PMX enabled specific and sensitive gram-negative bacterial molecular imaging. Importantly, we retained the positively charged diaminobutyric-acid (DAB) residues on the cyclic ring which are critical for overall cationic charge and bacterial binding (*33, 34*). Structure–activity relationship studies showed that the linker length between the PMX and NBD was crucial for binding, while the amphipathic properties of NBD-PMX were maintained by the hydrophobic residues which remained in position 6 and 7 (D-phe and Leu respectively)(*16*). Mechanisms of PMX resistance remain largely confined to alterations to the Lipid A component of LPS(*35, 36*) and as PMX resistance in nosocomial gram-negative infections is rare, even amongst multi-drug resistance strains(*32, 37, 38*), NBD-PMX will have wide applicability.

To extend the possibility of using such a technology platform to monitor treatment response, bioburden of bacteria in the distal lung was clearly distinguishable. GNPI is emerging as one of the most difficult infections to treat due to rising multidrug resistance(*39, 40*), thus the ability for repeated visualization of bacterial bioburden in the distal lung to serially monitor pathogen bioburden may help to refine/escalate and deescalate therapy. Although, we have showed here that the use of OEM was safe, repeated visualization does require repeat bronchoscopy and consequently the associated risks of repeated use of this technology will need to be weighed against the information it could provide.

 The challenge of distinguishing de novo bacterial infection vs resolving infection or indeed colonization is an important clinical question and necessitates novel technology development. In the six

bronchiectasis patients, we clearly observed Gram-specific imaging of bacteria and in the ICU patients we observed qualitative signatures of infection in two patients. In this small clinical study, NBD-PMX did not detect *Proteus Mirabilis* which is known to be PMX-resistant. *P. mirabilis* is an uncommon cause of nosocomial pneumonia(*41*) and opportunistic pulmonary infection in immunosuppressed patients. This observation did however show the potential of the platform to detect AMR.

The study has several limitations at this stage of development including; the small cohort of patients, the availability of the technology for clinical applicability and the anticipated requirement of additional fluorescent probes for polymicrobial infections. To date, the technology platform has demonstrated early stage safety and feasibility in the ICU; however, the future use of such platforms requires large well-constructed trials to demonstrate efficacy and safety alongside health economic evaluation based upon STARD guidelines(*42*). As histopathological confirmation is not a routine test for pneumonia, given potential morbidity and mortality, this technology platform will need to be assessed against the current accepted reference standards for pneumonia diagnosis in the ICU. These multicenter studies will be essential to support the utility of this approach, distinguish colonization from infection and improve and validate the imaging signature through further refinements of the imaging analyses algorithms.

Secondly, regarding clinical applicability, the imaging systems are commercially available but the fibre optic confocal fibres currently cost $300-400 per procedure and are restricted to limited uses and require sterilization between uses. These implementation costs are significant and alongwith the paucity of molecular imaging agents that are clinically compatible, have limited the widespread clinical use of this technology platform. However, single use low-cost disposable fibres, which dramatically reduce cost have now been developed(*43*). Similarly, the cost of goods of the imaging fluorescent probes is low and with the advent of frugal innovation and technology such as widefield OEM(*44*), this approach is potentially cost-effective following validation in larger trials. However, as with any novel technology platform, there is an expected time-lag prior to widespread applicability including the time for the regulatory approval of imaging fluorescent probes.

Finally, whilst GNPI are the predominant cause of pulmonary infections in lung transplantation(*45*) and in nosocomial infections in the ICU(*41*), pneumonia may be caused by gram-positive or multiple bacterial species(*46*). Hence in the longer term, this diagnostic platform will require a matching gram-positive imaging agent, such as fluorescently labelled Vancomycin(*29*) or other gram-positive selective ligands, or alongside other bacterial and inflammation specific probes(*47, 48*) as OEM has the potential to be multiplexed (*25, 44*). It is also feasible to exploit other properties to further distinguish bacterial labelling from intrinsic lung autofluorescence such as fluorescence lifetime imaging or a spectrally distinct fluorophore.

The proof-of-concept study was performed to determine if Gram-negative bacteria could be labelled *in situ* in the distal human lung; consequently, although our ICU cohort demonstrates promise in this regard, larger studies are needed to fully address the distinction between colonization or true infection with this modality. However, the absence of a true gold standard for bacterial infection in the mechanically ventilated patient, makes this challenging. Nevertheless, as demonstrated, high resolution OEM coupled with a fluorescent probe was able to delineate bacterial presence in distinct segments of the pulmonary tree (such as the acinar gas exchanging unit) and associated imaging parameters such as cellular infiltrates. The specificity, safety and rapid readout of this technology platform represent potentially significant advantages over current technologies, and now require further large scale validation.

Methods

Study Design: The primary objective of the clinical study was to determine if NBD-PMX could label gram negative bacteria *in situ* in the human lung and be imaged using OEM over human autofluorescence. This was an early stage single arm exploratory study assessing the ability to microdose the compound topically in the lung and image in the intended manner. No randomisation or blinding was undertaken as all patients received the compound, though all investigators were unaware of the final microbial results at the time of the procedure. Informed consent was taken from all patients or from a personal legal representative. Adverse events and serious adverse events were recorded. The study (ClinicalTrials.gov identifier: NCT02491164) was approved by Regional Ethics Committee (REC no: 15/SS/0126).

Ethics Statement: All experiments using human samples in vitro were performed following approval of the appropriate regional ethics committee (REC) and with informed consent of the patients. BAL for alveolar macrophages: (REC no: 07/S1102/20), blood for isolation of neutrophils and mononuclear cells: (REC no: 08/S1103/38) and human lung tissue: (REC no: 13/ES/0126). Animal experiments were performed under UK Home Office Animals Scientific Procedures Act 1986 (Project License Number 60/4434). Ovine lungs were from ewes destined for cull and were euthanized under Schedule 1 of Animals (Scientific Procedures) Act 1986.

Patient Cohorts and procedure: Two cohorts were assessed; i) an outpatient population of patients with bronchiectasis and ii) a cohort of mechanically ventilated patients with suspected pneumonia in the ICU. Patients with known bronchiectasis were recruited from the Regional Specialist Bronchiectasis service. All patients underwent written and informed consent and underwent pre-bronchoscopy peripheral blood sampling and X-ray imaging and had previously performed Computerized Tomography (CT) imaging. Bronchoscopy was undertaken in a dedicated endoscopy suite and a flexible fibreoptic bronchoscope (Olympus BF-1T260 or Olympus BF-260) with conscious sedation using intravenous benzodiazepine and/or short acting opiate, in keeping with routine practice in our institution. Patients were monitored continuously throughout the procedure and subsequently in a

clinical environment for 4 hours, following which they were discharged home. All patients were contacted at 24 hours to assess for any adverse events. For the ICU cohort, consent was obtained from a personal legal representative as patients did not have capacity. These patients were identified by the attending ICU consultant where there was suspected pneumonia, and the patient was due to undergo bronchoscopy and lavage. In ICU, all procedures were done at the point-of care and the APACHE II score(*49*) and associated Hospital Mortality Probability Score calculated. The segment for NBD-PMX administration and OEM was chosen based upon the most affected region based on CT (for bronchiectasis patients) or guided by the chest x-ray changes and endobronchial examination and targeting of the segment with the greatest degree of mucopurulence. If the changes were widespread, then the posterior segment of the right lower lobe would be targeted. For all patients, the bronchoscope was navigated to an affected segment where baseline (pre-fluorescent probe) imaging was undertaken using a clinically approved 488nm laser excitation source OEM imaging system (Cellvizio, Mauna Kea Technologies) and 1.4 mm Alveoflex fibre recording at 12 frames per second, through the working channel of the bronchoscope. This clinical system recorded a circular field of view of ~600 μm diameter. GMP manufactured NBD-PMX was instilled into the bronchopulmonary segment via a flexible catheter (1.5mm APC catheter, ERBE). Following removal of the flexible catheter, the Alveoflex was re-inserted into the working channel and images were recorded for up to 2 minutes. BAL was subsequently undertaken in the same segment and bacteria were enumerated and identified. Sputum culture was also obtained where possible for the bronchiectasis cohort. Patients were monitored continuously in ICU post procedure and were discharged from the study at 4 hours. **Bacterial Culture:** Bacterial strains were grown and counterstained as previously described(*47*). Strains used include *P. aeruginosa* (PA01- ATCC 47085), *P. aeruginosa* (J3284-clinical isolate), *A. baumannii* (J3433 Clinical Isolate), *S. maltophilia* (J3270 Clinical Isolate), *K. pneumoniae* (ATCC BAA1706), *E. coli* (ATCC 25922), *H. influenzae* (Clinical Isolate), *MRSA* (ATCC 25923), *MSSA* (ATCC 252), *S. pneumoniae* (D39 NCTC 7466), *B. cenocepacia* (J2315 and K56-2 both clinical isolates) and GFP fluorescent *S. aureus* (RN6390-Gfp-EryR). Bacterial cultures were reconstituted to 0.5 OD_{595}nm for confocal assays, 0.01 OD_{595}nm for flow cytometry or 2 OD_{595}nm for ovine ex vivo lung experiments. Colony forming units per milliliter (CFU/mL) were enumerated by plating serial dilution to 8th log_{10} dilution on

broth/blood agar plates and incubation at 37 °C for 16 hours (for *S. pneumoniae* supplemented with 5% CO_2). Identification of bacterial species was confirmed through the bacterial diagnostic laboratories, Royal Infirmary of Edinburgh, Edinburgh, UK.

Neutrophil and monocyte isolation: Cells were isolated from the peripheral blood of healthy human volunteers as previously described[50]. Briefly, peripheral venous blood was mixed with 3.8% citrate, centrifuged (350g for 20 minutes) with removal of the plasma layer, mixed with 6% dextran and rested to allow sedimentation. The upper layer was removed and layered on discontinuous Percoll gradients of 55%/68%/81%; the monocyte layer was isolated from the 55%/68% interface and neutrophil predominant layer at 68%/81% interface.

BAL Macrophage Isolation: Bronchoalveolar lavage (BAL) was collected from patients with pulmonary fibrosis undergoing routine bronchoscopy for a non-infective indication. The bronchoscope was navigated to the right middle lobe and wedged, a BAL was undertaken with 40ml instillations of 0.9% NaCl. Retrieved BALF was was filtered through a 40μm cell strainer and centrifuged at 1200rpm for 10 mins at 4°C. The cell pellet was resuspended and used in co-culture experiments. .

Emission Spectra: The fluorescence emission of NBD-PMX solutions were measured in a Synergy H1 Multi-Mode Spectrophotometer (BioTek) upon excitation at 450 nm in increasing concentrations of DMSO (Sigma-Aldrich).

In vitro bacterial labelling and confocal assessment: 8-Well Lab-Tek II Confocal Chambers (VWR were coated in fibronectin (for mammalian cell experiments) or poly-D-lysine (for bacteria) at 37 °C for 20 minutes, then washed in PBS. Bacteria were counterstained and co-incubated with NBD-PMX at the required concentrations in a single 8-well chamber. For co-culture assays, neutrophils, mononuclear cells or alveolar macrophages were seeded in each well (1 x 10^5 per well) for 20 minutes with Syto 60 (Invitrogen) (5 μM) and non-adherent cells aspirated prior to bacterial and NBD-PMX inoculation. For imaging in human lung tissue, lung tissue was dissected into thin sections and incubated in a 48 well plate with bacteria. To each well, 5 μM Syto 82 (Invitrogen) and NBD-PMX at final

concentration was added, incubated for 15 minutes and without a wash step was placed on a glass slide and a 13 mm coverslip was placed over the sample and edges sealed. A laser-scanning confocal imaging system (LSM510; Carl Zeiss), incorporating an upright Axioskop FS2 microscope (63× objective) was used. 'Green' fluorescence (for NBD) was excited with a dedicated 488 nm line (detected with meta detector at 500-530 nm), Syto 82 nuclear acid dyes were excited with a dedicated 543 nm line (detected with meta detector at 570-610 nm) and Syto 60 nuclear acid dyes were excited with a dedicated 633nm line (detected with a meta detector at 660-700 nm). Analysis was performed with ImageJ (version 1.46r, National Institutes of Health); the Syto channel was thresholded and the ROI generated was quantified on the NBD channel. All experiments were performed at least three times unless otherwise stated. For analyses where direct comparison of fluorescence was made, the confocal settings for the NBD channel were kept identical between experiments.

Flow Cytometry: NBD-PMX (50µL) or PBS were added to 50µL of counterstained bacteria and incubated for 5 minutes at 37ºC. Samples were diluted to 500µL immediately prior to analysis using BD FACSCalibur (Becton Dickenson, San Jose) flow cytometer capturing 50,000 of counterstained gated events. Voltages remained constant throughout the experiment and data was collected on a logarithmic scale. Analysis was performed using FlowJo version 7.6.5 (TreeStar Inc.) where the counterstain was gated to eliminate debris and artefact, followed by histogram analysis of the FL-1 (probe) channel. For quantification, the mean of the FL-1 channel was recorded and data presented as the mean fluorescence from independent experiments.

GLP Toxicity Study: A single dose, intra-tracheal rat toxicity study was undertaken by Covance Laborotories (Harrogate, UK) under GLP conditions and conducted in accordance with the requirements of the Animals (Scientific Procedures) Act 1986. Crl:WI(Han) strain rats (Charles River Laboratories) were acclimatised for 2 weeks prior to a single intratracheal dose of 100 µg NBD-PMX or PBS control. Animals were monitored daily, provided with *ad libitum* water and maintenance diet. Animals were weighed daily, and sub-groups were sacrificed at 48 hours or day 14 post dosing (dosing day as day 1). Assessments were undertaken by the contract research organization (CRO). Blood

sampling from the abdominal aorta of animals at necroscopy was obtained for haematology (by flow cytometry methods), coagulation (by turbidometry methods) and clinical chemistry (by optimized UV, colorimetric, bromocresol green or Ion-selective electrode methods) and analysed by the CRO under GLP conditions.

Ovine model: Ewes which were destined for cull were terminally euthanized with an overdose of anaesthetic. The right pulmonary artery was identified, cannulated and perfused with 1000ml 0.9% NaCl with free drainage from the left ventricle. The trachea was intubated with an 8.0 endotracheal tube and lungs were placed in a neonatal incubator with an ambient temperature of 37°C, humidity of 65% and ventilated using a Pressure Controlled Ventilator (Vivo PV 403, Breas Medical). For positive control data GFP expressing *S. aureus* were instilled via a flexible catheter (1.5mm APC catheter, ERBE) and bronchoscopic guidance into a naive segment and subsequently imaged with a clinically approved OEM imaging system (Cellvizio, Mauna Kea Technologies,) capturing images at 12 frames per second with a field of view of ~305x430 μm. Imaging was undertaken with a 1.4 mm Alveoflex fibre, using a 488nm laser excitation source with constant laser power between experiments. For in situ bacterial labelling following 1 hour of optimal ventilation, bronchoscopy (Pentax EB-1530 T3 Video Bronchoscope) was undertaken with proximal wedging of the bronchoscope at individual segments followed by instillation of 2 mL of bacteria or PBS control via a flexible catheter (1.5mm APC catheter, ERBE). One hour later, a separate catheter was introduced into the working channel and NBD-PMX instilled into the distal bronchopulmonary segments. Then the Alveoflex fibre was passed down the working channel and the same segment was imaged with up to 5 transbronchial passes and recorded (recorded at 12 frames per second for minimum of two minutes). For BAL, the bronchoscope was wedged distally and 20 mL of 0.9% NaCl instilled and carefully withdrawn with lavage yields of 40-50%. Control segments were anatomically distinct and/or in the contralateral lung and images prior to the introduction of bacterial cultures to the lung to prevent contamination. The bronchoscope was decontaminated between each bacterial segment imaged.

Image analysis: Bespoke image processing algorithms were developed to detect bacteria (file S1 and S2), which appear as bright speckles in OEM imaging, utilizing two commonly used image processing algorithms, namely Laplacian of Gaussian (LoG) and Difference of Gaussian (DOG) filtering[51-53]. The algorithm was developed in the Matlab software environment (MathWorks Inc.) and all digital videos were transferred offline in the manufacturer's proprietary file format (.mkt) from Cellvizio (Mauna Kea Technologies) to a PC workstation. Each video file was deconstructed into individual frames, with each frame being processed independently. For each frame, irrelevant background content was suppressed by subdividing the image into multiple parts, calculating the average regional pixel signal intensity, and then suppressing all pixels within the region, which were below an experimentally derived cut-off value of between 4 to 6 times the standard deviation. Each frame then underwent LoG and DoG filtering to enhance bright spots (combined filter applied); and the center of each bright spot was localized, and the size and coordinates of its location recorded. A further criterion defined the maximum spot size (range from 1-7) so as to discard clusters of unresolvable bright/enhanced regions (size 4 or more) which could not be attributed to the detection of bacteria. Frames were considered positive if there were >80 dots per frame for ex vivo data. This arbitrary cut off value was selected based upon ex vivo negative (PBS segments) and positive control segments (GFP bacteria). Data is presented as the proportion of frames over a video sequence containing >80 dots per frame. For in vivo human imaging, the field of view was doubled and consequently a dot per frame of >160 was assigned. Furthermore, as there was a higher proportion of background noise due to increased elastin autofluorescence in aged humans, a second size criterion was applied excluding small single pixel dots (excluding size 1) to diminish noise. As not all frames contained relevant information, due to positioning of the OEM fiber or movement, redundant frames from the in vivo study were not included in the analysis. For inclusion, a target must have been present in a sequence of greater than 5 continuous frames. Frames were excluded if any of the following were present; i) absence of background fluorescence, ii) motion blur present, iii) >50% of image obscured due to fluid bubbles or iv) large airway or bronchus imaging.

Statistical Analysis: All experiments were performed at least three times and results expressed as mean ± SEM, unless otherwise stated. Data was analysed by Student's t-test, two-sample t-test or ANOVA and analyses are decribed in each figure legend. Binary classifications are demonstrated as a reciever operator characteristics curve with the area under the curve (AUC) and 95% confidence intrevals. Significance was determined as $p < 0.05$ and all analyses were undertaken on GraphPad Prism (version 5.01 for Windows, GraphPad Software).

Supplementary Materials

Supplementary Methods

Figure S1: Progressive lengthening of the linker leads to loss of gram-selectivity

Figure S2: NBD-PMX has improved signal-to-noise at lower concentrations in the presence of pulmonary surfactant

Figure S3: Binding with polymyxin resistant strains of *B. cenocepacia* is reduced compared to *P. aeruginosa*

Figure S4: NBD-PMX demonstrates no cell membrane toxicity and no in vivo toxicity after intratracheal instillation in mice

Figure S5: OEM coupled with NBD-PMX enables bioburden estimation in the distal ovine lung

Figure S6: NBD-PMX labels gram-negative bacteria endobronchially

Figure S7: Ex vivo confocal labelling of species from BAL in patient D11

Figure S8: Synthetic route of NBD-PMX probe.

Figure S9: Analytical high performance liquid chromatography of NBD-PMX.

Figure S10: Analytical high resolution mass spectrometry of NBD-PMX.

Table S1: NBD-PMX demonstrates no changes in weight, haematological, coagulation or clinical chemistry parameters in GLP toxicity study

Table S2: NBD-PMX is stable in aqueous formulation over 24 months

Table S3: Bronchiectasis patient demographics, blood results, adverse events and microbiological growth

Table S4: ICU patient demographics for six patients included in the analysis, blood results, adverse events and microbiological growth

Supplementary File S1: Matlab file containing image analysis algorithm for pre-clinical images.

Supplementary File S1: Matlab file containing image analysis algorithm for clinical images.

Movie S1: GFP expressing S. aureus instilled in ovine bronchopulmonary segment and imaged with OEM.

Movie S2: Patient D1 imaged post administration of NBD-PMX (no bacterial signal seen).

Movie S3: Patient D2 imaged post administration of NBD-PMX (bacterial signal seen).

Movie S4: Patient D3 imaged post administration of NBD-PMX (no bacterial signal seen).

Movie S5: Patient D4 imaged post administration of NBD-PMX (no bacterial signal seen).

Movie S6: Patient D5 imaged post administration of NBD-PMX (bacterial signal seen).

Movie S7: Patient D6 imaged post administration of NBD-PMX (bacterial signal seen).

Movie S8: ICU patient D7 imaged post administration of NBD-PMX (bacterial signal seen).

Movie S9: ICU patient D8 imaged post administration of NBD-PMX (no bacterial signal seen).

Movie S10: ICU patient D9 imaged post administration of NBD-PMX (no bacterial signal seen).

Movie S11: ICU patient D11 imaged post administration of NBD-PMX (bacterial signal seen).

Movie S12: ICU patient D12 imaged post administration of NBD-PMX (no bacterial signal seen).

Movie S13: ICU patient D13 imaged post administration of NBD-PMX (no bacterial signal seen).

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Author Contributions: SVC, AMF, TA, NA and MB performed chemical design and synthesis. ARA, NM, ES, BM, CH, MB and KD designed and performed the in vitro biological assays. ES and KD designed and performed the murine work. ARA, NM, TC, DSC, ES and KD designed, set-up and performed the ex vivo ovine lung experiments. ARA, TC, AM, ATH, AMS, TW, CH and KD designed and undertook the in vivo study. ARA, NM and KD undertook data analysis. NH and JG provided cells from BALF and clinical isolates of bacterial strains and microbiology guidance respectively. CG designed the image analysis algorithm with input from ARA, NM and KD. CH, MB, TW and KD conceived and supervised the project. ARA and KD wrote the manuscript. All authors approved the manuscript.

Competing Interests: CH, MB and KD have a shareholding in Edinburgh Molecular Imaging Ltd. KD has received travel and attendance fees from Mauna Kea Technologies as a consultant for an advisory board. ARA, TC, NM and KD have received travel fees for attendance at educational conferences supported by an unrestricted educational grant from Mauna Kea Technologies. TC has received travel fees for attendance at an educational conference from Edinburgh Molecular Imaging Ltd. KD, MB, CH, TW, NM, NA are inventors on a patent (WO2016075483A1) held by The University Court Of The University Of Edinburgh that covers the probe and method of use.

Figure 1: NBD-PMX labels gram-negative bacteria in a concentration dependent manner, with fluorescence amplification in hydrophobic environments. A) Structure of NBD-PMX; B) NDB-PMX fluorescence in relation to increasing concentrations of DMSO (NBD-PMX at 5µM), n=3. C) Left: Fluorescence quantification of *P. aeruginosa* imaged on a benchtop confocal microscope in the continued presence of increasing concentrations of NBD-PMX. Images show representative images at denoted concentration of NBD-PMX, scale bar represents 5µm. Right: Each point on graph represents the mean (+/- SEM) of three independent experiments (n=3) where at least three fields of view were quantified with a single site non-linear fit of data. D) Representative flow histograms for unstained bacteria (grey histograms) or NBD-PMX stained bacteria (5µM) (dotted line). Graph shows quantification of flow cytometry data for unstained bacteria (white bars, normalized) and NBD-PMX (grey bars). Bars represent means (+/- SEM) from n=3, analysis by Student's t-test, ns-not significant, *=p<0.05, **=p<0.01. E) Bacterial panel with NBD-PMX 1µM (green) and counterstain with Syto-82 (red). Gram-positive bacteria are indicated by the dotted box and are shown with their counterstain to demonstrate correct focal plane, scale bar represents 5µm. F) Quantification of bacterial panel with NBD-PMX (1µM) with gram-positive bacteria (white bars) and gram-negative (black bars). Bars show mean fluorescence (+/- SEM) from n=3, where at least three fields of view were assessed. Analysis by Student's t-test, *=p<0.05, **= p<0.01, ***=p<0.001.

Figure 2: NBD-PMX showed selectivity for bacteria over mammalian cells. Co-culture experiments of freshly isolated human neutrophils (A), freshly isolated human mononuclear cells (B) and human alveolar macrophages retrieved on bronchoalveolar lavage (C) with *P. aeruginosa* imaged in the continued presence of with NBD-PMX (5µM). Green panels demonstrate NBD fluorescence, middle panels demonstrate nuclear counterstain (Syto-60) and merge images shown on right. Plot profiles corresponding to yellow arrows shown on right. D) Confocal image of human lung tissue co-cultured with bacteria and imaged following labelling with NBD-PMX (5µM). 2 panels; left panel shows NBD and autofluorescence with excitation at 488nm and right panel shows merge with counterstain. White arrows indicate bacterial labelling, blue arrows demonstrate epithelial cells and yellow arrows demonstrate elastin autofluorescence. All experiments n=3, representative images shown, Scale bars represent 10µm.

Figure 3: NBD-PMX labelled gram-negative, but not gram-positive bacteria in situ in ex vivo ovine lungs. A) Experimental set-up. Image demonstrating the anatomically distinct pulmonary segments of the ovine lung and timeline outlining the experimental protocol of retrieval, ventilation, bacterial instillation and NBD-PMX instillation and OEM imaging. B) Representative OEM images of control, gram-positive, in the positive control (GFP *S. aureus*) and in gram-negative segments . C) Lavage was enumerated for bacterial counts, n=4 for all bacteria except *S. pneumoniae* where n=3, analysis by one-way ANOVA, ns=not significant. D) Analysis of entire videos for control segments (n=7), MSSA (n=5), MRSA (n=4), *S. pneumoniae* (n=4), *P. aeruginosa* (n=4), *K. pneumoniae* (n=5) and *E.coli* segments (n=5) showing the proportion of frames with >80 dots per frame for the gram-negative (grey bars) , gram-positive segments (white bars) and control; bars represent mean (+/- SEM), *=p<0.05, **=p<0.01, ns=not significant, Student's t-test. Receiver operator characteristics of image analysis videos; E) For control (n=7) or all gram-negative videos (n=14) the area under the curve was 1.0 (95% Confidence intervals (95%CI) 1.0-1.0), p=0.0002592. F) For gram-negative segments (n=14) compared to gram-positive segments (n=13) the AUC was 0.9945 (95%CI 0.9768-1.012), p<0.001.

Figure 4: NBD-PMX labels gram-negative bacteria in vivo in humans when administered endobronchially and imaged with OEM. A) Representative alveolar images of baseline imaging (left) and post administration of NBD-PMX (middle) in six patients with bronchiectasis, with single frame analysis shown on right. B) Graph demonstrates summary image analysis of individual frames from baseline imaging and post NBD-PMX. Mean number of frames per video analysis was 443 frames, data shown as proportion of frames with >160 dots detected per frame.

Figure 5: NBD-PMX administration and imaging in six mechanically ventilated patients in ICU demonstrated a gram-negative bacterial signal in two patients. Representative alveolar images of baseline imaging (left) and post administration of NBD-PMX in six patients with pulmonary infiltrates and suspected pneumonia. Cellular infiltrates are indicated by white arrows. Graph demonstrates the frame-by-frame analysis of each video sequence post NBD-PMX administration. Each point represents analysis of a single frame and the vertical line represents the mean, with the % above the threshold of 160 dots per frame shown on the right in red.