Susceptibility of Pseudomonas aeruginosa recovered from cystic fibrosis patients to murepavadin and thirteen comparator antibiotics

Susceptibility of *Pseudomonas aeruginosa* recovered from cystic fibrosis patients to murepavadin and thirteen comparator antibiotics

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Running title: Murepavadin susceptibility of *P. aeruginosa* in CF

Synopsis

Objectives: To determine the *in vitro* antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates cultured from cystic fibrosis (CF) patients and explore associations between strain sequence type and susceptibility.

Patients and Methods: Fourteen antibiotics and antibiotic combinations, including the novel antibacterial peptide murepavadin, were tested for activity against 414 *Pseudomonas aeruginosa* isolates cultured from respiratory samples of CF patients. The complete genomes of the isolates were sequenced and minimum spanning trees were constructed based on the sequence types (STs).

Results: Percentage resistance (R) according to CLSI-2019 breakpoints were as follows: cefepime 14%, ceftazidime 11%, ceftazidime-avibactam 7%, ceftolozane-tazobactam 3%, piperacillin-tazobactam 12%, meropenem 18%, imipenem 32%, aztreonam 23%, ciprofloxacin 30%, gentamicin 30%, tobramycin 12%, amikacin 18%, and colistin 4%. Murepavadin MIC_{50} and MIC_{90} were 0.12 mg/L and 2 mg/L, respectively. There were no apparent clonal clusters associated with resistance, but higher MICs did appear to occur more often in STs with multiple isolates than in single ST isolates. In general the CF isolates showed a wide genetic distribution.

Conclusions: *P. aeruginosa* CF isolates exhibited the lowest resistance rates against ceftolozane-tazobactam, ceftazidime-avibactam and colistin. Murepavadin demonstrated the highest activity on a per weight basis and may therefore become a valuable addition to the currently available antibiotics for treatment of respiratory infection in people with CF.

Introduction

Cystic fibrosis (CF) is a life-limiting inherited disease with a frequency of approximately 1 in 2500 live births. Chronic bacterial pulmonary infection leads to irreversible damage of the lung structure and to a decline in lung function, which is the main cause of mortality and morbidity (1). *Pseudomonas aeruginosa* is the most frequently isolated pathogen, chronically infecting up to 80% of adult CF patients (1). Pulmonary exacerbations occur frequently in people with CF chronically infected with *P. aeruginosa* and are associated with reduced survival and quality of life. Inhaled suppressive antibiotic therapy has been fundamental in improving quality of life, preserving lung function and reducing exacerbation frequency in CF patients chronically infected with *P. aeruginosa* (1,2). As the median predicted survival of CF birth cohorts now exceeds 40 years and the number of surviving adults is increasing, treatment with inhaled antibiotics may be required for decades (3,4). Unfortunately, CF pathogens are progressively more resistant to available antibiotics and up to 45% of CF patients are colonised with multidrug-resistant (MDR) isolates (5). Novel antibiotic agents for intravenous treatment of exacerbation and for inhalation therapy are therefore urgently needed.

Murepavadin, a 14-amino-acid cyclic peptide antibiotic, represents the first member of a novel class of antibacterials targeting the outer membrane protein. The drug binds to the lipopolysaccharide transport, protein D, in the outer membrane of *P. aeruginosa*, causing lipopolysaccharide alterations and ultimately killing the bacterium (6). The drug exhibits specific bactericidal activity against *P. aeruginosa*, and it has activity against isolates resistant to all or virtually all other commercially available antibiotics (6). Murepavadin has little to no effect on other bacterial species, which could possibly lead to a lower risk of eliciting cross-resistance with other antibiotics. Its development as intravenous formulation was recently halted due to

unexpected kidney injury findings. However, the development of a formulation for inhaled therapy is ongoing and may prove a valuable addition to the therapeutic options for treating *P. aeruginosa* lung infection in people with CF or other patients with chronic bronchial colonization with this organism, such as those with bronchiectasis.

In this study, we determined the activity of murepavadin and thirteen licensed antipseudomonal antibiotics used in CF care, against clinical CF *P. aeruginosa* isolates. To ensure that a sufficiently diverse and representative population was tested, the isolates were typed using a multi-locus sequence typing (MLST) approach with whole genome sequencing (WGS) data.

Materials and methods

Isolates

Pulmonary *P. aeruginosa* isolates from CF patients were selected as follows: The Netherlands (n=238): 130 isolates with unique sequence types (STs), collected in 2007 (7) and 2013, 20 isolates from 10 patients collected with intervals of 5 to 10 years between 2007 and 2014, and 88 prospectively collected isolates in 2015-16 which could include multiple isolates with different morphotypes from one specimen. Spain (n=114): 100 isolates collected in a multicenter national CF study in 2013 and 2014 (8), and 14 isolates from three patients with changing morphotypes over a four-year period. Northern Ireland (n=58): 38 isolates collected in previous multicenter CF studies (9,10), 19 clinical isolates collected in 2015-16, and one clinical strain commonly used in animal models of infection (Q502). Australia (n=4): clinical isolates from 2015-16. Mucoid and small colony morphotypes were recorded. Age group (pediatric versus adult) was available for 399/414 isolates. Identification of all isolates was confirmed by matrix-assisted

laser desorption/ionisation time-of-flight (MALDI-TOF, Bruker Daltonics, Germany) and by whole genome sequencing.

Susceptibility testing

Minimal inhibitory concentrations (MICs) were determined in cation adjusted Mueller-Hinton broth by standard ISO broth microdilution with two frozen panels, one of which was supplied by TREK Diagnostic Systems (Westlake, Ohio) with ceftazidime, aztreonam, meropenem, imipenem, ciprofloxacin, tobramycin and colistin; and one which was made in-house by Polyphor AG (Basel, Switzerland), with ceftazidime-avibactam, cefepime, piperacillin-tazobactam, gentamicin, amikacin and murepavadin. The decision to produce the second set of frozen panels was prompted by the prolonged time required for commercial production. The antibiotics and their ranges tested were: cefepime (0.12-128 mg/L), ceftazidime (0.25-256 mg/L), ceftazidime-avibactam (0.25-256 mg/L), piperacillin-tazobactam (0.25-256 mg/L), meropenem (0.06-64 mg/L), imipenem (0.125-128 mg/L), aztreonam (0.25-256 mg/L), gentamicin (0.125-128 mg/L), tobramycin (0.125-128 mg/L), amikacin (0.125-128 mg/L), ciprofloxacin (0.03-32 mg/L), colistin (0.25-16 mg/L), and murepavadin (0.016-16 mg/L); tazobactam and avibactam were tested at fixed doses of 4 mg/L. Susceptibility to ceftolozane-tazobactam (range 0.016-256 mg/L) was determined by gradient diffusion testing using the Liofilchem MIC Test strip (Liofilchem, Abruzzi, Italy) as per the manufacturer's instructions. (At the time of the study this antibiotic combination was not available as pure compound for use in the frozen plates). *Escherichia coli* ATCC strain 25922, *P. aeruginosa* ATCC strain 27853, and MDR *P. aeruginosa* strain PA3140 (with a murepavadin MIC of 0.25 mg/L) were included as run controls. MIC_{50} and MIC_{90} values were determined and percentages susceptible,

intermediate and resistant isolates were calculated using CLSI and EUCAST breakpoints (11,12).

Whole genome sequencing (WGS) and analysis

Bacterial DNA was purified using the Qiacube with the DNeasy Blood & Tissue kit with the enzymatic lysis protocol (Qiagen, Carlsbad, CA) and used to prepare a library for sequencing with the MiSeq or Nextseq (Illumina, San Diego, CA) platforms, using the NexteraXT library prep kit (Illumina). Contigs were assembled with SPAdes genome assembler v.3.6.2. The assembled contigs were used to determine the STs with the MLST 2.0 (Multi-locus Sequence Typing) module from the Center for Genomic Epidemiology (DTU, Copenhagen, Denmark, accessed October 28, 2018) (13) and PubMLST (https://pubmlst.org/; accessed January 11, 2019) (14).

Results

Susceptibility testing

MIC_{50}, MIC_{90} and percentage susceptibility for the antibiotics tested are listed in Table 1. Of all drugs tested, murepavadin expressed the highest activity on a per weight basis, with an MIC_{50} of 0.12 mg/L and an MIC_{90} of 2 mg/L (supplemental Figures S1A-B). Eleven strains (2.7%) had MICs exceeding 16 mg/L, but this was not specifically associated with elevated MICs for the other antipseudomonal antibiotics. The second lowest MIC_{50} value was that of meropenem (0.25 mg/L); however, the MIC_{90} of meropenem was higher: 16 mg/L. Applying both EUCAST and CLSI breakpoints, 76% of the strains were susceptible to meropenem. Ceftolozane-tazobactam

and colistin had identical MIC_{90} values to that of murepavadin (2 mg/L), but higher MIC_{50} values.

Ceftolozane-tazobactam was the drug with highest susceptibility rate: 95% of the isolates had MICs ≤4 mg/L, the breakpoint for susceptibility of both EUCAST and CLSI. Colistin and ceftazidime-avibactam demonstrated susceptibility greater than 90%. Susceptibility to ceftazidime-avibactam was higher than to ceftazidime alone (Table 1 and supplemental Figure S2), indicating a role for β-lactamases in the resistance of *P. aeruginosa* isolates to this cephalosporin. High resistance rates were found in particular for ciprofloxacin, gentamicin and aztreonam. Of the aminoglycosides tested, tobramycin was the most active, with MIC_{50} and MIC_{90} values fourfold lower than those of gentamicin and amikacin (Table 1 and supplemental Figure S3).

MIC_{50} and MIC_{90} values were, in general, equal or higher for isolates from the 288 adult patients than for those from the 111 pediatric patients; this difference was significant for ten of the fourteen antibiotics (p-value<0.05 by Mann Whitney U test). Sixty-three isolates were typed as small colony variants and 112 isolates were mucoid. The MICs of the mucoid isolates were generally comparable or lower to those of the total population (p<0.05 for ceftazidime-avibactam, piperacillin-tazobactam, aztreonam and colistin by Mann Whitney U test); small colony variants displayed higher MIC values, in particular higher MIC_{90} values (p<0.05 for murepavadin and the three aminoglycosides). No differences in MIC_{50} and MIC_{90} were observed between the different countries.

Whole genome sequencing

A whole genome sequence was obtained for 412 of the 414 tested isolates, which fell into 165 different STs. Figure 1 shows the relationship between the numbers of isolates with different STs, the genetic relation between the STs, and the countries where the strains were isolated. Isolates from The Netherlands, Spain and Northern Ireland largely overlapped, although some small clusters could be observed that differed between the three countries, such as a cluster of ST406 isolates in The Netherlands (the Dutch epidemic strain) (15), of ST508 isolates in Spain, and of ST146 isolates in Northern Ireland (Liverpool epidemic strain) (15). A limited clustering of MICs $\geq MIC_{90}$ could be observed in certain STs with a higher number of isolates; small clusters of up to three isolates and single isolates appeared to have a higher likelihood of a MIC $\leq MIC_{50}$ ($p<0.05$ by Mann Whitney U test for eight of the fourteen antibiotics; see also supplemental Figures S4A-N).

Discussion

In this study, the novel antimicrobial cyclic peptide, murepavadin, was highly active against *P. aeruginosa* recovered from CF patients, displaying a higher activity on a per weight basis than comparator antibiotics. Ceftolozane-tazobactam displayed the lowest percentage resistance of the tested antibiotics; this may be due in part to the higher intrinsic activity of this combination in *P. aeruginosa* and in part to the lack of exposure of CF patients to this relatively novel drug combination (16,17).

Susceptibility of the CF isolates to murepavadin was lower than previously reported for non-CF clinical *P. aeruginosa* isolates, including MDR *P. aeruginosa*; the MIC_{90} value (2 mg/L) was higher than the previously reported 0.12-0.25 mg/L (6,18), even more so in isolates recovered from adult patients. The driving mechanism behind the increased MIC values cannot

be explained by exposure of the bacteria to the drug, nor were there indications for cross-resistance with other antibiotics. Further analysis of the genetic data of these CF isolates may yield an explanation for this phenomenon.

Pharmacokinetic/pharmacodynamic (PK/PD) studies in neutropenic mouse models found that the efficacy of murepavadin correlated best with the concentration-time curve for the unbound fraction of the drug (fAUC)/MIC. The mean fAUC/MIC required for stasis was 27.78 mg/L with the mean fAUC/MIC required for a 1-log reduction 39.85 mg/L. The corresponding values for the epithelial lining fluid (ELF) were 34.5 and 49.4, respectively (19). For non-CF clinical isolates, these PK/PD targets were readily attained with the applied dosage regimens in previous clinical studies (20). The development of murepavadin as an intravenous formulation was halted due to adverse findings in clinical trials. However, the development of murepavadin by inhalation is continued and is supported by recent *in vivo* studies which investigated the pharmacokinetics, tolerability and efficacy of murepavadin administered by intratracheal application in *P. aeruginosa* lung infection models (21).

CF patients generally have chronic polymicrobial respiratory infection with an array of bacterial species, which may be symbiotic or compete with one another. It has been reported that 40-51% of CF patients colonized with *P. aeruginosa* harbor multiple phenotypes of the micro-organism, but that these mostly constitute different growth forms of the same strain (7). In the same report, only 8-11% of colonized patients had more than a single *P. aeruginosa* ST. A number of epidemic strains have been described, which have been associated with lower susceptibility to antibiotics and in some cases with increased virulence (15). However, up to half of CF patients are colonized with unique STs, and this fraction may increase even further as segregation policies in CF centres continue to have an effect.

In order to provide a relevant insight into susceptibility of *P. aeruginosa* in CF patients, a diverse collection of isolates from different countries, some with previously determined STs, was selected. The wide variety of isolates and the fact that the distribution appears to be similar in The Netherlands, Spain and Northern Ireland, suggests that this sample is representative for *P. aeruginosa* isolates from CF patients, at least for those in Western Europe.

Analysis of the distribution of the MICs for the different antipseudomonal antibiotics did not yield clear patterns of clonal clusters associated with resistance. This, and the fact that isolates from pediatric patients were more susceptible than those from adults, is consistent with CF patients mostly acquiring unique *P. aeruginosa* isolates from the environment – isolates which during chronic infection gradually develop resistance. Furthermore, it appeared that STs with only a single isolate more frequently had MICs lower or equal to the MIC_{50}, while STs with multiple isolates may represent epidemic CF isolates that express more antibiotic resistance.

The host and microbe interspecies interactions are an area of much interest, where the response to antibiotics is likely due to factors additional to bacterial killing (1). For example, studies in which sputa from CF patients were analyzed by deep sequencing of 16S rRNA genes have failed to establish differences between the microbiota of CF patients during chronic infection, during acute exacerbations and after systemic antibiotic treatment (22). The correlation between susceptibility test results and outcome of antibiotic treatment is also not clearly established for pulmonary infection in CF (23). Nevertheless, the use of antipseudomonal antibiotics for early eradication of *P. aeruginosa*, for treatment of exacerbations and for chronic suppressive inhalation therapy has significantly contributed to improving the quality of life and life expectancy of CF patients (24). Furthermore, in practice, the choice of antibiotic regimens is guided by susceptibility testing results, particularly when patients fail to respond to first line

220 therapy. Therefore, the results from susceptibility tests in this study suggest that drugs such as
221 ceftolozane-tazobactam and ceftazidime-avibactam may be useful for the treatment of CF
222 pulmonary infections. In addition, murepavadin may be added to this list, if an inhaled
223 formulation is developed.

224 **Acknowledgements**: Dr Tim Kidd kindly provided the Australian isolates for this study. Judith
225 Vlooswijk and Sjoukje van Gorkum performed susceptibility testing, Barry Benaissa-Trouw
226 performed WGS.
227
228 **Funding**: This research project received support from the Innovative Medicines Initiative Joint
229 Undertaking under Grant Agreement no 115721, resources of which are composed of financial
230 contribution from the European Union Seventh Framework Program (FP7/2007-2013) and
231 EFPIA companies in kind contribution.
232
233 **Transparency declarations**: Francesca Bernardini and Glenn Dale were employed by Polyphor
234 AG. All other authors: no conflict of interest to report.
235

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312

313 **Table 1: Minimal inhibitory concentrations and susceptibility of 414 *Pseudomonas aeruginosa* isolates from cystic fibrosis**

314 **patients**

315

	All strains (n=414)		Pediatric (n=111)		Adult (n=288)		Mucoid (n=112)		Small colony (n=63)		EUCAST (all strains n=414)			CLSI (all strains n=414)		
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	%S	%I	%R	%S	%I	%R
Murepavadin	0.12*	2	0.12	1	0.25	4	0.12	2	0.25	8	ND	ND	ND	ND	ND	ND
Cefepime	4	32	4	16	8	64	8	32	8	>128	75	.	25	75	11	14
Ceftazidime	2	64	2	8	2	128	2	8	2	128	80	.	20	80	4	11
Ceftazidime-avibactam	2	8	2	4	2	8	1	8	2	8	93	.	7	93	.	7
Ceftolozane-tazobactam	1	2	0.5	2	1	4	1	2	1	4	95	.	5	95	1	3
Piperacillin-tazobactam	4	128	4	32	4	>256	2	128	2	>256	81	.	19	81	7	12
Meropenem	0.25	16	0.25	4	0.5	16	0.5	16	0.5	16	76	12	12	76	6	18
Imipenem	2	32	2	16	2	32	2	32	1	32	68	.	32	59	8	32
Aztreonam	8	128	8	32	4	256	2	64	2	>256	77	.	23	62	14	23
Ciprofloxacin	1	8	0.5	4	2	8	1	8	2	16	39	.	61	53	17	30

Gentamicin	4	64	4	32	8	64	4	16	8	>128	53	.	47	53	17	30
Tobramycin	1	16	0.5	8	2	16	1	4	2	64	84	.	16	84	5	12
Amikacin	16	64	8	64	16	128	16	64	16	>128	47	21	32	68	14	18
Colistin	1	2	1	2	1	2	0.5	1	1	4	93	.	7	93	3	4

316

317 MIC_{50} / MIC_{90}= Minimal Inhibitory Concentration for 50%/90% or the tested strains; S= susceptible; I= intermediately susceptible; R= resistant; ND = no

318 breakpoint defined. *mg/L

Figure 1: Minimum-spanning tree of 412 *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis patients. Each color represents a different country.

Legend to figure 1:

Minimum spanning tree of 412 *Pseudomonas aeruginosa* respiratory isolates from cystic fibrosis patients, indicating the distribution of isolates over geographic regions. Sequence types (STs) based on seven housekeeping genes as determined by whole genome sequencing. The numbers in the circles indicate the STs assigned by PubMLST (www.pubmlst.org). Xx and xy are isolates with partially deleted housekeeping genes which could not be assigned an ST. The circle size indicates the number of isolates with the same ST. The numbers on the lines between the circles indicate the number of allele differences between two STs.