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Rifampicin potentiation of aminoglycoside activity against cystic fibrosis isolates of *Pseudomonas aeruginosa*

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Objectives: Rifampicin potentiates the activity of aminoglycosides (AGs) versus *Pseudomonas aeruginosa* by targeting the AmgRS two-component system. In this study we examine the impact of rifampicin on the AG susceptibility of cystic fibrosis (CF) lung isolates of *P. aeruginosa* and the contribution of AmgRS to AG resistance in these isolates.

Methods: *amgR* deletion derivatives of clinical isolates were constructed using standard gene replacement technology. Susceptibility to AGs ± rifampicin (at ½ MIC) was assessed using a serial 2-fold dilution assay.

Results: Rifampicin showed a variable ability to potentiate AG activity versus the CF isolates, enhancing AG susceptibility between 2- and 128-fold. Most strains showed potentiation for at least two AGs, with only a few strains showing no AG potentiation by rifampicin. Notably, loss of *amgR* increased AG susceptibility although rifampicin potentiation of AG activity was still observed in the Δ*amgR* derivatives.

Conclusions: AmgRS contributes to AG resistance in CF isolates of *P. aeruginosa* and rifampicin shows a variable ability to potentiate AG activity against these, highlighting the complexity of AG resistance in such isolates.

Introduction

Pseudomonas aeruginosa is a common lung pathogen and a major cause of morbidity and mortality in patients with cystic fibrosis (CF).[1] Aminoglycosides (AGs) are commonly employed in the management of *P. aeruginosa* lung infections in CF[2] although their use is complicated by the toxicity of these agents[3] and development of resistance in the infecting *P. aeruginosa*.[4] To address these issues, AG-potentiating agents have previously been sought, with a focus on those that target AG resistance mechanisms.[5] One AG-potentiating agent, rifampicin, was found to target the envelope stress-responsive AmgRS two-component system (TCS),[6,7] a demonstrated determinant of AG resistance in laboratory[6] and clinical[8] isolates of *P. aeruginosa* that also mediates the AG induction of the AG resistance-promoting MexXY multidrug efflux system.[7] Interestingly, rifampicin only reduced resistance to a subset of AGs, the 4,5-linked AGs neomycin and paromomycin, in a WT laboratory strain and some clinical isolates, although it did increase susceptibility to all AGs, including the more clinically relevant 4,6-linked AGs tobramycin, amikacin and gentamicin, in a clinical isolate in which resistance was attributable to AmgRS-dependent up-regulation of MexXY.[5] To better understand its utility in potentiating AG activity versus clinical strains of *P. aeruginosa*, the impact of rifampicin on the AG susceptibility of a large collection of AG-resistant CF lung isolates was assessed, as was the contribution of AmgRS to AG resistance in these isolates.

Materials and methods

Bacterial strains

Strains K3518–K3691 are tobramycin-resistant CF lung isolates of *P. aeruginosa*, either internationally collected epidemic strains or strains obtained (with consent) from patients attending the Calgary Adult Cystic Fibrosis Clinic (see Table S1, available as Supplementary data at *JAC* Online). Permission from the Conjoint Health Region Ethics Board of the University of Calgary was granted for the collection and analysis of these strains (REB 15-0854). Strains K3716, K3710, K3712, K3715, K3713 and K3714 are Δ*amgR* derivatives of CF strains K3519, K3524, K3527, K3528, K3530 and K3533, respectively.

Construction of ΔamgR derivatives of P. aeruginosa

Despite repeated attempts, construction of *amgR* deletion derivatives of several tobramycin-resistant CF isolates of *P. aeruginosa* using the previously described pEX18Tc::Δ*amgR* suicide vector[6] was unsuccessful. Difficulties in using pEX18Tc to generate deletions in clinical strains has been noted previously[9] and likely stems from the need to simultaneously select for conjugation of the vector into the strain being mutated and its integration into the chromosome (pEX18Tc lacks a *P. aeruginosa* origin of

replication), either of which may be less efficient in clinical strains. To address this issue, a pEX18Tc::Δ*amgR* derivative carrying a temperature-sensitive *P. aeruginosa* origin of replication was constructed, permitting vector conjugation and integration to be carried out separately and sequentially, the former at the permissive temperature and the latter at the non-permissive temperature. Thus, a 1.9 bp PstI fragment carrying a temperature-sensitive origin of replication, mSF^{ts1}, was excised from plasmid pSS255[10] and inserted into the PstI site of pEX18Tc::Δ*amgR*, yielding pEX18Tc::Δ*amgR*-Ts. The vector was introduced into *Escherichia coli* strain S17-1 (selected on 10 mg/L tetracycline) for subsequent mobilization into the clinical strains using a previously described protocol,[8] with modifications. Briefly, aliquots of overnight cultures of S17-1 carrying pEX18Tc::Δ*amgR*-Ts (700 μL) and individual clinical isolates (300–600 μL) were mixed and spotted onto L-agar plates and incubated for 6 h at 30 °C. Following resuspension of the mating mixtures, transconjugants were selected at 30 °C on L-agar plates containing tetracycline (20–100 mg/L) and chloramphenicol (5 mg/L; to counter-select *E. coli*), the exact tetracycline concentration being determined empirically for each strain being mutated. Transconjugants showing the expected temperature-sensitive phenotype (i.e. growth in tetracycline-containing medium at 30 °C, but not at 42 °C) were cultured overnight in tetracycline-containing L-broth at 30 °C, plated on tetracycline-containing L-agar plates and then cultured for 1–2 days at 42 °C to select for plasmid integration into the chromosome. Tetracycline-resistant colonies were streaked onto sucrose-containing [10% (v/v)] L-agar plates, incubated at 42 °C for 1–2 days and sucrose-resistant colonies were then screened for deletion of *amgR* using colony PCR as described previously.[8]

Antibiotic susceptibility testing

The susceptibility of *P. aeruginosa* to AGs was assessed using the 2-fold serial microtitre broth dilution method described previously.[11] MICs were recorded as the lowest concentration of antibiotic inhibiting visible growth after 18 h of incubation at 37 °C. Where indicated, rifampicin was included at ½ MIC for the strains being tested.

Results and discussion

Rifampicin potentiates AG activity versus clinical strains of P. aeruginosa

In a previous study, rifampicin was shown to potentiate the activity of 4,5-linked AGs versus the few clinical strains that were examined, with additional potentiation of 4,6-linked AGs seen in a single isolate.[5] To get a better sense of rifampicin's utility in potentiating AG activity versus clinical *P. aeruginosa* strains, the impact of rifampicin on AG activity versus 45 CF lung isolates provisionally identified as tobramycin resistant was assessed. This included several internationally recognized epidemic strains of *P. aeruginosa* and multiple isolates (median 2/patient) from 12 patients with genotypically distinct infections followed by the Calgary Adult Cystic Fibrosis Clinic.[12] Initially, the susceptibility of these strains to AGs, including tobramycin, was determined in the absence of rifampicin, generally confirming the original tobramycin resistance and, indeed, revealing elevated MICs of multiple AGs (Table 1). At ½ MIC, rifampicin had a variable impact on AG susceptibility that varied with strain and AG (Table 1). As expected, rifampicin had the greatest impact on neomycin, a representative 4,5-linked AG, both in terms of the number of strains for which rifampicin yielded a ≥4-fold decline in MIC (36/45) and the magnitude of the fold decrease (20/45 showed decreases ≥16-fold, with decreases of up to 128-fold seen). Indeed, in several instances it was the only AG whose activity was potentiated ≥4-fold (9/45), reflecting what was seen earlier in the WT strain K767.[5] Instances of rifampicin-promoted reductions in AG MICs ≥4-fold were less common though still substantial for the 4,6-linked AGs (tobramycin, 22/45 strains; amikacin, 22/45; gentamicin, 24/45). The magnitude of the 4,6-linked AG MIC decrease prompted by rifampicin was also generally lower than that seen for neomycin (typically <16-fold; Table 1). For most strains, there was ≥4-fold potentiation of at least two AGs (32/45) and a few examples where no AG potentiation was seen (4/45), with instances of rifampicin potentiation ≥4-fold for all of the 4,6-linked AGs less common (12/45) and for all AGs even less so (10/45). While rifampicin appears to have a substantial impact on AG susceptibility in the CF isolates, with a marked impact on all clinically relevant 4,6-linked AGs in about a quarter of the isolates examined, the wide variability in magnitude and AG impacted in each strain doubtless speaks to the multiplicity of resistance mechanisms that can contribute to AG resistance in *P. aeruginosa*[13] and their presumably variable susceptibility to rifampicin action.

AmgRS contributes to AG resistance in clinical strains of P. aeruginosa

Rifampicin potentiation of AG activity was previously shown to be dependent on the presence of AmgRS,[5] an indication that it was directly or indirectly targeting this TCS. To gain some insights into the nature of the rifampicin potentiation of AGs seen in the current study, as well as to assess the contribution of AmgRS to AG resistance in CF isolates generally, attempts were made to delete *amgRS* in the CF isolates and to determine the impact on both AG resistance and rifampicin potentiation of AG activity. In focusing initially on strains K3518–K3540, and despite repeated attempts, *amgR* deletions were achieved in only six isolates (Table 2). This reflects an ongoing problem with deletion construction in clinical isolates and may be explained by conjugation and/or recombination deficiencies in such isolates, the latter potentially related to DNA sequence variations between the plasmid-borne deletion construct and the corresponding genomic region of the clinical isolates. Nonetheless, loss of *amgR* had a substantive impact on AG resistance in the CF isolates for which deletions could be engineered, with MIC decreases ≥4-fold seen for at least one AG in 6/6 strains and for all 4,6-linked AGs in 3/6 strains (Table 2), an indication that AmgRS is contributing to AG resistance in these isolates. In one case in particular, K3710, the MICs of all four AGs tested decreased a minimum of 16-fold upon loss of *amgR* (Table 2). Surprisingly, however, given the earlier link between rifampicin potentiation and AmgRS, in most cases and for most AGs, rifampicin still reduced AG MICs for the deletion derivatives, in several instances more so than for the AmgR$^+$ parental strains. Thus, rifampicin can clearly impact AG susceptibility independent of AmgRS. The observation, too, that in some instances rifampicin only modestly potentiated AG activity in an AmgR$^+$ strain despite a major contribution of AmgRS to AG resistance [e.g. strain K3524 where rifampicin increased susceptibility to amikacin and gentamicin 2- and 4-fold, respectively, while loss of *amgR* in this strain (see K3710) reduced MICs of these AGs 16- and 32-fold] indicates that rifampicin is unable to reverse all examples of AmgRS-mediated AG resistance. Thus, while rifampicin does have a broad ability to increase susceptibility to individual AGs in clinical strains of

Table 1. Impact of rifampicin on AG susceptibility of CF lung isolates of *P. aeruginosa*[a]

	MIC (mg/L)[c]							
	TOB		AMK		GEN		NEO	
Strain[b]	−RIF	+RIF[d]	−RIF	+RIF	−RIF	+RIF	−RIF	+RIF
K3518	32	**8** (4)	128	64 (2)	256	**64** (4)	512	256 (2)
K3519	32	**2** (16)	128	**8** (16)	128	**4** (32)	256	**64** (4)
K3520	16	8 (2)	32	64 (0.5)	64	64 (1)	256	**64** (4)
K3522	16	**4** (4)	32	16 (2)	32	**8** (4)	1024	**8** (128)
K3524	128	**16** (8)	512	256 (2)	512	**128** (4)	1024	**64** (16)
K3525	16	16 (1)	1	2 (0.5)	64	64 (1)	32	16 (2)
K3526	1	2 (0.5)	8	8 (1)	8	8 (1)	512	**64** (8)
K3527	4	2 (2)	64	**8** (8)	64	**16** (4)	512	**64** (8)
K3528	4	**1** (4)	16	**4** (4)	16	**2** (8)	256	**16** (16)
K3529	32	**4** (8)	4	4 (1)	512	**32** (16)	32	**1** (32)
K3530	16	16 (1)	64	64 (1)	64	64 (1)	1024	**16** (64)
K3531	32	**8** (4)	64	32 (2)	128	**32** (4)	1024	**128** (8)
K3532	16	16 (1)	32	32 (1)	16	32 (0.5)	256	**64** (4)
K3533	16	16 (1)	128	128 (1)	64	64 (1)	1024	**8** (128)
K3534	4	4 (1)	32	**8** (4)	16	8 (2)	512	**8** (64)
K3535	16	16 (1)	64	32 (2)	128	**32** (4)	128	**32** (4)
K3536	32	32 (1)	256	256 (1)	128	64 (2)	1024	**64** (16)
K3537	16	8 (2)	32	16 (2)	64	32 (2)	512	**64** (8)
K3538	64	**8** (8)	512	**128** (4)	512	**64** (8)	512	**4** (128)
K3539	16	8 (2)	128	**32** (4)	256	**64** (4)	2048	**128** (16)
K3540	8	8 (1)	64	64 (1)	64	32 (2)	1024	**16** (64)
K3641	32	16 (2)	32	32 (1)	64	64 (1)	128	128 (1)
K3642	64	**16** (4)	256	**64** (4)	128	**32** (4)	256	128 (2)
K3645	16	**4** (4)	64	**16** (4)	64	**16** (4)	256	**2** (128)
K3647	16	8 (2)	64	**16** (4)	64	32 (2)	32	**8** (4)
K3649	16	8 (2)	64	32 (2)	64	**8** (8)	512	**32** (16)
K3651	16	8 (2)	64	64 (1)	64	32 (2)	128	64 (2)
K3653	32	**8** (4)	128	**32** (4)	64	32 (2)	256	**64** (4)
K3656	64	32 (2)	128	64 (2)	128	64 (2)	512	**128** (4)
K3657	64	32 (2)	256	**64** (4)	256	**64** (4)	512	256 (2)
K3659	128	**32** (4)	512	**64** (8)	256	**32** (8)	512	**64** (8)
K3660	128	64 (2)	512	256 (2)	256	256 (1)	512	256 (2)
K3665	16	**4** (4)	128	**32** (4)	128	**16** (8)	1024	**8** (128)
K3667	16	**4** (4)	64	32 (2)	64	64 (1)	512	**32** (16)
K3669	32	**8** (4)	128	64 (2)	128	64 (2)	256	**8** (32)
K3673	512	256 (2)	4096	**512** (8)	≥4096	**256** (≥16)	512	512 (1)
K3675	64	**16** (4)	256	128 (2)	256	**64** (4)	2048	**256** (8)
K3677	8	4 (2)	32	**8** (4)	32	16 (2)	256	**16** (16)
K3679	32	**4** (8)	128	**16** (8)	128	**16** (8)	512	**8** (64)
K3681	128	**8** (16)	256	**16** (16)	256	**16** (16)	512	**4** (128)
K3683	128	**16** (8)	256	**32** (8)	256	**32** (8)	1024	**128** (8)
K3685	1024	**128** (8)	1024	**128** (8)	2048	**256** (8)	512	256 (2)
K3687	128	**8** (16)	512	**32** (16)	512	**16** (32)	1024	**128** (8)
K3689	16	**4** (4)	128	**16** (8)	16	8 (2)	256	**16** (16)
K3691	16	8 (2)	128	**32** (4)	64	32 (2)	256	**64** (4)

TOB, tobramycin; AMK, amikacin; GEN, gentamicin; NEO, neomycin; RIF, rifampicin.

[a]Susceptibility of *P. aeruginosa* CF isolates to the indicated AGs was assessed in the absence (−) and presence (+) of rifampicin at ½ MIC.

[b]Strains where rifampicin enhanced susceptibility to all tested AGs ≥4-fold are underlined. Strains where rifampicin enhanced susceptibility to all AGs except neomycin ≥4-fold are italicized.

[c]Fold change in MIC of the indicated AG in the presence versus absence of rifampicin is indicated in parentheses. MIC values ≥4-fold lower in the presence of rifampicin are in bold.

[d]Rifampicin was included in AG susceptibility assays at 2 mg/L (K3526), 4 mg/L (K3528, K3532, K3536, K3641, K3656, K3667), 8 mg/L (K3518, K3519, K3522, K3525, K3529, K3530, K3531, K3533, K3534, K3535, K3537, K3538, K3539, K3540, K3651, K3653, K3657, K3660, K3669, K3675, K3677, K3679, K3685, K3687, K3691) or 16 mg/L (K3520, K3524, K3527, K3642, K3645, K3647, K3649, K3659, K3665, K3673, K3681, K3683, K3689).

Table 2. Impact of loss of *amgR* on AG susceptibility of CF lung isolates of *P. aeruginosa*[a]

		MIC (mg/L)[c]							
		TOB		AMK		GEN		NEO	
Strain[b]	AmgR	−RIF	+RIF[d]	−RIF	+RIF	−RIF	+RIF	−RIF	+RIF
K3519	+	32	2	128	8	128	4	256	64
K3716	−	**4** (8)	1	**16** (8)	16	**32** (4)	4	256 (1)	64
K3524	+	128	16	512	256	512	128	1024	64
K3710	−	**8** (16)	4	**32** (16)	16	**16** (32)	8	**16** (64)	8
K3527	+	4	2	64	8	64	16	512	64
K3712	−	4 (1)	0.5	**16** (4)	1	**16** (4)	2	**64** (8)	8
K3528	+	4	1	16	4	16	2	256	16
K3715	−	**1** (4)	0.25	**4** (4)	1	**4** (4)	1	**16** (16)	8
K3530	+	16	16	64	64	64	64	1024	16
K3713	−	8 (2)	4	32 (2)	8	32 (2)	8	**256** (4)	16
K3533	+	16	16	128	128	64	64	1024	8
K3714	−	16 (1)	4	64 (2)	16	32 (2)	16	**256** (4)	8

TOB, tobramycin; AMK, amikacin; GEN, gentamicin; NEO, neomycin; RIF, rifampicin.

[a]Susceptibility of *amgR* deletion derivatives of *P. aeruginosa* CF isolates to the indicated AGs was assessed in the absence (−) and presence (+) of rifampicin at ½ MIC. Results for the AmgR$^+$ parent strains are shown for comparison purposes.

[b]K3716, K3519 Δ*amgR*; K3710, K3524 Δ*amgR*; K3712, K3527 Δ*amgR*; K3715, K3528 Δ*amgR*; K3713, K3530 Δ*amgR*; K3714, K3533 Δ*amgR*. Strains where loss of *amgR* enhanced susceptibility to all tested AGs ≥4-fold are underlined. Strains where loss of *amgR* enhanced susceptibility to all AGs except neomycin ≥4-fold are italicized.

[c]Fold change in MIC of the indicated AG in the absence versus presence of *amgR* is indicated in parentheses. MIC values 4-fold lower in the absence of *amgR* are in bold.

[d]Rifampicin was included in AG susceptibility assays at 2 mg/L (K3715), 4 mg/L (K3528, K3716), 8 mg/L (K3519, K3530, K3533, K3712, K3713, K3714) or 16 mg/L (K3524, K3527, K3710).

P. aeruginosa, its mode(s) of action appear to be varied and, for the most part, undefined.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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