AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Potentiation of Aminoglycoside Activity in *Pseudomonas aeruginosa* by Targeting the AmgRS Envelope Stress-Responsive Two-Component System

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A screen for agents that potentiated the activity of paromomycin (PAR), a 4,5-linked aminoglycoside (AG), against wild-type *Pseudomonas aeruginosa* identified the RNA polymerase inhibitor rifampin (RIF). RIF potentiated additional 4,5-linked AGs, such as neomycin and ribostamycin, but not the clinically important 4,6-linked AGs amikacin and gentamicin. Potentiation was absent in a mutant lacking the AmgRS envelope stress response two-component system (TCS), which protects the organism from AG-generated membrane-damaging aberrant polypeptides and, thus, promotes AG resistance, an indication that RIF was acting via this TCS in potentiating 4,5-linked AG activity. Potentiation was also absent in a RIF-resistant RNA polymerase mutant, consistent with its potentiation of AG activity being dependent on RNA polymerase perturbation. PAR-inducible expression of the AmgRS-dependent genes *htpX* and *yccA* was reduced by RIF, suggesting that AG activation of this TCS was compromised by this agent. Still, RIF did not compromise the membrane-protective activity of AmgRS, an indication that it impacted some other function of this TCS. RIF potentiated the activities of 4,5-linked AGs against several AG-resistant clinical isolates, in two cases also potentiating the activity of the 4,6-linked AGs. These cases were, in one instance, explained by an observed AmgRS-dependent expression of the MexXY multidrug efflux system, which accommodates a range of AGs, with RIF targeting of AmgRS undermining *mexXY* expression and its promotion of resistance to 4,5- and 4,6-linked AGs. Given this link between AmgRS, MexXY expression, and pan-AG resistance in *P. aeruginosa*, RIF might be a useful adjuvant in the AG treatment of *P. aeruginosa* infections.

Pseudomonas aeruginosa is a common nosocomial pathogen (1) and a major cause of morbidity and mortality in patients with cystic fibrosis (CF) (2, 3). Treatment of *P. aeruginosa* infections is complicated by the organism's innate resistance to many antimicrobials, a product of its impressive intrinsic resistome (4) and its access to an array of acquired resistance mechanisms (5, 6), with difficult-to-treat multidrug-resistant (MDR) (7) and extremely drug-resistant (8, 9) *P. aeruginosa* organisms becoming increasingly common. In the face of this intrinsic and acquired multidrug resistance, the use of agents historically used less commonly owing to issues of toxicity (e.g., the polymyxins) (10, 11) and the use of drug combination therapy (12, 13) are increasingly promoted. Still, despite much *in vitro* evidence for synergistic drug combinations being effective against MDR *P. aeruginosa* (14–20), the clinical benefits of drug combinations are less obvious (13, 21, 22).

Aminoglycosides (AGs) have a long history in the management of *P. aeruginosa* infections, particularly in the case of lung infections in patients with cystic fibrosis (23, 24), and are often used in combination with β-lactams (25–27) owing to a well-established synergy between these two antimicrobial classes (18, 20, 26–29). β-Lactam synergy with AGs has been suggested to result from β-lactam-promoted AG uptake owing to cell wall damage or lessening of this barrier. Still, although a β-lactam-promoted increase in the uptake of the AG streptomycin has been seen in various bacteria (30–32), including *P. aeruginosa* (33), synergy between these agents in the absence of β-lactam-enhanced AG uptake has also been noted (32). Fosfomycin, another cell wall synthesis inhibitor, has also been shown to potentiate AG activity against Gram-negative bacteria, including *P. aeruginosa* (34, 35). Still, AGs are ototoxic (36) and nephrotoxic (37), which has hitherto limited their use in treating *P. aeruginosa* infections more generally.

Given the increasing prevalence of multidrug-resistant *P. aeruginosa* and the paucity of useful antipseudomonal agents, AGs may become increasingly important in managing *P. aeruginosa* infections (38). To possibly limit issues with toxicity, these agents can, perhaps, more routinely be partnered with compounds that potentiate their activity and thus enable the use of lower doses of AGs, which should be less toxic (39, 40). In addition to the aforementioned antimicrobials, a number of AG potentiators have been described in the literature. AG potentiation as a result of metabolite-promoted (41) and alkaline pH-promoted (42) generation of a proton motive force that drives AG uptake has, for example, been reported for *Escherichia coli* and *P. aeruginosa*, respectively. Methylxanthines (a class of bronchodilators that includes caffeine) (43, 44), inhibitors of quorum sensing (45–47), a quorum-sensing signal (diffusible signal factor) from *Xanthomo-*

Received 23 December 2015 **Returned for modification** 3 February 2016
Accepted 17 March 2016

Accepted manuscript posted online 28 March 2016

Citation Poole K, Gilmour C, Farha MA, Mullen E, Lau CH-F, Brown ED. 2016. Potentiation of aminoglycoside activity in *Pseudomonas aeruginosa* by targeting the AmgRS envelope stress-responsive two-component system. Antimicrob Agents Chemother 60:3509–3518. doi:10.1128/AAC.03069-15.

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nas campestris (48), and green light (49) have all been shown to potentiate AG activity against *P. aeruginosa*, although the mechanistic details of potentiation are unknown.

In attempting to potentiate AG activity, however, AG resistance mechanisms will be a confounding problem, and therefore, potentiators that target these mechanisms would be of particular interest. AG-modifying enzymes (AMEs) are major determinants of AG resistance in a variety of bacteria, including *P. aeruginosa* (6, 50), and there are a number of reports describing AME inhibitors (51, 52). Still, there is little, if any, indication that these are effective in enhancing AG susceptibility in intact organisms, particularly AG-resistant strains. AMEs occur infrequently in CF lung isolates (53–55), however, where the AG-exporting (56) MexXY-OprM multidrug efflux system (57, 58) and the AmgRS two-component system (TCS) (59, 60) that responds to and protects *P. aeruginosa* from the adverse effects of AG-generated membrane-damaging aberrant polypeptides (60, 61) are major determinants of AG resistance (6, 50, 62; K. Poole, H. Fetar, and M. G. Surette, unpublished data). Thus, AG potentiators that target these might be useful adjuvants for antipseudomonal therapy of CF lung infections. In the current study, we identified a compound, the antimicrobial rifampin (RIF), which potentiates AG activity against laboratory and clinical isolates of *P. aeruginosa*. Moreover, this potentiation is absent in a mutant lacking AmgRS, an indication that RIF somehow targets this TCS and compromises its contribution to AG resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pEX18Tc and its derivatives were maintained in *E. coli* with 5 (in L broth) or 10 (on L agar) μg/ml of tetracycline and selected in *P. aeruginosa* with 50 μg/ml of tetracycline. A Δ*amgR* derivative of CF lung isolate K2156 was engineered using the pEX18Tc::Δ*amgR* plasmid pCG005 (Table 1) and a previously described protocol (60). A RIF-resistant *rpoB* derivative of *P. aeruginosa* PAO1 strain K767, K3696, was selected by plating an overnight culture (100 μl) on L agar containing 32 μg/ml of RIF (2× MIC) and picking a colony that grew up overnight at 37°C. The *rpoB* gene was amplified in two parts from the mutant using Phusion DNA polymerase (New England BioLabs, Ltd., Pickering, ON, Canada) and primer pairs rpoBFor1 (5′-GAGTGGGCAATGCAGGCC-3′) and rpoBRev1 (5′-CTGCTTCGGCG ACACGTC-3′) and rpoBFor2 (5′-GAAGGGTCAACTGGTGGACG-3′) and rpoBRev2 (5′-CAAGGCCTTTCCTCCTCACG-3′). Reaction mixtures (50 μl) contained 1 μg of chromosomal *P. aeruginosa* K767 DNA as the template, 0.5 μM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 1 U of Phusion DNA polymerase in 1× Phusion GC (part 1) or HF (part 2) buffer. Following an initial denaturation step at 98°C for 30 s, the mixture was subjected to 30 cycles of heating at 98°C for 30 s, 65°C for 30 s, and 72°C for 60 s, before finishing with a 5-min incubation at 72°C. The amplified gene was then sequenced using a variety of custom primers, and a single mutation, yielding a D521Y substitution, was identified in *rpoB*. D521 mutations are seen in several highly RIF-resistant *Pseudomonas* spp., including *P. aeruginosa*, with D521Y seen in RIF-resistant *Pseudomonas putida* (63).

Compound library screen for potentiators of aminoglycoside activity. Wild-type *Pseudomonas aeruginosa* PAO1 strain K767 was screened against the Pharmakon-1600 library (Microsource Discovery Systems, Inc.) for agents that rendered the organism sensitive to one-quarter MIC of the AG paromomycin (PAR; 64 μg/ml) according to CLSI guidelines. Duplicate screens were carried out in 100 μl of Mueller-Hinton broth in 96-well microtiter plates, with a Biomek FX liquid handler (Beckman Coulter Inc.) used to dispense both PAR and the library compounds (dissolved in dimethyl sulfoxide [DMSO]; 10 μM final concentration). Following compound addition, K767 was added to all wells and the plates were incubated for 18 h at 37°C. To control for potential inhibitory effects of the compounds on their own, each was tested as a single agent against strain K767, in duplicate. Background controls, containing only broth and DMSO, and growth controls, containing broth, DMSO, and K767 (8 wells/plate each), were also tested. Using an EnVision plate reader (PerkinElmer), the optical density at 600 nm (OD_{600}) was then determined for each well and the percentage growth for each test well (compound + PAR) was calculated as (OD_{600} for test − mean OD_{600} for background control)/(mean OD_{600} for growth control − mean OD_{600} for background control) × 100. Following normalization to the percent growth determined for a Pharmakon-1600 compound alone, a growth ratio was obtained. A ratio of <1.0 is suggestive of compound potentiation of PAR activity, although a hit cutoff of 0.6 was chosen to identify PAR potentiators.

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference
E. coli strains		
DH5α	ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* F⁻Δ(*lacZYA-argF*) *U169*	84
S17-1	*thi pro hsdR recA* Tra^+	85
P. aeruginosa strains		
K767	PAO1 prototroph	86
K3159	K767 Δ*amgR*	60
K1525	K767 Δ*mexXY*	87
K3162	K767 ΔPA2798	60
K3696	K767 $rpoB_{D521Y}$ (Rif^r)	This study
K3249	K767 $amgS_{R182C}$	62
K3260	K767 $amgS_{V121G}$	62
K3584	K3260 Δ*amgR*	62
K3585	K3249 Δ*amgR*	62
K3589	K767 Δ*yccA*	61
K3590	K767 Δ*htpX*	61
K3591	K767 ΔPA5528	61
K3593	K767 Δ*yccA* Δ*htpX*	61
K3594	K767 Δ*htpX* ΔPA5528	61
K3595	K767 ΔPA5528 Δ*yccA*	61
K3596	K767 Δ*htpX* ΔPA5528 Δ*yccA*	61
K2154	Aminoglycoside-resistant CF isolate	68
K2156	Aminoglycoside-resistant CF isolate	68
K2157	Aminoglycoside-resistant CF isolate	68
K2158	Aminoglycoside-resistant CF isolate	68
K2162	Aminoglycoside-resistant CF isolate	68
K3630	K2156 Δ*amgR*	This study
K2167	K2156 Δ*mexXY*	68
Plasmids		
pEX18Tc	Broad-host-range gene replacement vector; *sacB* Tc^r	88
pCG005	pEX18Tc::Δ*amgR*	60

[a] RIF^r, rifampin resistant; Tc^r, tetracycline resistant.

Antibiotic susceptibility testing. The susceptibility of *P. aeruginosa* to antimicrobial agents was assessed using the 2-fold serial microtiter broth dilution method described previously (64), with an inoculum of ~5×10^5 cells per ml. MICs were recorded as the lowest concentration of antibiotic inhibiting visible growth after 18 h of incubation at 37°C.

qRT-PCR. RNA was isolated from log phase cells exposed or not to 1× MIC PAR (K767, 256 μg/ml; K2154, 512 μg/ml; added at early log phase, 90 min prior to harvesting of cells for RNA isolation) and converted to cDNA as described previously (65). Expression of *mexX* (65), *htpX* (62),

armZ (PA5471) (65), and *rpoD* (62) was assessed using a previously detailed quantitative real-time PCR (qRT-PCR) protocol (65). Expression of *yccA* was also determined as described previously (65) using the primer pair *yccA*-F (5′-GTTCTGCGCAATACCTACGG-3′) and *yccA*-R (5′-GAACACGTTCGGATAGGGC-3′), which had an amplification efficiency of 95% (r^2 = 0.994). In some experiments, *P. aeruginosa* was treated with rifampin (8 μg/ml) for 30 min prior to PAR addition (or prior to RNA isolation in instances where cells were not exposed to PAR).

Membrane depolarization assay. A previously described fluorometric assay (60), involving the membrane potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4$ [3]), was employed to measure the degree of cytoplasmic membrane depolarization promoted by AG treatment of *P. aeruginosa* and the impact of RIF on this. Briefly, early-logarithmic-phase (OD_{600} = 0.3 to 0.5) L broth subcultures of *P. aeruginosa* were treated with either of the AGs gentamicin (GEN; final concentration, 2 or 5 μg/ml) and PAR (final concentration, 256 or 640 μg/ml). Samples (5 ml) of the AG-treated and untreated control cultures were taken immediately and then hourly over 3 h and exposed to $DiBAC_4$ (3) (Invitrogen) at 37°C for 5 min in the dark at a final concentration of 10 μg/ml. Bacteria were then pelleted and resuspended in phosphate-buffered saline (66) to a final OD_{600} of 0.1. Membrane depolarization-dependent fluorescence emitted by cells was then measured using a Varian (now Agilent) Cary Eclipse fluorescent spectrophotometer with excitation and emission wavelengths of 490 and 518 nm, respectively. To assess the impact of chloramphenicol (CAM) or RIF on AG-promoted membrane depolarization, *P. aeruginosa* was pretreated with CAM (128 μg/ml for 15 min [61]) or RIF (8 μg/ml for 30 min) prior to the addition of the AGs.

RESULTS

Rifampin potentiates AG activity against *P. aeruginosa* dependent on AmgRS. A number of chromosomal genes contribute to aminoglycoside (AG) resistance in *P. aeruginosa*, including the uncharacterized regulatory gene pair PA2797-PA2798 (60), the AmgRS two-component system (TCS) (59, 60) and the MexXY-OprM multidrug efflux system (57, 60, 67), all of which have been shown to contribute to AG resistance in clinical isolates (60, 62, 68, 69). In an effort to identify inhibitors of these AG resistance determinants, and so improve AG activity against *P. aeruginosa*, a preliminary screen of a limited compound library was undertaken, looking initially for potentiators of AG activity. Paromomycin (PAR) was chosen as a representative AG since it demonstrated the greatest difference in MIC between strains carrying versus lacking the aforementioned AG resistance determinants (60) and, thus, provided a suitably sensitive screen for AG potentiators that targeted these determinants. Using wild-type *P. aeruginosa* PAO1 strain K767, a 1,600-compound library was screened for agents that rendered K767 susceptible to one-quarter MIC of PAR for this strain but had no intrinsic antimicrobial activity (at the concentrations being screened). A number of putative AG-potentiating compounds were identified, including the rifamycin-related compound rifaximin. We subsequently tested the related rifamycin compound, rifampin (RIF), and demonstrated that it, too, potentiated PAR activity, increasing the PAR susceptibility of strain K767 8-fold (Table 2).

To ascertain whether RIF acted via one of the above-mentioned AG resistance determinants that are linked to resistance in clinical isolates, deletion strains individually lacking these resistance determinants were assessed for RIF potentiation of PAR activity. The mutants lacking *mexXY* or PA2797-PA2798, as expected, showed increased susceptibility to PAR, consistent with their known contributions to intrinsic AG resistance (Table 2). Nonetheless, RIF reduced PAR MICs (2- to 4-fold) in these mutants (Table 2), an indication that it was still potentiating PAR activity in the absence of these resistance determinants and, so, not targeting them. In contrast, while the Δ*amgR* mutant also showed enhanced PAR susceptibility, consistent with its known contribution to intrinsic AG resistance, RIF did not impact the PAR MIC for this strain (Table 2), suggesting that RIF acts via AmgRS in its potentiation of PAR activity. Significantly, the PAR MIC for the Δ*amgR* mutant was the same as that observed for wild-type strain K767 treated with RIF (Table 2), an indication that RIF was effectively blocking the contribution of this TCS to AG resistance in wild-type *P. aeruginosa*. RIF also potentiated PAR activity against derivatives of K767 harboring AmgRS-activating *amgS* gain-of-function mutations and showing elevated AG resistance, in one instance (K3249) rendering the mutant as susceptible as an *amgR* knockout strain (Table 2), evidence that it was fully reversing the AmgRS-promoted increase in PAR resistance in this mutant. As expected, loss of *amgR* in the *amgS* mutants reduced PAR MICs, and RIF failed to potentiate PAR activity in these $AmgR^-$ derivatives (Table 2, strains K3584 and K3585), confir-

TABLE 2 AmgRS-dependent rifampin potentiation of paromomycin activity against *P. aeruginosa*[a]

Strain	Relevant genotype	Rifampin	Paromomycin MIC (μg/ml)
K767	Wild type	−	256
		+	32
K3159	Δ*amgR*	−	32
		+	32
K1525	Δ*mexXY*	−	16
		+	8
K3162	ΔPA2798	−	16
		+	4
K3249	$amgS_{R182C}$	−	512
		+	32
K3584	$amgS_{R182C}$ Δ*amgR*	−	32
		+	32
K3260	$amgS_{V121G}$	−	512
		+	128
K3585	$amgS_{V121G}$ Δ*amgR*	−	32–64
		+	32
K3696	$rpoB_{D521Y}$ (RIF^r)	−	256
		+	256
K3590	Δ*htpX*	−	512
		+	64
K3589	Δ*yccA*	−	128–256
		+	64
K3591	ΔPA5528	−	128
		+	64
K3593	Δ*yccA* Δ*htpX*	−	64
		+	16
K3594	Δ*htpX* ΔPA5528	−	512
		+	32
K3595	ΔPA5528 Δ*yccA*	−	64
		+	32
K3596	Δ*htpX* ΔPA5528 Δ*yccA*	−	8
		+	8–16

[a] The paromomycin MICs were determined for the indicated *P. aeruginosa* strains in the absence (−) and presence (+) of one-half MIC of rifampin (RIF) for each strain (8 μg/ml). While the RIF MIC for the *rpoB* mutant K3696 was >1,024 μg/ml, RIF was also used at 8 μg/ml for this strain in order to assess whether its potentiation of PAR in wild-type strain K767 was via RpoB or independent of this RIF target. RIF^r, RIF resistant.

TABLE 3 Rifampin potentiates the activity of 4,5-linked aminoglycosides against wild-type *P. aeruginosa*[a]

Antimicrobial	MIC (μg/ml)	
	−Rifampin	+Rifampin
Paromomycin	256	32
Neomycin	32	4
Ribostamycin	1,024	256
Gentamicin	2	2
Amikacin	2	2
Kanamycin	64	64
Streptomycin	32	32
Erythromycin	512	512
Chloramphenicol	32	16–32
Nalidixic acid	128	128
Carbenicillin	64	32–64

[a] The MICs for the indicated antimicrobials were determined for wild-type *P. aeruginosa* PAO1 strain K767 in the absence (−) and presence (+) of one-half MIC of rifampin (8 μg/ml).

mation that RIF was targeting AmgRS in potentiating PAR activity against the mutants. RIF potentiation of PAR was lost in a RIF-resistant *P. aeruginosa* stain (K3696) harboring a mutation in the *rpoB* gene that encodes the RNA polymerase β subunit, which is the target of RIF (70) (Table 2), an indication that its PAR-potentiating activity is dependent on its interaction with and disruption of RNA polymerase and not some other possible activity of this compound.

Rifampin specifically potentiates the activity of 4,5-linked aminoglycosides. Having confirmed AmgRS-dependent RIF potentiation of PAR in *P. aeruginosa*, it was of interest to assess whether this was, as expected, limited to AGs (AmgRS is linked to AG resistance only). Surprisingly, in screening a number of AGs and non-AGs, RIF showed an ability to potentiate a limited subset of AGs (and no non-AGs) that included only neomycin (NEO) and ribostamycin in addition to PAR (Table 3), while failing to potentiate the more traditional antipseudomonal AGs such as gentamicin and amikacin (Table 3). Intriguingly, PAR, NEO, and ribostamycin are examples of 4,5-linked AGs whose structures differ substantially from those of the other AGs listed in Table 3, which are all 4,6-linked AGs (except streptomycin, which has its own unique structure) (71). These results suggest that the 4,5-linked AGs have some unique effects on *P. aeruginosa* relative to the 4,6-linked AGs and that the mechanism of AmgRS protection against the effects of the 4,5-linked AGs is unique and specifically targeted by RIF.

Rifampin targeting of AmgRS does not impact the membrane-protective role of this TCS. AmgRS was previously shown to protect *P. aeruginosa* from membrane damage resultant from exposure to AGs and the resultant production of membrane-damaging aberrant polypeptides (61). Thus, one possibility to explain the RIF potentiation of AG activity via AmgRS was that it compromised this TCS's membrane protective activities but only for the 4,5-linked subset of AGs. The earlier studies showing AmgRS-mediated protection against AGs examined the 4,6-linked AGs gentamicin and tobramycin only. Thus, to assess whether 4,5-linked AGs such as PAR also promote membrane damage and whether this is ameliorated by AmgRS and exacerbated by RIF, PAR-promoted membrane damage was first assessed using a membrane depolarization assay. As seen in Fig. 1A, exposure of wild-type *P. aeruginosa* K767 to PAR at 1× MIC resulted in a time-dependent increase in membrane damage, as was seen previously with gentamicin- and tobramycin-treated cells (61). As reported previously for gentamicin-exposed cells (61), too, PAR-promoted membrane damage was abrogated when *P. aeruginosa* was first treated with CAM to block translation and, so, production of membrane-perturbing aberrant mistranslated polypeptides (Fig. 1A), consistent with PAR treatment ultimately generating these membrane-damaging polypeptides. Strikingly, however, loss of *amgR* in mutant strain K3159 did not increase PAR-promoted membrane damage (Fig. 1B), in contrast to previous results with GEN, where GEN-promoted membrane damage was enhanced in the absence of this TCS (61). For the 4,5-linked AG, PAR, then, AmgRS-mediated protection is not manifest via a membrane-protective mechanism, in contrast with the 4,6-linked

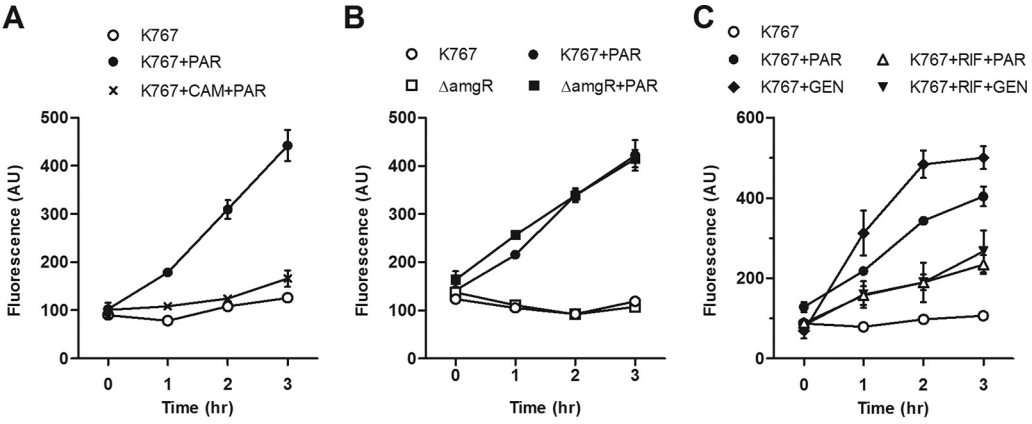

FIG 1 Impact of chloramphenicol and rifampin on paromomycin-promoted cytoplasmic membrane depolarization in wild-type *P. aeruginosa*. Cytoplasmic membrane depolarization, as assessed by DiBAC (4) fluorescence, was measured over time following exposure of *P. aeruginosa* to various antimicrobials added at 0 h. (A) Wild-type *P. aeruginosa* K767 exposed to 1× MIC of paromomycin (PAR; 256 μg/ml) in the absence or presence of 128 μg/ml of chloramphenicol (CAM). Untreated K767 was included as a control. (B) *P. aeruginosa* K767 and its *amgR* deletion derivative exposed to 1× MIC of paromomycin (PAR; 256 μg/ml). Untreated controls are also shown. (C) *P. aeruginosa* K767 exposed to 1× MIC of paromomycin (PAR; 256 μg/ml) or 1× MIC of gentamicin (GEN; 2 μg/ml) in the absence or presence of 8 μg/ml of rifampin (RIF). Untreated K767 was included as a control. The data are means ± standard errors of the means (SEMs) from 3 independent experiments.

AGs. Not surprisingly, RIF treatment did not enhance PAR-promoted membrane damage and, indeed, was seen to reduce PAR-promoted membrane damage (Fig. 1C). A similar result was seen for the 4,6-linked GEN (Fig. 1C). This reduction in AG-promoted membrane damage likely reflects the noted connection between transcription and translation and the potential, therefore, for perturbation of the former to compromise the latter. Indeed, RIF has been shown to limit protein as well as RNA synthesis (72). Therefore, its ability to reduce AG-promoted membrane damage may simply reflect a RIF-driven reduction in the synthesis of membrane-damaging AG-generated mistranslation products. In any case, it is apparent that AmgRS controls some additional AG-protective activity independent of membrane damage and that this is related to some unique effect(s) of the 4,5-linked AGs.

Role of AmgRS target genes in rifampin potentiation of AG activity. A possible explanation for RIF's potentiation of 4,5-linked AGs is that it compromises AmgRS activity or its activation by these AGs and, ultimately, expression of an AmgRS-dependent 4,5-linked AG resistance mechanism. A number of genes, including *htpX*, whose homologue in *E. coli* encodes a cytoplasmic membrane-associated protease (72), are AG inducible, dependent on AmgRS (59, 61). Therefore, *htpX* expression is a good measure of AmgRS activation or activity. Consistent with previous results, exposure of K767 to PAR increased *htpX* expression (3-fold) Fig. 2A). Strikingly, this was largely absent in K767 treated with RIF (Fig. 2A), an indication that RIF was interfering with PAR-mediated AmgRS activation. To ensure that this was not a general effect of this RNA polymerase inhibitor, which might be expected to reduce global gene expression, the impact of RIF on expression of another AG-inducible but AmgRS-independent gene, *armZ* (61, 73), was also assessed. As seen in Fig. 2E, *armZ* was strongly PAR inducible, and this was unaffected by RIF treatment. Thus, RIF was specifically compromising the PAR-induced expression of the AmgRS-regulated gene *htpX*. In light of this result, it was possible that the loss of *htpX* expression was responsible for the increased PAR and NEO susceptibility of RIF-treated cells and that this AmgRS target gene was, thus, specifically responsible for AmgRS-promoted resistance to this AG subset. Still, loss of *htpX* in strain K3590 did not render *P. aeruginosa* as susceptible to PAR as an *amgR* knockout, and RIF still showed potentiation of PAR activity in strain K3590 (Table 2), an indication that RIF was not acting solely on this AmgRS target in potentiating PAR activity. A previous study (59) identified 3 AmgRS-dependent genes as the major contributors to AmgRS-promoted AG resistance, *htpX*, PA5528, and *yccA*, and it may be that collectively, these are responsible for the AmgRS-dependent 4,5-linked AG resistance that is compromised by RIF. The *yccA* homologue in *E. coli* encodes a modulator of the FtsH protease that is implicated in membrane protein quality control (74), while PA5528 encodes a predicted cytoplasmic membrane-associated protein of unknown function. A mutant stain lacking these three genes, K3596, showed the expected substantial increase in PAR susceptibility, and RIF had no additional effect on PAR susceptibility in this mutant (Table 2). Mutant strains individually lacking *yccA* (K3589) or PA5528 (K3591), like the *htpX* mutant, still showed RIF potentiation of PAR activity, as did strains lacking any 2 of these (Table 2, strains K3593 to K3595), further support for *htpX*, *yccA*, and PA5528 contributing collectively to RIF-targeted AG resistance. In examining the impact of RIF on PAR induction of PA5528 and *yccA* in strain K767, however, RIF did not compromise PAR-inducible PA5528 expres-

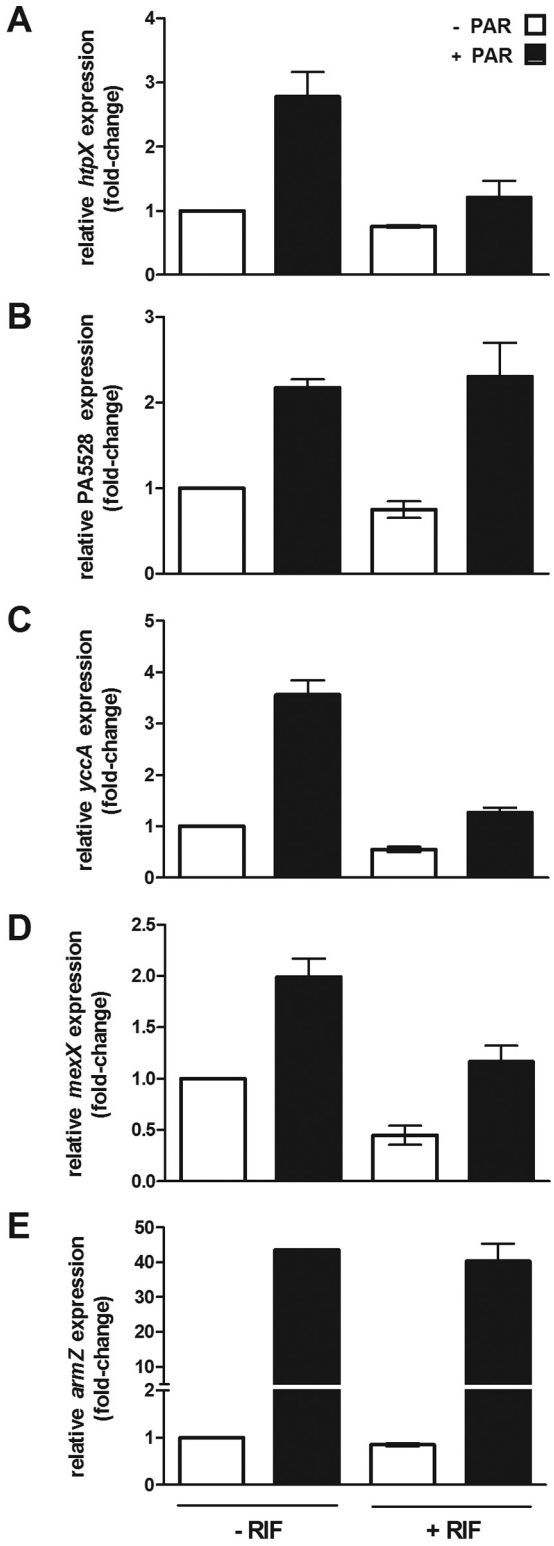

FIG 2 Impact of rifampin on aminoglycoside-promoted expression of AmgRS-regulated genes in wild-type *P. aeruginosa*. The impact of paromomycin (256 μg/ml) on expression of *htpX* (A), PA5528 (B), *yccA* (C), *mexXY* (D), and *armZ* (E) was assessed in log-phase cultures of *P. aeruginosa* strain K767 with or without prior exposure to rifampin (8 μg/ml) using quantitative real-time PCR. Expression was normalized to *rpoD* and is reported relative to that for the wild-type *P. aeruginosa* strain K767 (fold change). Values are means ± SEMs from at least three independent determinations, each performed in triplicate.

TABLE 4 Rifampin potentiation of aminoglycoside activity against aminoglycoside-resistant clinical isolates of *P. aeruginosa*[a]

Strain	RIF	MIC (μg/ml) for[b,c]:											
		PAR	NEO	AMI	KAN	GEN	STR	CAM	ERY	NAL	CAR	SPC	NOR
K2154	−	512	128	16	256	512	4,096	256	256	2,048	>4,096	>4,096	64
	+	64	16	4	64	64	1,024	256	256	2,048	>4,096	1,024	64
K2156	−	512	64	16	128	8	256	128	256	2,048	1,024	1,024	4
	+	32	8	1	32	1	8	16	256	2,048	512	32	4
K2157	−	2,048	256	64	—	—	—	—	—	—	—	—	—
	+	256	32	32	—	—	—	—	—	—	—	—	—
K2158	−	2,048	256	64	—	—	—	—	—	—	—	—	—
	+	256	32	32	—	—	—	—	—	—	—	—	—
K2162	−	>4,096	512	256	—	—	—	—	—	—	—	—	—
	+	2048	32	128	—	—	—	—	—	—	—	—	—

[a] The MICs for the indicated antimicrobials were determined for the indicated clinical (cystic fibrosis) isolates of *P. aeruginosa* in the absence (−) and presence (+) of one-half MIC of rifampin (RIF) for each strain (8 μg/ml for strains K2154, K2156, and K2162 and 16 μg/ml for strains K2157 and K2158).
[b] PAR, paromomycin; NEO, neomycin; AMI, amikacin; KAN, kanamycin; GEN, gentamicin; STR, streptomycin; CAM, chloramphenicol; ERY, erythromycin; NAL, nalixidic acid; CAR, carbenicillin; SPC, spectinomycin; NOR, norfloxacin.
[c] —, not determined.

sion (Fig. 2B), and while it did not fully block PAR induction of *yccA* (Fig. 2C), PAR-induced *yccA* levels in RIF-treated K767 were markedly below that for RIF-untreated K767 (Fig. 2C), an indication that RIF was adversely impacting *yccA* expression. Interestingly, PAR-inducible expression of *mexXY*, shown previously to be dependent on *htpX*, PA5528, and *yccA* (61), was also somewhat compromised by RIF treatment (Fig. 2D). Taken together, these results are consistent with RIF somehow disrupting AmgRS activation and/or operation.

Pan-aminoglycoside potentiation by rifampin in clinical isolates of *P. aeruginosa*. Clinical isolates of *P. aeruginosa*, particularly CF lung isolates, often exhibit elevated resistance to multiple AGs. It was of interest, therefore, to assess whether RIF might potentiate AG activity against such isolates and whether such potentiation would also be limited to the 4,5-linked AGs. Five previously studied CF isolates showing elevated pan-AG resistance (68) all showed markedly enhanced susceptibility (8- to 16-fold) to the 4,5-linked AGs PAR and NEO in the presence of one-half MIC RIF (Table 4). Unexpectedly, while some of the isolates showed minimal RIF potentiation of a representative 4,6-linked AG, amikacin (2-fold) (Table 4), consistent with what was seen with the wild-type laboratory strain K767, RIF significantly increased amikacin susceptibility (4- to 16-fold) in two isolates, K2154 and K2156 (Table 4). RIF also substantially increased the susceptibility of these isolates to additional 4,6-linked AGs (KAN and GEN; 8-fold) as well as streptomycin (4- to 32-fold) (Table 4). To ascertain whether the ability of RIF to potentiate a broad range of AGs in K2154 and K2156 was still via an action on AmgRS, deletions of *amgR* were engineered into these strains and the impact of RIF on AG MICs was again determined. Despite repeated attempts, an *amgR* knockout could not be obtained in K2154, consistent with previous failed attempts to generate deletions in this clinical isolate (68). An *amgR* deletion was, however, successfully engineered into K2156. The K2156 AmgR$^-$ derivative showed the expected increase in susceptibility to 4,5- and 4,6-linked AGs (Table 5), consistent with AmgRS contributing to the broad-range AG resistance of K2156, although RIF did not further influence AG MICs for this strain (Table 5), an indication that RIF potentiation of AGs in K2156 was via AmgRS. Significantly, the impact of the *amgR* deletion on AG MICs was nominally equivalent to the impact of RIF treatment of K2156, further support for RIF acting via this TCS in promoting enhanced AG susceptibility.

Surprisingly, in examination of the AG specificity of RIF's potentiation activity in K2154 and K2156, RIF was shown to also

TABLE 5 AmgRS- and MexXY-dependent rifampin potentiation of aminoglycoside activity against an aminoglycoside-resistant clinical isolate of *P. aeruginosa*[a]

Strain	Genotype	RIF	MIC (μg/ml) for[b]:					
			PAR	NEO	AMI	KAN	GEN	STR
K2156	Wild type	−	512	64	16	128	8	256
		+	32	8	1	32	1	8
K3630	Δ*amgR*	−	16	8	1	16	1	16
		+	16	8	1	16	1	16
K2167	Δ*mexXY*	−	32	16	1	32	2	4
		+	16	8	1	32	1	2

[a] The MICs for the indicated antimicrobials were determined for the clinical (cystic fibrosis) *P. aeruginosa* isolate K2156 and its AmgR$^-$ and MexXY$^-$ derivatives in the absence (−) and presence (+) of one-half MIC of rifampin (RIF) for each strain (8 μg/ml).
[b] PAR, paromomycin; NEO, neomycin; AMI, amikacin; KAN, kanamycin; GEN, gentamicin; STR, streptomycin.

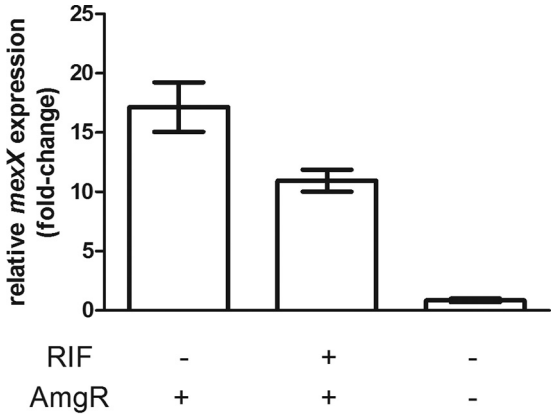

FIG 3 Impact of rifampin and *amgR* loss on *mexXY* expression in an aminoglycoside-resistant clinical isolate of *P. aeruginosa*. *mexX* expression was measured in log-phase cells of clinical strain K2156 without or with RIF exposure (8 μg/ml for 30 min) and log-phase cells of its Δ*amgR* derivative, K3630, using quantitative real-time PCR. Expression was normalized to *rpoD* and is reported relative to that for the wild-type *P. aeruginosa* strain K767 (fold change). Values are means ± SEMs from at two independent determinations, each performed in triplicate.

potentiate CAM and spectinomycin (SPC) activity in K2156, though not the other non-AGs that were tested (Table 4). AmgRS is a membrane-protective TCS that contributes to resistance to mistranslation-causing AGs but not CAM and SPC, which do not promote mistranslation. Indeed, the antimicrobials impacted by RIF in K2156 are those typically associated with the MexXY-OprM multidrug efflux system. In light of the recent report that AG-promoted or mutational activation of AmgRS can drive *mexXY* expression, it may be that AmgRS somehow promotes *mexXY* expression in K2156, with MexXY-OprM responsible for much of the AG (and CAM and SPC) resistance of this isolate that is, in turn, negatively impacted by RIF. Consequently, RIF interference with AmgRS would compromise *mexXY* expression and, so, resistance to AGs as well as CAM and SPC. In agreement with this, *mexXY* expression in strain K2156 was elevated markedly relative to the K767 wild-type laboratory strain and was reduced by RIF exposure K2156 (Fig. 3). RIF potentiation of AGs was largely absent in a *mexXY* deletion derivative of K2156 (Table 5), further support for RIF ultimately acting on this efflux system in potentiation of AG activity in strain K2156. Notably, with the exception of STR, the impact of the loss of *mexXY* was nominally the same as that of loss of *amgR*, consistent with AG resistance in K2156 being mediated by MexXY-OprM but dependent on AmgRS. Consistent with this, the elevated expression of *mexXY* seen in K2156 was lost upon deletion of *amgR* (Fig. 3). That RIF treatment had a much more modest negative effect on *mexXY* expression than the *amgR* knockout likely reflects the failure of RIF inactivation of AmgRS to eliminate those AmgRS-dependent *mexXY* transcripts formed prior to RIF treatment. If one assumes that the steady-state *mexXY* transcript levels reflect a balance between new synthesis and turnover of existing transcripts, RIF treatment of strain K2156 would only limit new synthesis, with *mexXY* transcript turnover responsible for the observed decline in *mexXY* expression. In any case, MexXY-OprM-mediated high-level pan-AG resistance in K2156 is clearly linked to AmgRS and, therefore, is inhibited by RIF. Sequencing of the *amgRS* genes failed to identify any mutations in this TCS operon, however, an indication that other mutations impact *mexXY* expression in K2156, in a manner that is dependent on AmgRS.

DISCUSSION

RIF potentiation of AG activity in *P. aeruginosa* is dependent on the presence of AmgRS, an indication that it acts on this envelope stress response TCS. In agreement with this, RIF was shown to compromise expression of the AmgRS target genes *htpX* and *yccA*. While expression of a third AmgRS target, PA5528, was not influenced by RIF treatment, it may be that this reflected some AmgRS-independent compensatory impact on PA5528 expression masking the possibly inhibitory effect of RIF inhibition on AmgRS and, ultimately, PA5528. Certainly, the impact of an *htpX*-PA5528-*yccA* triple deletion on AG susceptibility was greater than that seen for an *amgRS* knockout, an indication that one or more of *htpX*, PA5528, and *yccA* is expressed and contributes to AG resistance independent of AmgRS. Moreover, deletion of *htpX* in *P. aeruginosa* has been shown to increase PA5528 expression more than 3-fold (C. H.-F. Lau, unpublished data), a result that is suggestive of PA5528 expression responding to defects in other AmgRS targets. Of note, deletion of PA5528 did not impact *htpX* expression (Lau, unpublished), an indication that *htpX* does not respond in a similarly compensatory fashion: i.e., this is possibly unique to PA5528. Thus, perturbation of AmgRS and/or its targets may stimulate a compensatory increase in PA5528 that is AmgRS independent, although the latter has yet to be tested. The observation that *mexXY* expression was compromised by RIF is also consistent with this agent somehow perturbing the operation of AmgRS and its AG resistance-promoting targets HtpX, PA5528, and YccA; AG-inducible expression of this multidrug efflux operon is dependent on AmgRS, specifically the *htpX*, PA5528, and *yccA* targets of this TCS. This effect of RIF on *mexXY* expression likely explains the more modest effect of RIF on AG MICs in a Δ*mexXY* strain (2-fold) versus that in the wild type (8-fold), since part of the effect of RIF on AG susceptibility in K767 is likely due to the loss of AmgRS-promoted *mexXY* expression.

The observation that RIF potentiates only 4,5-linked AGs in wild-type strain K767 is striking and speaks to some unique property of this class of AG. Possibly, 4,5-linked AG perturbation of the ribosome generates some unique (relative to 4,6-linked AGs) cell-damaging products that can both activate AmgRS and be "neutralized" by the products of AmgRS-regulated AG resistance genes such as *htpX*, PA5528 and *yccA*. Of note, 4,5-linked AGs are better activators of AmgRS (as determined by the level of induction of *htpX* and PA5528) (62), suggesting that these unique products are better sensed by and/or are preferred targets of the AmgRS TCS and its AG resistance determinants. It may be, therefore, that RIF acts by somehow limiting the 4,5-linked AG generation of these deleterious products, thereby compromising the activation of AmgRS and the resultant recruitment of its AG resistance determinants. Consequently, resistance to these AGs but not the 4,6-linked AGs would be compromised by RIF. Consistent with the two classes of AGs having different detrimental effects on *P. aeruginosa* that could be differentially sensed by AmgRS, loss of the TCS increases susceptibility to both 4,5- and 4,6-linked AGs but increases only membrane damage caused by the 4,6-linked AGs. Differences between 4,5- and 4,6-linked AGs have also been noted in terms of their impact on ribosome conformation (75), which might manifest as different downstream effects and, so, different products of ribosome perturbation. Presumably, since a

RIF-promoted reduction in AmgRS-activating, 4,5-linked AG-generated deleterious products might be expected to enhance and not reduce AG resistance, 4,5-linked AGs must have additional deleterious effects on the cell that are ameliorated as a result of AmgRS activation. Therefore, RIF limitation of 4,5-linked AG products that activate this TCS would still render cells sensitive to 4,5-linked AGs.

How RIF might limit production of AG-generated AmgRS-activating products is unclear, though it must do so ultimately as a downstream effect of its action on its known target, RNA polymerase, since it failed to potentiate AG activity in a RIF-resistant *rpoB* mutant. The noted connection between transcription and translation, with the rate of one impacting the rate of the other (76), does suggest, however, that RIF may ultimately impact ribosome function. Indeed, RIF has been shown to reduce protein as well as RNA synthesis (77), and RIF treatment of *E. coli* is known to alter ribosome structure and composition (78). How this might manifest specifically in fewer products of 4,5-linked AG perturbation of ribosomes is unknown. It is likely, however, that these products act on AmgRS and are substrates for the AmgRS-regulated *htpX*, *yccA*, and PA5528 gene products. These gene products are collectively required for AG induction of *mexXY* expression, a result interpreted as their activities, two of three of which are linked to proteolysis, ultimately generating the *mexXY* inducing signal(s) (61). The observation that RIF fails to block PAR-inducible PA5528 expression in wild-type *P. aeruginosa* even as it compromises PAR-inducible *mexXY* expression argues that PA5528 function must somehow be perturbed by RIF. Since there is no reason to suspect that RIF specifically hampers PA5528 translation or operation, apparent loss of PA5528 function might simply reflect a RIF-promoted lack of 4,5-linked AG-generated substrates on which PA5528 (and HtpX and YccA) must apparently act in yielding the *mexXY* inducer molecule(s) (61).

Apparent synergy between AGs and RIF has been noted previously (79–81) and in one case was attributed to RIF suppressing what was then known as AG adaptive resistance (81). Still, in these instances synergy was demonstrated with 4,6-linked AGs, in contrast to results shown here. The link to adaptive AG resistance is, however, interesting, inasmuch as we now recognize this to involve the AG-inducible MexXY multidrug efflux system (82), which has been shown to be AmgRS dependent (61) and whose expression is, as we show here, limited to some extent by RIF. It may be that in these earlier studies RIF was, in fact, targeting AmgRS and this was compromising *mexXY* expression such that resistance to 4,6-linked AGs was affected (resistance to 4,5-linked was not examined). The genetic background and *mexXY* status of the earlier *P. aeruginosa* strains are unknown, though all were clinical strains and, so, not wild type. As such, they may have resembled our clinical isolate K2156, in which the AmgRS-dependent upregulation of MexXY was promoting resistance to both classes of AG, with RIF targeting of AmgRS ultimately compromising MexXY-mediated AG resistance. In any case, while the results of the current study suggest that in wild-type laboratory strains, RIF may be of limited utility since it only potentiated lesser-used 4,5-linked AGs, it substantially potentiated the more commonly used 4,6-linked AGs (e.g., amikacin and gentamicin) in some AG-resistant clinical isolates, an indication that it might prove to be of some use in the clinic. Indeed, a randomized trial involving the treatment of *P. aeruginosa* bacteremias with an AG-penicillin combination with or without RIF showed a significantly increased rate of bacteriologic cure in the arm with RIF and a marked reduction in relapsing bacteremias (83).

ACKNOWLEDGMENTS

This work was supported by operating grants from Cystic Fibrosis Canada to K.P. and to E.D.B. E.D.B. was also supported by a salary award from the Canada Research Chairs program.

FUNDING INFORMATION

This work, including the efforts of Keith Poole and Eric D. Brown, was funded by Cystic Fibrosis Canada (Fibrose kystique Canada).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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