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Potentiation of Antibiotic Activity by a Novel Cationic Peptide: Potency and Spectrum of Activity of SPR741

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ABSTRACT Novel approaches for the treatment of multidrug-resistant Gram-negative bacterial infections are urgently required. One approach is to potentiate the efficacy of existing antibiotics whose spectrum of activity is limited by the permeability barrier presented by the Gram-negative outer membrane. Cationic peptides derived from polymyxin B have been used to permeabilize the outer membrane, granting antibiotics that would otherwise be excluded access to their targets. We assessed the *in vitro* efficacies of combinations of SPR741 with conventional antibiotics against *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. Of 35 antibiotics tested, the MICs of 8 of them were reduced 32- to 8,000-fold against *E. coli* and *K. pneumoniae* in the presence of SPR741. The eight antibiotics, azithromycin, clarithromycin, erythromycin, fusidic acid, mupirocin, retapamulin, rifampin, and telithromycin, had diverse targets and mechanisms of action. Against *A. baumannii*, similar potentiation was achieved with clarithromycin, erythromycin, fusidic acid, retapamulin, and rifampin. Susceptibility testing of the most effective antibiotic-SPR741 combinations was extended to 25 additional multidrug-resistant or clinical isolates of *E. coli* and *K. pneumoniae* and 17 additional *A. baumannii* isolates in order to rank the potentiated antibiotics. SPR741 was also able to potentiate antibiotics that are substrates of the AcrAB-TolC efflux pump in *E. coli*, effectively circumventing the contribution of this pump to intrinsic antibiotic resistance. These studies support the further development of SPR741 in combination with conventional antibiotics for the treatment of Gram-negative bacterial infections.

KEYWORDS Gram-negative bacteria, microbiology, potentiation

The emergence of Gram-negative bacteria with increasingly multidrug-resistant (MDR) profiles has become a serious public health concern worldwide (1), and the rapidity with which resistance determinants disseminate globally (2), along with the increasing frequency of reports of clinical isolates that are resistant to carbapenems (3), has led to the reintroduction of older, potentially more toxic antibiotics such as colistin (4, 5) into clinical practice. Despite the fact that no antibiotic class has been spared by the evolution and dissemination of resistance, no totally new class of antibiotics active against Gram-negative bacteria has been introduced for over 40 years due to the manifold challenges of antibacterial drug discovery (6–8). One of the most significant obstacles to developing agents targeting Gram-negative bacteria is the bacterial outer membrane (9), and one novel therapeutic approach to solve the current problem of limited effective treatment options against Gram-negative bacteria is to combine existing antibiotics with a "potentiator" molecule that permeabilizes the outer membrane, granting antibiotics that would otherwise be ineffective access to their intracellular targets (10, 11). SPR741, formerly NAB741 (12), is a cationic peptide derived from

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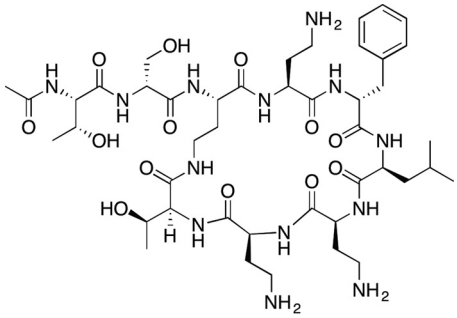

FIG 1 Structure of SPR741.

polymyxin B and is one such potentiator molecule that is under development for the treatment of serious Gram-negative bacterial infections. Unlike polymyxin B, SPR741 exhibits minimal intrinsic Gram-negative antibacterial activity (12). It retains the cyclic peptide portion of polymyxin B but differs in two important areas: first, SPR741 lacks two cationic diaminobutyryl residues from the linear portion of the peptide, reducing the net positive charge of the molecule to +3, compared to the net charge of +5 in polymyxin B, and second, SPR741 lacks the 6-methyloctanoyl or 6-methylheptanoyl fatty acid tail found in polymyxin B (12). Despite these changes, SPR741 retains the ability to permeabilize the outer membrane of Gram-negative bacteria, thus sensitizing them to hydrophobic antibiotics (12). Importantly, these structural changes also significantly improve the safety profile of SPR741 compared to that of polymyxin B, which suffers severe, dose-limiting nephrotoxicity in humans. In multiday rodent and nonhuman primate studies, polymyxin B exhibited nephrotoxicity at a far-lower exposure-normalized dose than SPR741 (T. Lister, unpublished data).

In the present study, we determine the ability of SPR741 to potentiate the activity of a broad range of conventional antibiotics from multiple classes against strains of *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* and demonstrate that specific combinations with SPR741 are efficacious against both clinical isolates and MDR reference collection isolates of these bacteria.

RESULTS AND DISCUSSION

SPR741 (Fig. 1) was previously shown to be synergistic with rifampin and clarithromycin (12). In the present work, we sought to understand the extent to which SPR741 was able to potentiate the activity of antibiotics more broadly against *E. coli*, *K. pneumoniae*, and *A. baumannii*. To this end, 35 antibiotics from 27 different classes, with diverse mechanisms of action, were tested in combination with SPR741 by using a checkerboard methodology. For each combination, the results were assessed in several ways: first, fractional inhibitory concentration indices (FICIs) were calculated to determine whether there was a synergistic interaction; second, the fold reduction in the MIC ("potentiation factor") (Table 1) in the presence of either 2, 4, or 8 μg/ml (*E. coli*) or 4, 8, or 16 μg/ml (*K. pneumoniae* and *A. baumannii*) SPR741 was calculated, relative to the MIC of the antibiotic alone; and third, the possible relevance of the MIC of the antibiotic in the presence of SPR741 was inferred by determining whether the MIC had been reduced to a level at or below the clinical breakpoint for susceptibility for each organism-antibiotic combination (13) or, where this information was not available due to the normal spectrum of activity of the antibiotic (i.e., minimal Gram-negative antibacterial activity), below the equivalent breakpoint for *Staphylococcus* spp. (13).

In combinations with SPR741, the MICs of 13 antibiotics (azithromycin, clarithromycin, erythromycin, fidaxomicin, fosfomycin, fusidic acid, mupirocin, novobiocin, quinupristin-dalfopristin, ramoplanin, retapamulin, rifampin, and telithromycin) against *E. coli* ATCC 25922 were reduced at least 32-fold (Fig. 2A). In line with previously reported results (12), in combination with SPR741, the MIC of rifampin was substantially reduced; in the present work, the MIC was reduced >8,000-fold to 0.002 μg/ml in the presence of

TABLE 1 MICs of rifampin, clarithromycin, and azithromycin, each in the presence and absence of SPR741, against *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *A. baumannii* NCTC 12156

Antibiotic	Strain	MIC (μg/ml) with SPR741 at concn (μg/ml) of:				Potentiation factor[a] at 8 μg/ml SPR741	FICI
		0	2	4	8		
Rifampin	*E. coli* ATCC 25922	16	0.008	0.004	0.002	8,192	0.06–0.25
	K. pneumoniae ATCC 43816	16	2	1	0.5	32	0.06–0.13
	A. baumannii NCTC 12156	2	0.5	0.25	0.06	32	0.09–0.27
Clarithromycin	*E. coli* ATCC 25922	64	0.06	0.03	0.016	4,096	0.03–0.13
	K. pneumoniae ATCC 43816	64	64	64	1	64	0.05
	A. baumannii NCTC 12156	16	4	1	0.25	64	0.08–0.27
Azithromycin	*E. coli* ATCC 25922	2	0.06	0.06	0.06	32	0.06–0.16
	K. pneumoniae ATCC 43816	2	0.06	0.06	0.06	32	0.06
	A. baumannii NCTC 12156	0.25	0.03	0.03	0.03	8	0.25–0.49

[a]Fold reduction in the MIC in the presence of 8 μg/ml SPR741 relative to the MIC in the absence of SPR741.

8 μg/ml SPR741 (Fig. 2A and Table 1), substantially below the Clinical and Laboratory Standards Institute (CLSI) breakpoint for susceptibility of 1 μg/ml for *Staphylococcus* spp. (13). Large reductions in MICs were also observed for the macrolide antibiotics erythromycin (1,024-fold, to 0.03 μg/ml) and clarithromycin (4,096-fold, to 0.016 μg/ml) in the presence of up to 8 μg/ml SPR741 (Fig. 2A and Table 1). Although neither antibiotic would typically be considered for the treatment of Gram-negative bacterial infections, these MIC values are well below the CLSI breakpoints for susceptibility of *Staphylococcus* spp. to these agents (13). The ketolide antibiotic telithromycin, which also targets protein synthesis, is typically used to treat respiratory pathogens (14) but is less active against members of the enterobacteriaceae and *Acinetobacter* spp. (15). In combination with SPR741, however, the MIC of telithromycin against *E. coli* ATCC 25922 was reduced up to 256-fold (Fig. 2A) to 0.06 μg/ml, indicating that the spectrum of activity of the antibiotic can be widened when used in combination with SPR741.

Other agents whose activities were substantially enhanced in the presence of SPR741 included retapamulin and fusidic acid (Fig. 2A). Retapamulin is a semisynthetic pleuromutilin antibiotic that targets the 50S subunit of the bacterial ribosome and is typically active against Gram-positive bacteria (16). In combination with SPR741, the MIC of retapamulin was reduced by 256-fold (Fig. 2A) to 0.03 μg/ml. Fusidic acid is an inhibitor of protein synthesis and typically regarded as a relatively narrow-spectrum antibiotic used for the treatment of skin and soft tissue infections and osteomyelitis caused by staphylococci (17). Fusidic acid alone lacked activity against *E. coli* ATCC 25922 (MIC $>$ 128 μg/ml), but in combination with 8 μg/ml SPR741, the MIC was reduced by 512-fold (Fig. 2A) to 0.5 μg/ml, again demonstrating the ability of SPR741 to extend the spectrum of activity of an antibiotic when the two agents are used in combination.

In addition to the impermeability of the outer membrane, another contributor to the intrinsic resistance of Gram-negative bacteria to several of the antibiotics tested in the present work is efflux, which prevents the intracellular accumulation of antibiotics (18). Clarithromycin is subject to efflux by the AcrAB-TolC efflux pump in *E. coli* (19); a *tolC* mutant, which is deficient in this efflux system, displayed increased susceptibility to clarithromycin relative to its isogenic parent strain (19) (Table 2). Because the disruption of *tolC* results in pleiotropic effects (20), we also included an *acrA* mutant, which produced similar results (Table 2). The combination of clarithromycin and 1 μg/ml SPR741 reduced the MIC of the antibiotic against parent strain BW25113 to levels similar to those observed for the *tolC* and *acrA* mutants (Table 2); a similar reduction was observed with the clarithromycin-SPR741 combination against *E. coli* clinical strain ATCC 25922 (Table 2). Fusidic acid, mupirocin, and retapamulin are all substrates of AcrAB-TolC (21, 22) (Table 2), and substantial reductions in the MICs of all three antibiotics against the wild-type strains were also achieved by combining them with

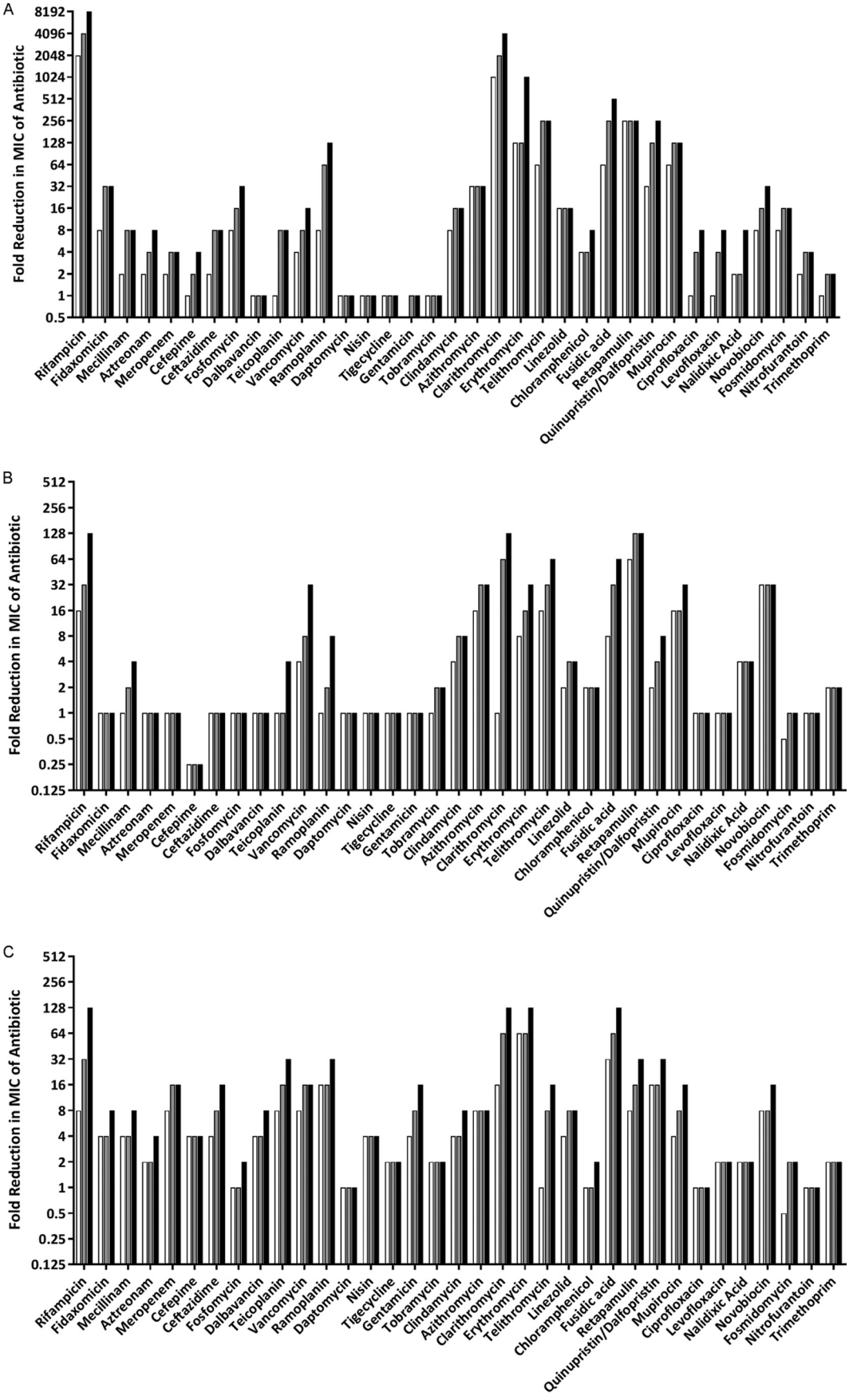

TABLE 2 Susceptibilities of *E. coli* strains to antibiotics that are substrates of the AcrAB-TolC efflux pump

Antibiotic	MIC (μg/ml) for *E. coli* strain					
	BW25113	BW25113Δ*tolC*::*kan*	BW25113Δ*acrA*::*kan*	BW25113 + 1 μg/ml SPR741	ATCC 25922	ATCC 25922 + 1 μg/ml SPR741
Clarithromycin	>32	0.5	1	0.25	32	0.125
Fusidic acid	512	1	4	<8	>128	4
Linezolid	>128	8	8	64	256	32
Mupirocin	128	0.5	1	2	64	8
Retapamulin	8	0.125	0.125	0.06	8	0.06

SPR741 (Table 2). The lack of intrinsic activity of the oxazolidinone antibiotic linezolid against *E. coli* is also due to efflux by AcrAB-TolC, but in this case, the outer membrane does not contribute to intrinsic resistance, since permeabilization by a polymyxin B nonapeptide does not reduce the MIC of this antibiotic against *E. coli* (21, 23). In agreement with these findings, there was little potentiation of the activity of linezolid when it was combined with SPR741 (Table 2 and Fig. 2A). Taken together, these data suggest that the intrinsic resistance of *E. coli* to certain antibiotics that is mediated by both the outer membrane and the AcrAB-TolC efflux system can be overcome, or circumvented, by combining the antibiotic with SPR741 but that potentiation of intrinsic resistance due primarily to efflux may be limited.

Against *K. pneumoniae*, the extent of the fold reduction in the MICs of antibiotics achieved by combining them with SPR741 was generally lower than that observed against *E. coli* ATCC 25922 (Fig. 2A and B), with a maximum reduction of 128-fold observed for rifampin, clarithromycin, and retapamulin (Fig. 2B). Indeed, SPR741 was itself intrinsically less active against *K. pneumoniae* ATCC 43816 (modal MIC of >128 μg/ml) than against *E. coli* ATCC 25922 (modal MIC of 16 μg/ml). The MICs of 10 antibiotics (azithromycin, clarithromycin, erythromycin, fusidic acid, mupirocin, novobiocin, retapamulin, rifampin, telithromycin, and vancomycin) were reduced at least 32-fold in combination with SPR741. The MICs of clarithromycin and rifampin were reduced to below their respective CLSI breakpoints for susceptibility against *Staphylococcus* spp. (13) (Table 1). The intrinsic MIC of azithromycin against *K. pneumoniae* ATCC 43816 was relatively low, at 2 μg/ml, although the MIC was reduced even further in the presence of SPR741 (Fig. 2B and Table 1). The MICs of both erythromycin and telithromycin were reduced to 2 μg/ml in the presence of 16 μg/ml SPR741. Similar to the results with *E. coli* ATCC 25922 (Fig. 2A), the MIC of retapamulin against *K. pneumoniae* ATCC 43816 was substantially reduced when combined with SPR741 (Fig. 2B).

A. baumannii NCTC 12156 was also less susceptible to SPR741 (modal MIC of 64 μg/ml) than was *E. coli* ATCC 25922. The MICs of eight antibiotics (clarithromycin, erythromycin, fusidic acid, quinupristin-dalfopristin, ramoplanin, retapamulin, rifampin, and teicoplanin) were reduced ≥32-fold against this strain (Fig. 2C). Of these antibiotics, the most well-potentiated antibiotics (MIC reductions of 128-fold relative to the MIC of the antibiotic alone) were clarithromycin, erythromycin, fusidic acid, and rifampin. *A. baumannii* NCTC 12156 was considerably more susceptible to rifampin (MIC of 0.5 μg/ml) than was either *E. coli* ATCC 25922 or *K. pneumoniae* ATCC 43816 (MIC of 16 μg/ml), although the MIC was reduced even further when rifampin was combined with SPR741 (Fig. 2C). Similarly, this strain of *A. baumannii* was substantially more susceptible to fusidic acid (MIC of 2 μg/ml) than was either of the other test strains (MIC > 128 μg/ml), but again, it was possible to reduce this value further in combination with SPR741 (Fig. 2C). The MICs of both clarithromycin and erythromycin were reduced to 0.125 μg/ml, below the CLSI breakpoints for susceptibility of *Staphylococcus* spp. to these agents (13).

FIG 2 SPR741 potentiates the activities of antibiotics against *E. coli* ATCC 25922 (A), *K. pneumoniae* ATCC 43816 (B), and *A. baumannii* NCTC 12156 (C). The fold reduction in the MIC of each of the 35 antibiotics (grouped by class between broken lines) is displayed at each of three concentrations of SPR741: 2 μg/ml (white bars), 4 μg/ml (gray bars), and 8 μg/ml (black bars) (A) or 4 μg/ml (white bars), 8 μg/ml (gray bars), and 16 μg/ml (black bars) (B and C).

The activities of several antimicrobial agents in combination with SPR741 were not consistently enhanced against all three species. For example, the activities of the aminoglycoside antibiotics gentamicin and tobramycin were not increased against *E. coli* or *K. pneumoniae*; the efficacy of the quinolone antibiotics ciprofloxacin and levofloxacin were enhanced against *E. coli* only (Fig. 2A to C). Simply sharing a mechanism of action or cellular target was therefore not predictive of the ability of SPR741 to potentiate the activity of a given antibiotic. Agents active against pathways involved in cell wall synthesis were potentiated to different extents: for example, dalbavancin lacked activity against *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 43816 irrespective of the addition of SPR741; the activity of ramoplanin, however, was substantially enhanced against *E. coli* ATCC 25922, with a 128-fold reduction in the MIC from 512 to 4 μg/ml. Interestingly, while there was little evidence of a potentiation of dalbavancin against these strains, when combined with SPR741, the activities of the glycopeptide antibiotics vancomycin and teicoplanin and the lipoglycopeptide antibiotic ramoplanin against *E. coli* and *A. baumannii* were all enhanced by between 8- and 128-fold (Fig. 2A and C). However, these reductions were not sufficient to reduce the MICs of vancomycin and teicoplanin below the breakpoint for susceptibility against *Staphylococcus* spp.

Taken together, these data show that SPR741 can potentiate the activities of antibiotics with diverse physicochemical properties and mechanisms of action that are normally ineffective against Gram-negative bacteria. In combination with SPR741, the MICs of clarithromycin, erythromycin, fusidic acid, retapamulin, and rifampin were reduced by at least 32-fold, relative to the MICs of the antibiotics alone, against *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *A. baumannii* NCTC 12156. Combination with SPR741 also resulted in substantial ($\geq$32-fold) reductions in the MICs of azithromycin, mupirocin, and telithromycin against *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 43816. SPR741 is hypothesized to increase the efficacy of antibiotics by permeabilizing the bacterial outer membrane, thereby facilitating the access of the antibiotic to its target; it is not expected that SPR741 would in any way modify the activity of the antibiotic or its interaction with the target. Therefore, point mutations that modify the target would be expected to render bacteria resistant to a given antibiotic irrespective of whether access to that target was enhanced by the presence of a potentiating molecule such as SPR741. Indeed, strains of *E. coli* bearing defined mutations in *rpoB* that confer resistance to rifampin were found not to be susceptible to the SPR741-rifampin combination (data not shown).

To determine whether these combinations would be more broadly useful against the three organisms for which a single example strain had already been tested, and in order to rank the combinations, the MICs of azithromycin, clarithromycin, fusidic acid, mupirocin, retapamulin, and rifampin against a panel of 25 *E. coli*, 25 *K. pneumoniae*, and 17 *A. baumannii* strains were determined in the presence and absence of SPR741 (Table 3). Meropenem and aztreonam were also included as examples of antibiotics whose activity was increased to a lesser extent by SPR741 (Fig. 2). MIC_{90} values were calculated for each panel-antibiotic combination in the absence or presence of 2, 4, or 8 μg/ml SPR741 (*A. baumannii* and *K. pneumoniae*) or 2 or 4 μg/ml SPR741 (*E. coli*; 8 μg/ml SPR741 alone was sufficient to inhibit the growth of 90% of this panel of *E. coli* strains).

By far the most effective combination against the *E. coli* panel was SPR741-rifampin: the MIC_{90} of rifampin was reduced by at least 4,096-fold, from $>$128 μg/ml in the absence of SPR741 to 0.06 μg/ml in the presence of 2 μg/ml SPR741 (Table 3). In agreement with the above-described results, against *E. coli* ATCC 25922 (Fig. 2A), the activities of azithromycin, fusidic acid, mupirocin, and retapamulin were all increased at least 32-fold (Table 3); the efficacy of meropenem was not increased by combining the antibiotic with SPR741 (Table 3). Interestingly, against this panel of *E. coli* strains, the efficacy of clarithromycin, as judged by the MIC_{90} results, was unchanged in combination with 2 μg/ml SPR741 (MIC_{90} of 32 μg/ml; range, 8 to $>$128 μg/ml) (Table 3). While this could be the result of the limited concentration of SPR741 used in these experi-

TABLE 3 MIC_{90}s of rifampin, mupirocin, azithromycin, fusidic acid, meropenem, aztreonam, retapamulin, and clarithromycin in the presence or absence of SPR741 against clinical and MDR isolate panels

Bacterium	Antibiotic	SPR741 concn (μg/ml)	No. of strains included[a]	MIC (μg/ml)			Potentiation factor[b] (MIC_{90})
				MIC_{50}	MIC_{90}	Range	
E. coli	Rifampin	0	25	16	>128	4–>128	
		2	20	0.016	0.06	0.004–8	>2,048
	Mupirocin	0	25	128	>128	32–>128	
		2	20	1	2	0.5–32	>64
	Azithromycin	0	25	8	128	1–>128	
		2	20	0.125	2	0.03–8	64
	Fusidic acid	0	25	>128	>128	>128	
		2	20	0.125	2	0.25–8	>64
	Meropenem	0	25	0.015	4	0.002–64	
		2	19	0.016	4	0.001–32	1
	Aztreonam	0	25	16	128	0.016–>128	
		2	19	0.5	16	0.004–64	8
	Retapamulin	0	25	8	16	4–16	
		2	19	0.06	0.5	0.03–1	32
	Clarithromycin	0	25	16	32	8–>128	
		2	19	0.25	32	0.016–32	1
K. pneumoniae	Rifampin	0	25	16	32	16–>128	
		2	25	4	16	0.125–64	2
		8	24	0.5	2	0.06	16
	Mupirocin	0	25	128	>128	32–>128	
		2	25	16	64	0.5–32	>2
		8	24	8	32	1–32	>4
	Azithromycin	0	25	32	>128	4–128	
		2	25	128	>128	4–>128	1
		8	24	2	8	0.125–16	>16
	Fusidic acid	0	25	>128	>128	>128	
		2	25	128	>128	4–>128	1
		8	24	16	64	1–64	>2
	Meropenem	0	25	4	64	0.016–64	
		2	25	16	128	0.03–128	0.5
		8	24	8	32	0.03–128	2
	Aztreonam	0	25	128	128	0.125–128	
		2	25	128	128	0.016–128	1
		8	24	128	128	0.06–128	1
	Retapamulin	0	25	64	128	16–128	
		2	25	4	32	1–128	4
		8	24	1	4	0.125–4	32
	Clarithromycin	0	25	64	>128	32–>128	
		2	25	16	64	0.25–64	>2
		8	24	1	32	0.06–64	>4
A. baumannii	Rifampin	0	17	2	4	1–4	
		2	16	0.03	0.06	0.016–0.125	64
		8	10	0.008	0.016	0.002–0.03	256
	Mupirocin	0	17	>128	>128	64–>128	
		2	16	64	128	16–128	>2
		8	10	16	32	2–32	>8
	Azithromycin	0	17	4	8	0.25–>128	
		2	16	0.5	1	0.03–64	8
		8	10	0.5	0.5	0.03–0.5	16
	Fusidic acid	0	17	128	>128	32–>128	
		2	16	1	2	0.5–4	>64
		8	10	0.25	0.5	0.125–1	>256
	Meropenem	0	17	4	128	0.06–128	
		2	16	1	8	0.06–16	16
		8	9	0.5	0.5	0.03–0.5	256
	Aztreonam	0	17	32	64	16–64	
		2	16	8	16	2–16	4
		8	10	1	2	1–4	32
	Retapamulin	0	17	64	128	1–128	
		2	16	1	8	0.25–8	16
		8	9	0.5	4	0.5–4	32

(Continued on next page)

TABLE 3 (Continued)

Bacterium	Antibiotic	SPR741 concn (μg/ml)	No. of strains included[a]	MIC (μg/ml)			Potentiation factor[b] (MIC_{90})
				MIC_{50}	MIC_{90}	Range	
	Clarithromycin	0	17	8	16	0.125–64	
		2	16	0.125	0.5	0.03–4	32
		8	9	0.03	1	0.016–1	16

[a]The growth of several strains in the panel was inhibited by 8 μg/ml SPR741, and so the number of strains included in the analysis under these conditions was reduced.
[b]Fold reduction in the MIC_{90} in the presence of SPR741 relative to the MIC_{90} in the absence of SPR741.

ments, three observations suggest that the efficacy of this combination could be limited by intrinsic resistance to clarithromycin: first, although the MIC_{90} of clarithromycin in the combination was unchanged, the range of MICs was widened considerably to 0.016 to 32 μg/ml; second, substantial potentiation of other antibiotics had been observed under the same conditions (Table 3); and third, in the presence of 2 μg/ml SPR741, the MIC of clarithromycin against *E. coli* ATCC 25922 was reduced 512-fold (data not shown). For the *A. baumannii* panel, 8 μg/ml SPR741 was able to effectively potentiate the activities of fusidic acid and rifampin (Table 3), with reductions in the MIC_{90}s of 512- and 256-fold, respectively, but also of meropenem (256-fold reduction in the MIC_{90}). Against the *K. pneumoniae* panel, reductions in the MIC_{90}s of each antibiotic were smaller than those against the *E. coli* and *A. baumannii* panels (Table 3). Retapamulin was the most effectively potentiated antibiotic in combination with 8 μg/ml SPR741, with a 32-fold reduction in the MIC_{90} compared to the value for the antibiotic alone (Table 3). SPR741 was also effective in combination with both azithromycin and rifampin (16-fold reductions in MIC_{90}s). Based on the reductions in the MIC_{90}s against each organism, it was possible to rank the effectiveness of each antibiotic-SPR741 combination. Overall, against the three species of bacteria, the magnitude of the potentiation of this series of antibiotics by SPR741 followed the descending order of rifampin, fusidic acid, retapamulin, azithromycin, mupirocin, meropenem, aztreonam, and clarithromycin. Interestingly, retapamulin was equally well potentiated by SPR741 against all three species (Table 3). There were differences in this order among the three species; importantly, these broader strain panels demonstrated that although several antibiotics benefited greatly from combination with SPR741 when assessed against susceptible isolates (Fig. 2), the broader panels tested here revealed that intrinsic resistance determinants that may be present in some strains could limit the effectiveness of certain combinations against clinical and MDR isolates (e.g., clarithromycin).

In summary, the data presented here demonstrate that SPR741 can be used effectively to potentiate and extend the spectrum of activity of several antibiotics with diverse targets and mechanisms of action against *E. coli*, *K. pneumoniae*, and *A. baumannii in vitro*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains were recovered from long-term storage at −80°C by culturing on Columbia agar and incubation at 37°C overnight. *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *A. baumannii* NCTC 12156 were used for checkerboard antibiotic susceptibility assays. For broader susceptibility testing, panels of 25 *E. coli*, 25 *K. pneumoniae*, and 17 *A. baumannii* strains were used, comprising reference collection isolates from the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC) (UK) as well as clinical isolates collected from the UK, United States, and India from 2006 to 2014. *E. coli* BW25113, JW0452-3 (BW25113Δ*acrA*::*kan*), and JW5503-1 (BW25113Δ*tolC*::*kan*) were sourced from the Coli Genetic Stock Centre (CGSC) (Yale University, USA). *E. coli* JW0452-3 and JW5503-1 were maintained on medium containing 25 μg/ml kanamycin.

Peptide synthesis. SPR741-sulfate salt is a synthetic cyclic peptide that was procured from Bachem AG (Bubendorf, Switzerland) via custom synthesis. Briefly, SPR741 was produced by using established polypeptide manufacturing techniques, using SPPS to assemble the peptide sequence. After solution-phase cleavage and selective deprotection, the peptide was cyclized and functionalized. After complete structural assembly, the peptide was deprotected to produce a crude product. The crude peptide was then purified via sequential reverse-phase chromatographic separations and ion exchange to give the desired sulfate salt. Finally, SPR741 was obtained after lyophilization to a sterile powder. Multiple batches of SPR741 were employed throughout these investigations.

Polymyxin B sulfate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Azithromycin, ceftazidime, chloramphenicol, ciprofloxacin, clarithromycin, erythromycin, fosfomycin, fusidic acid, levofloxacin, amdinocillin, mupirocin, nalidixic acid, nisin, nitrofurantoin, ramoplanin, novobiocin, rifampin, teicoplanin, and tigecycline were obtained from Sigma-Aldrich (Dorset, UK). Aztreonam, cefepime, gentamicin, tobramycin, trimethoprim, and vancomycin were purchased from Alfa Aesar (Lancashire, UK). Dalbavancin, daptomycin, fidaxomicin, fosmidomycin, quinupristin-dalfopristin, and telithromycin were purchased from Santa-Cruz Biotechnology (Dallas, TX, USA). Meropenem was purchased from Melford Laboratories Ltd. (Suffolk, UK). Clindamycin was purchased from Duchefa Biochemie BV (Netherlands). Linezolid was purchased from Fluorochem Ltd. (Derbyshire, UK). Retapamulin was purchased from Combi-Blocks, Inc. (San Diego, CA, USA).

Antibacterial susceptibility testing and checkerboard assays. The MICs of SPR741 and antibiotics were determined by using cation-adjusted Mueller-Hinton II broth (caMHB; Fluka), according to CLSI guidelines M7-A10 (24) and M100-S26 (13). SPR741 was prepared in sterile water. Antibiotics were prepared according to CLSI guidelines (13). Assay plates were incubated at 37°C for 16 to 20 h and read both visually and by using spectrophotometer at 600 nm. The MIC was defined as the lowest concentration of test articles resulting in the complete inhibition of visible bacterial growth.

To determine whether there was an interaction between SPR741 and a given antibiotic, the MICs of combinations were determined in a checkerboard format based on previously reported guidelines (25), except that flat-bottomed polystyrene plates (catalog number 3370; Corning) and an assay mixture volume of 200 μl were used throughout. The FICI was determined for each combination (i.e., each well) for which the MIC of the combination was different from that of each compound in isolation and was calculated and interpreted as described previously (26, 27).

MIC_{90} determination. The MICs of aztreonam, azithromycin, clarithromycin, fusidic acid, meropenem, mupirocin, retapamulin, and rifampin were determined against a panel of 25 *E. coli*, 25 *K. pneumoniae*, and 17 *A. baumannii* MDR and clinical isolates in the presence or absence of 2 μg/ml SPR741 (all strains) or 2 and 8 μg/ml SPR741 (*K. pneumoniae* and *A. baumannii*). Sterile, round-bottomed, untreated polystyrene plates (catalog number 3788; Corning) and an assay mixture volume of 200 μl were used throughout. Assay mixtures were incubated for the appropriate length of time for each organism as specified by CLSI guidelines (24). The MICs of SPR741, antibiotics, and combinations thereof were defined as the lowest concentration that inhibited visible bacterial growth. The MIC_{90} represents the MIC value at which the growth of 90% of the strains within the test panel was inhibited, calculated by using the formula $n \times 0.9$ (where the resulting number was not an integer, the next integer following the respective value was used).

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