


***In vitro* activity of rifabutin against 293 contemporary carbapenem-resistant *Acinetobacter baumannii* clinical isolates and characterization of rifabutin mode of action and resistance mechanisms**

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Background: Rifabutin, an oral drug approved to treat *Mycobacterium avium* infections, demonstrated potent activity against *Acinetobacter baumannii* in nutrient-limited medium enabled by rifabutin cellular uptake through the siderophore receptor FhuE.

Objectives: To determine rifabutin *in vitro* activity and resistance mechanisms in a large panel of *A. baumannii* isolates.

Methods: Two hundred and ninety-three carbapenem-resistant *A. baumannii* clinical isolates collected from Europe, the USA and Asia during 2017–19 were used for MIC determination. Sequencing/genotyping of *fhuE*, *rpoB* and *arr-2* genes in isolates with elevated rifabutin MIC combined with genetic engineering and gene expression quantification was used to characterize rifabutin's mode of action and resistance mechanisms.

Results: Rifabutin showed excellent activity on the strain panel, with an MIC_{50/90} of 0.008/1 mg/L, and was superior to all other antibiotics tested, including colistin, tigecycline and cefiderocol (MIC₉₀ of 8 mg/L). Rifabutin remained active on resistant subpopulations, including strains resistant to the siderophore–drug conjugate cefiderocol (MIC₉₀ of 2 mg/L, *n* = 23). At least two independent resistance mechanisms were required to abolish rifabutin activity, which is in line with the dose-dependent mutational resistance frequency reaching 10^{−9} at rifabutin concentrations at or above 2 mg/L.

Conclusions: This study demonstrated the potent activity of rifabutin against carbapenem-resistant *A. baumannii*. We propose that FhuE-mediated active uptake of rifabutin enables activity against rifampicin-resistant isolates. To achieve clinically meaningful strain coverage and to avoid rapid resistance development, rifabutin concentrations ≥2 mg/L are required, something rifabutin oral formulations cannot deliver.

Introduction

Infections caused by Gram-negative bacteria are increasingly difficult to treat due to the emergence and global spread of MDR or XDR strains.^{1,2} Of particular concern according to the WHO priority list is carbapenem-resistant *Acinetobacter baumannii*, against which novel antibiotics are urgently needed.³

Rifabutin is a spiro-piperidyl rifamycin of the ansamycin antimicrobial drug class. Rifabutin (Mycobutin[®]) was approved by the FDA in 1992 as an oral formulation for the prevention of disseminated *Mycobacterium avium* complex disease in patients with

advanced HIV infection. Rifabutin exerts its antibacterial activity by selective inhibition of the bacterial DNA-dependent RNA polymerase that catalyses RNA synthesis.⁴ Rifabutin was previously shown to have only minimal activity against Gram-negative bacteria when determined in standard nutrient-rich cationic-adjusted Mueller–Hinton broth (CA-MHB) because of the Gram-negative outer membrane that represents a permeability barrier.⁵ We found that rifabutin exerts potent antibacterial activity against *A. baumannii* when tested in the nutrient-limited Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with FCS.⁶

This increased activity is mediated by cellular uptake of the antibiotic through the TonB-dependent siderophore receptor FhuE, which is up-regulated in RPMI supplemented with FCS but not in standard CA-MHB medium.⁶ Importantly, this novel *in vitro* activity correlated with excellent *in vivo* efficacy in murine systemic and lung infection models with *A. baumannii*.⁶ In this study we intended to determine the *in vitro* activity of rifabutin on a panel of recently isolated carbapenem-resistant *A. baumannii* (CRAB) isolates.

Materials and methods

Bacterial isolates

A. baumannii isolates ($n = 293$) used in this study were collected between 2017 and 2019 in various clinical laboratories from Europe ($n = 144$), the USA ($n = 99$) and Asia-West Pacific ($n = 50$) regions (JMI Laboratories, North Liberty, IA, USA). All the isolates were carbapenem resistant, and most of the isolates were from patients with bloodstream infection ($n = 82$), pneumonia ($n = 174$) or skin/soft tissue infection ($n = 33$) (Table S1, available as [Supplementary data](#) at JAC Online).

Susceptibility testing

Rifabutin MICs were determined using broth microdilution and agar dilution methods following the CLSI guidelines but using RPMI supplemented with 10% (v/v) FCS (IC-RPMI) as assay medium for broth microdilution and Mueller–Hinton agar (MHA) supplemented with 0.1 mM pyridoxal isonicotinoyl hydrazone (PIH) (IC-MHA) for agar dilution. MICs of the comparator antibiotics meropenem, ceftazidime, minocycline, colistin, tigecycline, tobramycin and cefiderocol were determined according to CLSI guidelines.⁷

Gene expression level quantification

Quantitative RT-PCR (qRT-PCR) was performed to quantify gene expression levels as previously described.⁸ Expression was normalized to that of the housekeeping gene *rpoD* using the comparative $\Delta\Delta CT$ method. The list of primers used in this study can be found in Table S2.

Genetic engineering

All genetic engineering (deletion and allelic exchange) manipulations on *A. baumannii* clinical isolates were performed using a two-step recombination method previously described, as it allows manipulation of multiple clinical strains independent of their resistance profile.⁸ Briefly, DNA fragments corresponding to 700 bp upstream and downstream genomic regions of the genes to be deleted were amplified by PCR and cloned in the multiple cloning site of the knockout plasmid pVT77. For FhuE allelic exchange, the entire sequence from 700 bp upstream to 700 bp downstream of *fhuE*^{HUMC1} (including the *fhuE*^{HUMC1} sequence) was cloned in pVT77. The resulting plasmids were conjugated in *A. baumannii* isolates, and transconjugants were selected on LB agar plates containing 100 mg/L tellurite. The second recombination was selected on 200 mg/L 3'-azido-3'-deoxythymidine and the desired genetic modifications were confirmed by PCR and sequencing (Table S2).

Plasmid-mediated expression

The *fhuE* gene (AWC45_RS10145) from *A. baumannii* HUMC1 and LAC-4 strains and the *arr-2* gene (ABAYE_RS17985) from the *A. baumannii* AYE strain were cloned into the *Escherichia coli*/*A. baumannii* shuttle plasmid pVT111, which contains the *A. baumannii* pWH1266 origin of replication, under the control of the IPTG-inducible promoter P_{trc}-lacO. The FhuE V38P mutation (codon GTC to CCC) was introduced in FhuE^{HUMC1} by PCR

site-directed mutagenesis. The resulting plasmids as well as the original pVT111 control plasmid were conjugated into *A. baumannii* ATCC 17978 and transconjugants were selected on LB agar plates containing kanamycin. The presence of the desired plasmids in *A. baumannii* was confirmed by PCR and sequencing (Table S2).

Genotyping of *fhuE*, *rpoB* and *arr-2*

A genomic DNA sequence including *fhuE* or the rifampicin resistance-determining regions (RRDRs) of *rpoB* was PCR amplified and the PCR products were sent for sequencing (Microsynth AG, Balgach, Switzerland). The full *rpoB* gene of strain BV667 was sequenced. The presence of *arr-2* and *fhuE*^{LAC-4} was assessed by PCR using primers specific for *fhuE*^{LAC-4} that do not anneal on the *fhuE*^{HUMC1} allele (Table S2).

Determination of spontaneous resistance frequencies

The frequency of mutational resistance (FoR) to rifabutin was determined on MHA plates supplemented with 0.1 mM PIH. Cells from a 20 h culture in 50 mL of Mueller–Hinton medium were concentrated in 1 mL of PBS by centrifugation and 100 μ L of 10-fold serial dilutions was inoculated on agar plates containing specified antibiotic concentrations. The plates were incubated at 35°C for 24 h and the FoR was calculated as the ratio between the number of colonies growing on plates with antibiotic and the total colony count of the inocula determined on non-selective agar plates.

Results

Iron chelation in standard Mueller–Hinton medium enables active rifabutin cellular uptake through FhuE overexpression

Potent rifabutin activity observed in RPMI supplemented with 10% FCS is enabled by FhuE overexpression in this specific nutrient- and iron-depleted medium.⁶ In contrast, the standard CA-MHB MIC testing medium is a complex and rich medium that does not promote FhuE expression (Figure 1a). Plasmid-mediated expression of FhuE in CA-MHB increased rifabutin activity to levels similar to those observed in RPMI supplemented with FCS, indicating that FhuE overexpression is a condition sufficient in CA-MHB to mediate rifabutin cellular uptake (Figure 2a, Figure S1). However, the activity of rifampicin and other known rifamycin antibiotics, including rifamycin CGP 4832, remained unchanged upon FhuE expression, indicating that FhuE is highly selective towards rifabutin transport (Figure S2). Moreover, the expression of a FhuE variant carrying a V38P mutation in the TonB box known to disrupt the interaction between FhuE and the energy transducer protein TonB, was not able to restore rifabutin susceptibility, indicating that the drug is actively transported via FhuE into *A. baumannii* (Figure 2a).^{9,10}

Previous transcriptomic data have shown that reduction of available iron in rich medium using iron chelation led to *fhuE* overexpression in *A. baumannii*.¹¹ We hypothesized that the iron present in CA-MHB must be chelated to achieve FhuE overexpression and potent rifabutin activity in order to develop a standard MIC testing medium that correlates with rifabutin *in vivo* efficacy.⁶ We used the iron chelator PIH to induce iron-chelated conditions in CA-MHB and demonstrated its ability to increase FhuE expression and rifabutin activity when added to CA-MHB (Figure 1a and b). PIH was non-toxic against *A. baumannii*, which was confirmed in a panel of diverse *A. baumannii* strains (Table S3). However, for some isolates with a very low rifabutin MIC, we noted the phenomenon

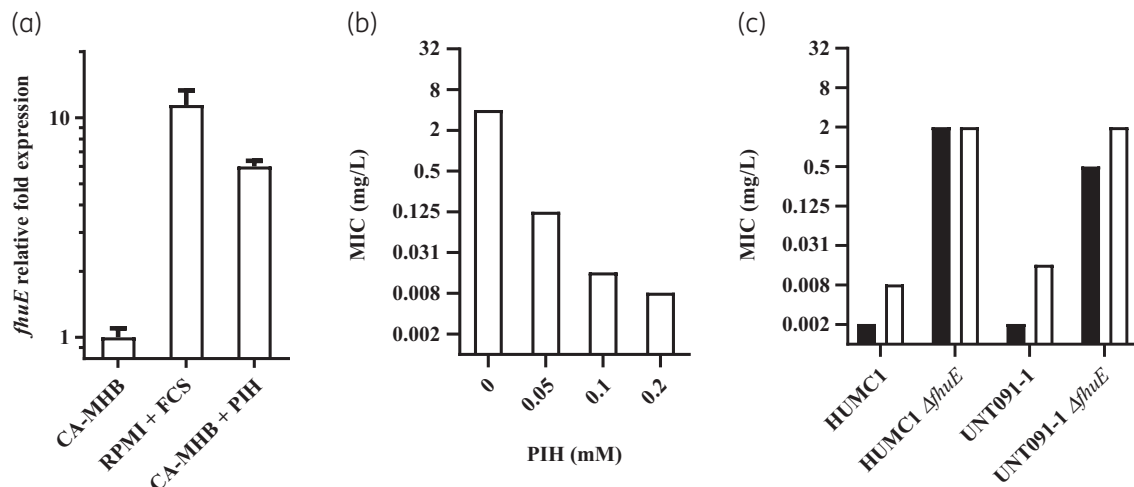


Figure 1. Quantification of *fhuE* expression level and rifabutin activity against *A. baumannii*. (a) *A. baumannii* HUMC1 *fhuE* transcript levels were determined in different media by quantitative real-time PCR and normalized to the *fhuE* expression of the strain grown in CA-MHB. CA-MHB and RPMI media were supplemented with 0.1 mM pyridoxal isonicotinoyl hydrazone (PIH) or 10% (v/v) FCS, respectively. (b) Rifabutin activity on *A. baumannii* HUMC1 determined in CA-MHB supplemented with PIH. (c) Rifabutin activity determined in RPMI supplemented with 10% (v/v) FCS (black) and MHA supplemented with 0.1 mM PIH (white) on WT *A. baumannii* strains and their $\Delta fhuE$ mutants.

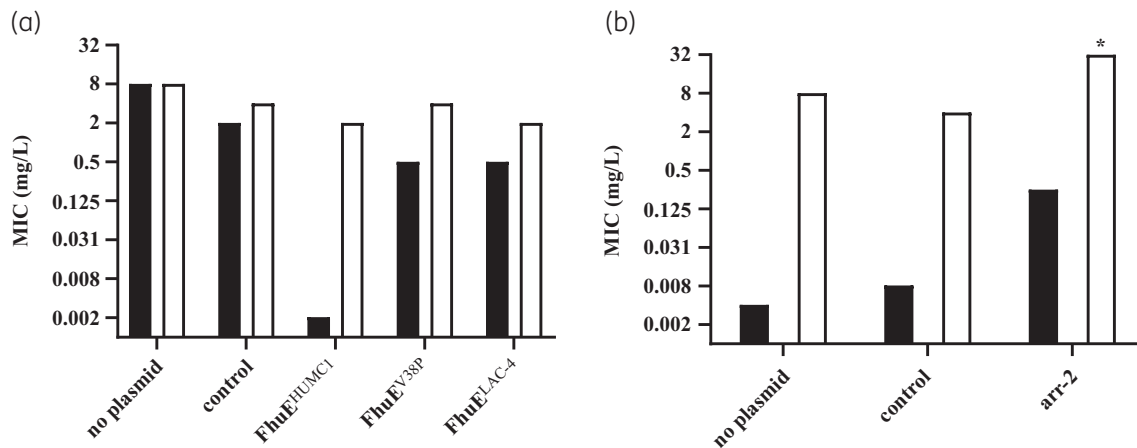


Figure 2. Activity of rifabutin (black bars) and rifampicin (white bars) upon plasmid-mediated expression of (a) different FhuE variants determined in CA-MHB medium and (b) Arr-2 determined in RPMI supplemented with 10% FCS. *A. baumannii* ATCC-17978 was used as host strain; gene expression from plasmids was induced with 1 mM IPTG, and a plasmid that did not encode *fhuE* or *arr-2* was used as control. *MIC >32 mg/L.

of skipped wells with MICs determined in CA-MHB supplemented with PIH or with other iron chelators (apo-transferrin, 2,2-bipyridyl and deferiprone) (Figure S3). The characterization of clones from MIC with skipped wells demonstrated that this phenomenon results from mutations in *fhuE* leading to a disrupted rifabutin uptake (Table S4). Specific uptake disruption *in vitro* was previously reported for the antibiotic fosfomycin, for which agar dilution MIC is the only approved method allowing robust MIC determination; according to CLSI guidelines, broth microdilution should not be performed.^{7,12} Therefore, we determined rifabutin agar dilution MICs on MHA supplemented with 0.1 mM PIH and confirmed that PIH addition to MHA triggers a FhuE-dependent uptake of rifabutin (Figure 1c). The results obtained on MHA supplemented with 0.1 mM PIH are in accordance with the MICs observed in RPMI supplemented with 10% FCS, indicating that this iron-chelated MHA

formulation (IC-MHA) allows a reliable determination of rifabutin MIC against *A. baumannii*. This standard agar dilution method may replace non-standard testing conditions using iron-chelated RPMI medium (IC-RPMI) to determine robust rifabutin MICs.

Rifabutin shows excellent activity on 293 CRAB isolates

A panel of 293 CRAB clinical isolates collected between 2017 and 2019 from Europe ($n = 144$), the USA ($n = 99$) and Asia-West Pacific ($n = 50$) regions was used for rifabutin MIC determination. Rifabutin MICs were determined by broth microdilution and agar dilution methods in IC-RPMI and IC-MHA, respectively (Table S1). Rifabutin showed excellent activity in both testing methods, with an MIC₅₀/MIC₉₀ of 0.002/2 mg/L and 0.008/1 mg/L using IC-RPMI and IC-MHA, respectively (Figure 3a). There was little difference

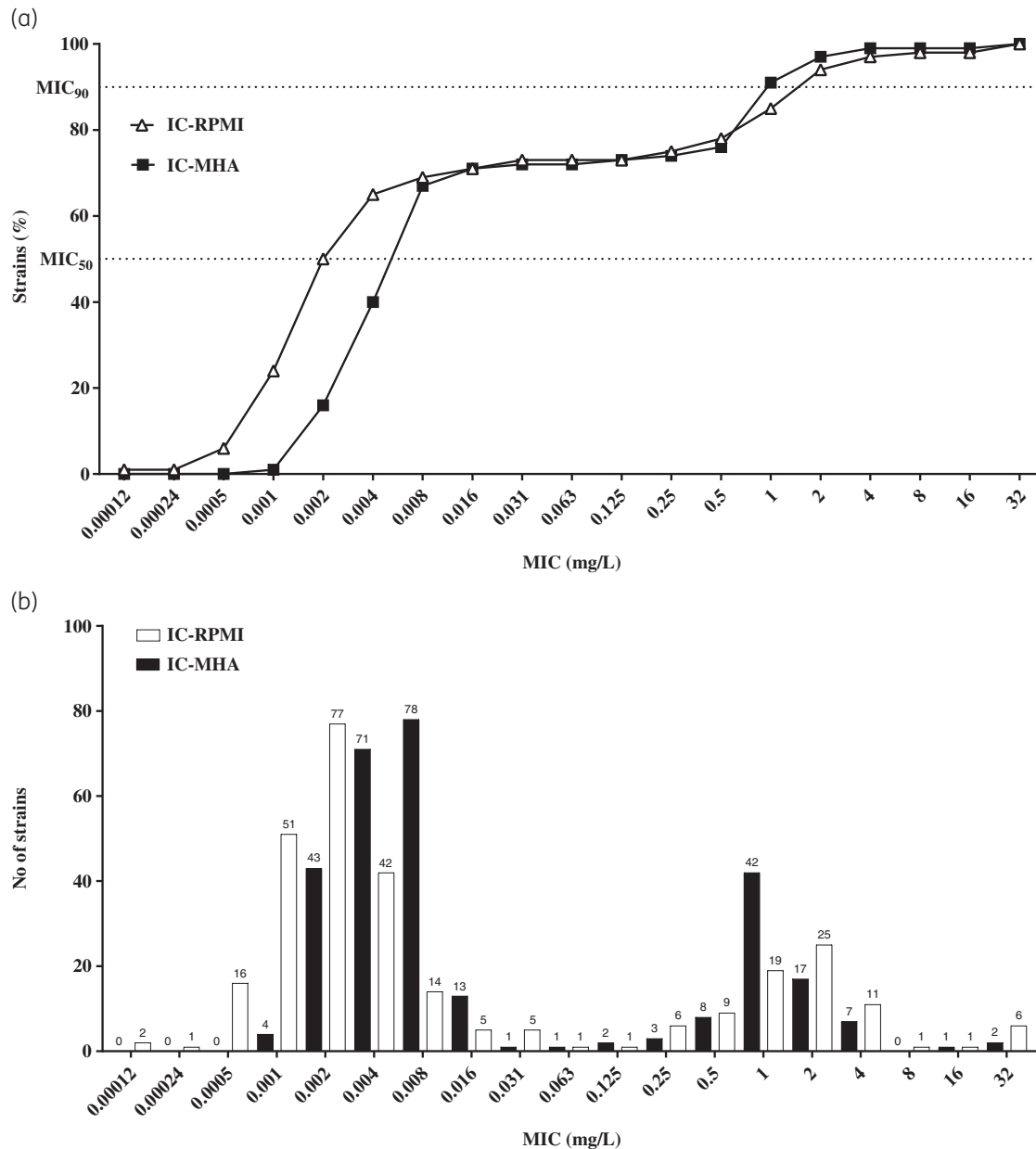


Figure 3. Comparison of the efficacy of rifabutin against 293 carbapenem-resistant clinical isolates of *A. baumannii* in two different media. (a) Cumulative susceptibility and (b) MIC distribution of rifabutin determined in IC-RPMI and IC-MHA.

between testing methods in the overall susceptibility of the isolates. However, for the isolates with a very low MIC (≤ 0.008 mg/L), a one-dilution shift towards lower MIC in IC-RPMI was observed, which may reflect the nutrient-limited conditions of this medium (Figure 3a). In addition, rifabutin activity was consistent across regions and infection types, with only a one-dilution MIC₉₀ shift for isolates from the USA or from soft/skin tissue infections (MIC₉₀ of 2 mg/L) (Table 1).

Rifabutin was superior to all comparator antibiotics tested, including colistin, tigecycline and cefiderocol (Figure 4, Table 2). Overall, rifabutin activity was not affected by resistance to specific antibiotics as the rifabutin MIC₅₀/MIC₉₀ remained stable on the

different antibiotic-resistant subpopulations (Table 3). Importantly, there was no cross-resistance between rifabutin and cefiderocol despite both drugs employing bacterial iron transport mechanisms for efficient cellular uptake.¹³

Inactive rifabutin cellular uptake confers elevated rifabutin MIC in *A. baumannii* clinical isolates

Further analysis of the rifabutin MICs revealed three *A. baumannii* subpopulations with distinct rifabutin susceptibilities, population 1 (Pop1, MICs < 0.125 mg/L, 72%), population 2 (Pop2, MICs ≥ 0.125 to < 16 mg/L, 27%) and population 3 (Pop3, MICs ≥ 16 mg/L, 1%)

Table 1. Cumulative susceptibility to rifabutin determined in IC-MHA against all *A. baumannii* isolates or by region and infection type

Rifabutin MIC (mg/L)	Cumulative percentage of isolates (%) ^a						
	Total (n = 293)	Europe (n = 144)	USA (n = 99)	Asia (n = 50)	BSI (n = 82)	HAP (n = 174)	SSI (n = 33)
0.0005	0	0	0	0	0	0	0
0.001	1	2	0	2	0	2	0
0.002	16	18	10	22	17	17	9
0.004	40	43	30	52	49	37	36
0.008	67	75	51	76	83	62	52
0.016	71	81	54	80	84	68	52
0.031	72	81	54	80	84	69	52
0.063	72	82	54	80	84	69	55
0.125	73	83	55	80	84	70	55
0.25	74	83	56	82	84	72	55
0.5	76	86	59	84	87	74	61
1	91	94	83	98	96	90	79
2	97	98	94	98	98	97	91
4	99	100	98	98	99	99	97
8	99	100	98	98	99	99	97
16	99	100	99	98	99	99	100
32	100	100	100	100	100	100	100

BSI, bloodstream infection; HAP, hospital-acquired infection; SSI, soft/skin tissue infection.

^aMIC₉₀ values are shown in bold for each MIC distribution.

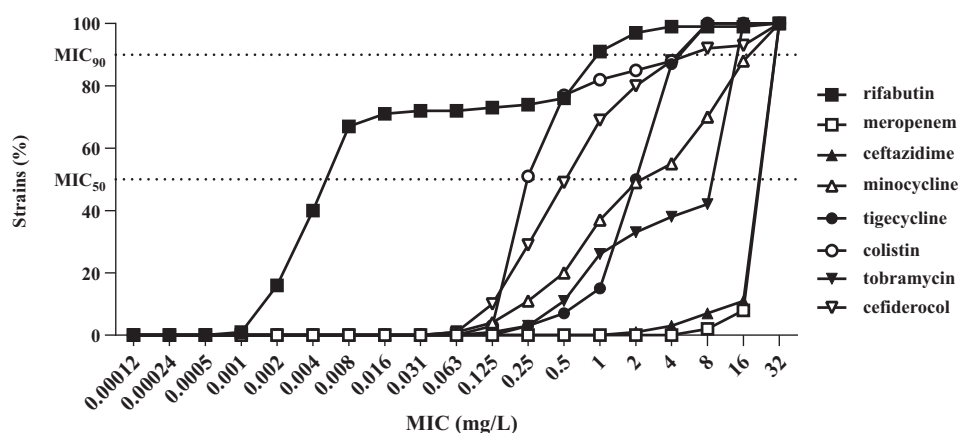


Figure 4. Cumulative susceptibility of rifabutin and comparator antibiotics against a panel of 293 carbapenem-resistant *A. baumannii*. Rifabutin MIC was determined in IC-MHA, whereas the MICs of meropenem, ceftazidime, minocycline, tigecycline, colistin, tobramycin and cefiderocol were determined according to CLSI guidelines.⁷

(Figure 3b, Table S5). The DNA sequence of the TonB-dependent siderophore receptor *fhuE*, responsible for rifabutin cellular uptake, was altered in 85% ($n = 70$) of the clinical isolates from Pop2 and Pop3 ($n = 82$) (Table 4, Table S6). Single mutation events inactivating *FhuE* were found in 50% ($n = 35$) of the strains with disrupted rifabutin uptake, including transposon insertions, nucleotide deletions/insertions and early stop codon mutations. The other 35 strains encoded a distinct *FhuE* variant (*FhuE*^{LAC-4}) with only 59% amino acid identity to the *FhuE* encoded in the reference strain HUMC1 (*FhuE*^{HUMC1}).⁶

Chromosomal replacement of *fhuE*^{LAC-4} by the *fhuE*^{HUMC1} allele in the LAC-4 strain restored the potent activity of rifabutin

(Figure 5a). In contrast, plasmid-mediated expression of *FhuE*^{LAC-4} did not improve rifabutin activity in CA-MHB, confirming that *FhuE*^{LAC-4} is unable to transport rifabutin (Figure 2a, Figure S1). To determine whether *FhuE*^{LAC-4} was still active in transporting siderophores, we evaluated the ability of siderophores to restore the growth of the *A. baumannii* ATCC 17978 mutant deleted for endogenous acinetobactin biosynthesis and expressing *FhuE*^{HUMC1} or *FhuE*^{LAC-4} in iron-limiting conditions, as previously described.⁹ Both *FhuE*^{HUMC1} and *FhuE*^{LAC-4} variants restored growth in the presence of siderophores, indicating that despite their sequence divergence both *FhuE* variants can transport siderophores (Figure 6). The *FhuE*^{LAC-4} variant may be derived from *Acinetobacter pittii*,

Table 2. Overview of antimicrobial susceptibility of 293 clinical *A. baumannii* isolates and clinical classification according to EUCAST and CLSI guidelines

Antimicrobial agent	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	EUCAST ^a		CLSI ^b		
			%S	%R	%S	%I	%R
Rifabutin ^c	0.008	1	–	–	–	–	–
Meropenem	>32	>32	0	100.0	0	0.3	99.7
Ceftazidime	>32	>32	–	–	6.8	4.5	88.7
Minocycline	4	32	–	–	55.3	14.7	30.0
Tigecycline	2	8	–	–	–	–	–
Colistin	0.25	8	84.6	15.4	84.6	–	15.4
Tobramycin	>16	>16	38.2	61.8	38.2	3.4	58.4
Cefiderocol	1	8	–	–	87.7	4.4	7.8

S, susceptible; I, intermediate; R, resistant.

^aEUCAST Clinical breakpoints version 9.0.³⁴

^bCLSI document M100-Ed30.⁷

^cRifabutin MIC determined on IC-MHA.

Table 3. Rifabutin shows strong activity against *A. baumannii* subpopulations resistant to different antibiotics

Resistant subpopulation	No. of isolates	Rifabutin MIC ₅₀ (mg/L) ^a	Rifabutin MIC ₉₀ (mg/L) ^a
Minocycline ≥16 mg/L	88	0.008	1
Colistin >2 mg/L	45	0.008	1
Tigecycline >2 mg/L	147	0.008	1
Tobramycin ≥16 mg/L	171	0.008	1
Cefiderocol ≥16 mg/L	23	0.008	2

^aRifabutin MICs determined on IC-MHA were used for calculations.

Table 4. Overview of the resistance mechanisms (based on FhuE) in relation to rifabutin and rifampicin activity on the 82 strains that belong to Pop2 and Pop3 rifabutin susceptibility subpopulations

FhuE mutations	No. of strains	MIC range (mg/L)		Additional resistance mechanisms (RpoB/Arr-2)
		Rifabutin ^a	Rifampicin	
WT	2	0.125–1	8	RpoB L542F
	4	0.125–1	>32	RpoB H535L
	1	>32	>32	RpoB H535L
	3	0.5–4	>32	RpoB H535C
	2	0.25–0.5	>32	Arr-2
LAC-4	33	0.5–4	1–8	none
	1	16	>32	RpoB H535Q
	1	>32	>32	Arr-2
Disrupted ^b	34	0.25–4	4–16	none
	1	4	>32	RpoB H535Q

^aRifabutin MIC determined on IC-MHA.

^bFhuE disruption due to transposon insertion, nucleotide deletion/insertion or an early stop codon. More information can be found in Table S6.

which encodes a FhuE variant with 96% amino acid identity to FhuE^{LAC-4}, while the other pathogenic *Acinetobacter* species (namely *A. nosocomialis*, *A. lactucae* and *A. seifertii*) encode FhuE variants similar to FhuE^{HUMC1} (93%–97% amino acid identity). In

addition, phylogenetic analysis revealed that each *A. baumannii* FhuE variant seems to be conserved in specific clonal lineages (Table S7). The FhuE^{HUMC1} variant is conserved in international clone (IC) 1 and IC2 strains, representing global clonal lineages,

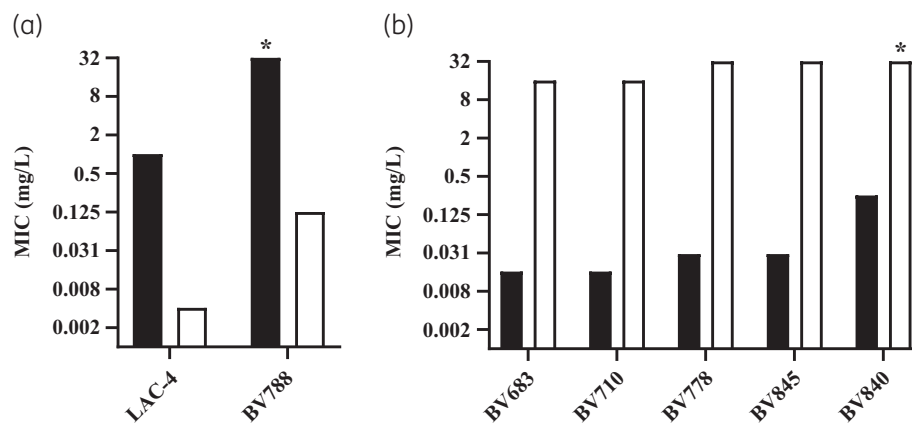


Figure 5. Rifabutin activity determined in IC-RPMI on *A. baumannii* strains with active or disrupted rifabutin uptake. (a) Rifabutin MIC on WT strains encoding the FhuE^{LAC-4} variant (black bars) and their isogenic mutants where *fhuE*^{LAC-4} was chromosomally exchanged for the *fhuE*^{HUMC1} allele (white bars). Strain BV788 carries an RpoB H535Q mutation while strain LAC-4 does not carry additional resistance mechanisms. *MIC >32 mg/L. (b) Rifabutin MIC on WT strains encoding the FhuE^{HUMC1} variant (black bars) and their $\Delta fhuE$ isogenic mutants (white bars). Strains BV683, BV710, BV778 and BV845 carry RpoB mutations I581M, S583L, H535Q and N527D, respectively, and strain BV840 encodes Arr-2 inactivation enzyme. *MIC >32 mg/L.

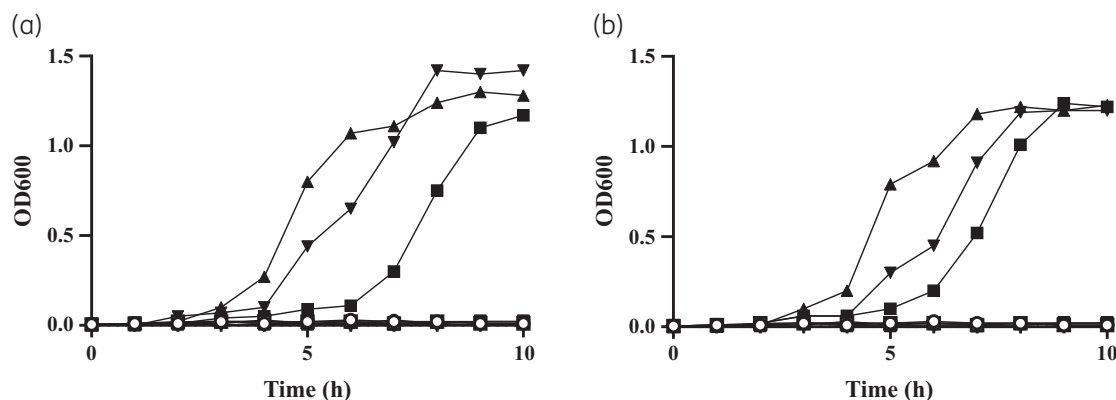


Figure 6. Growth complementation of the *A. baumannii* mutant strain depleted for endogenous siderophore production to determine the ability of FhuE^{HUMC1} and FhuE^{LAC-4} variants to transport siderophores. *A. baumannii* ATCC 19798 $\Delta basD\Delta fhuE$ double mutant strains complemented with (a) FhuE^{HUMC1}-expressing plasmid or (b) FhuE^{LAC-4}-expressing plasmid were grown in LB medium containing 225 μ M 2,2-bipyridyl supplemented with 20 μ M desferricoprogen (square), rhodotorulic acid (triangle), desferrioxamine B (inverted triangle) or no siderophores (circle), as previously described.⁹ *A. baumannii* ATCC 19798 $\Delta basD\Delta fhuE$ double mutant strains complemented with an empty plasmid were used as controls (open symbols).

with IC2 being the most prevalent worldwide.¹⁴ In contrast, FhuE^{LAC-4} is conserved in strains from IC5, which is a pan-American clonal lineage. This is in accordance with our strain panel where 80% of the strains encoding the FhuE^{LAC-4} variant were isolated from the USA (Table S6).

Rifabutin overcomes RpoB mutations and Arr-2 expression conferring rifampicin resistance in *A. baumannii* clinical isolates

Mutations in the DNA-dependent RNA polymerase β -subunit (RpoB) and expression of the *arr-2* gene coding for rifampicin ADP-ribosyltransferase are mechanisms that confer resistance to rifamycin antibiotics, and those resistance mechanisms have previously been identified in *A. baumannii*.^{15–18} Mutations in RpoB RRDR cluster I and II were identified in 86% ($n = 19$) of the strains

with an elevated rifampicin MIC (MIC >32 mg/L, $n = 22$) while the other 14% ($n = 3$) of the strains with an elevated rifampicin MIC encoded the *arr-2* gene (Table 5, Table S8).

The most frequent RpoB mutation, found in 16 isolates (73%), was the substitution of a histidine at position 535 by a glutamine ($n = 8$), leucine ($n = 5$) or cystine ($n = 3$). Among the isolates with RpoB mutations, nine strains belonged to Pop1 rifabutin susceptibility (MICs <0.125 mg/L), eight strains belonged to Pop2 (MICs ≥ 0.125 to <16 mg/L) and only two strains belonged to Pop3 (MICs ≥ 16 mg/L), suggesting that most RpoB mutations present in *A. baumannii* clinical isolates do not abolish rifabutin activity, in contrast to rifampicin loss of activity. Rifabutin uptake deletion in the Pop1 strains carrying RpoB mutations converted them to Pop3 strains, suggesting that potent rifabutin activity on RpoB mutants is due to FhuE-mediated active transport of rifabutin (Figure 5b). Interestingly, the two Pop3 strains (BV667 and BV788) carried

Table 5. Overview of the resistance mechanisms (based on RpoB) in relation to rifabutin and rifampicin activity on the 22 strains with rifampicin MIC above 32 mg/L

RpoB mutations	No. of strains	MIC range (mg/L)		Additional resistance mechanisms (FhuE/Arr-2)
		Rifabutin ^a	Rifampicin	
WT	2	0.25–0.5	>32	Arr-2
	1	>32	>32	FhuE ^{LAC-4} and Arr-2
I581M	1	0.016	>32	none
S583L	1	0.06	>32	none
N527D	1	0.008	>32	none
H535Q	6	0.008–0.016	>32	none
	1	4	>32	FhuE disruption
	1	16	>32	FhuE ^{LAC-4}
H535L	4	0.125–1	>32	none
	1	>32	>32	none
H535C	3	0.5–4	>32	none

More information can be found in Table S8.

^aRifabutin MIC determined on IC-MHA.

RpoB mutations identical to other Pop1 and Pop2 strains, suggesting that an additional mechanism must be involved in the elevated rifabutin MIC of these two strains (Table 5). The strain BV788 encoded a FhuE^{LAC-4} variant in addition to the RpoB H535Q mutation, and chromosomal replacement of *fhuE*^{LAC-4} by *fhuE*^{HUMC1} in this strain confirmed that rifabutin overcomes resistance conferred by this RpoB mutation (Figure 5a). The second Pop3 strain (BV667) carried an RpoB H535L mutation but did not encode additional *arr-2* or *fhuE* disruption, indicating that another rifabutin resistance mechanism may exist. The entire *rpoB* gene of BV667 was sequenced to rule out additional mutations outside RRDR clusters, and the induction of *fhuE* expression upon iron chelation was confirmed (Figure S4).

Among the *arr-2*-expressing isolates, two strains belonged to rifabutin susceptibility Pop2 and only one strain belonged to Pop3 (Table 5). The Pop3 strain encoded a FhuE^{LAC-4} variant in addition to the *arr-2* gene. Deletion of *fhuE* in a strain carrying *arr-2* (BV840) as its only resistance mechanism, as well as plasmid-mediated *arr-2* expression, demonstrated that the *arr-2* resistance mechanism alone does not abolish rifabutin activity, in contrast to loss of rifampicin activity (Figures 2b and 5b).

The frequency of mutational resistance of clinical *A. baumannii* strains to rifabutin is dose dependent, reaching 10⁻⁹ at drug concentrations at or above 2 mg/L

The mutational FoR to rifabutin determined on the Pop1 strain UNT091-1 ranged from 10⁻⁵ to 10⁻⁶ with rifabutin concentrations up to 1 mg/L and decreased to 10⁻⁹ at rifabutin concentrations at or above 2 mg/L (Table 6). No difference was observed between rifabutin FoR determined on IC-MHA and IC-RPMI agar plates. This dose-dependent FoR suggests that a multistep mutational process is required to acquire full rifabutin resistance (MIC >32 mg/L), which is in accordance with the identification of at least two independent resistance mechanisms in Pop3 clinical isolates (Tables 4 and 5). In addition, the median FoR determined at 4-fold MIC on

Table 6. *A. baumannii* UNT091-1 mutational resistance frequencies towards rifabutin

Rifabutin concentration	Frequency of resistance	Rifabutin MIC (mg/L) ^a
UNT091-1 wildtype	–	0.001
0.02 mg/L	1.1 × 10 ⁻⁵	1
0.2 mg/L	7.7 × 10 ⁻⁶	1
0.5 mg/L	7.5 × 10 ⁻⁶	ND
1 mg/L	6.0 × 10 ⁻⁶	ND
2 mg/L	4.2 × 10 ⁻⁹	>32
20 mg/L	4.3 × 10 ⁻⁹	>32

ND, not determined.

^aRifabutin MIC determined in IC-RPMI.

10 Pop2 strains was 6.7 × 10⁻⁹, indicating that intermediate resistance levels do not affect the FoR to rifabutin (Table S9). Finally, FoRs to rifabutin were 1 log lower than FoRs to meropenem and minocycline and in the same range as the FoR to tobramycin (Table S10). Our data suggest that rifabutin resistance development in *A. baumannii* is low at or above 2 mg/L, and similar or better than resistance development to standard of care antibiotics.

Discussion

Rifabutin demonstrated potent *in vitro* activity on clinical CRAB isolates collected between 2017 and 2019 from three different continents. Rifabutin was equally active across geographical regions and infection types, including bloodstream infection and hospital-acquired pneumonia, with an MIC₉₀ of 1–2 mg/L. The clonal nature of *A. baumannii* may lead to a considerable number of identical strains derived from clinical outbreaks. However, the tested isolates were collected from 72 different clinical sites within 22 different countries to reduce a potential bias of the study. In terms of MIC₉₀, rifabutin was superior to all tested comparator

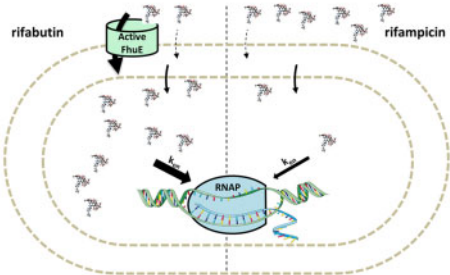
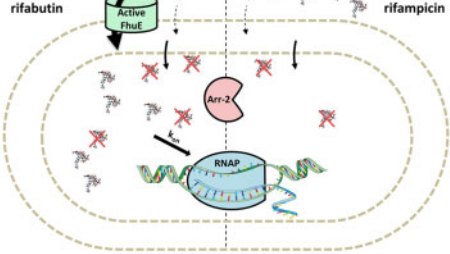
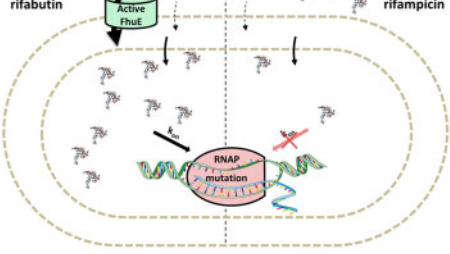
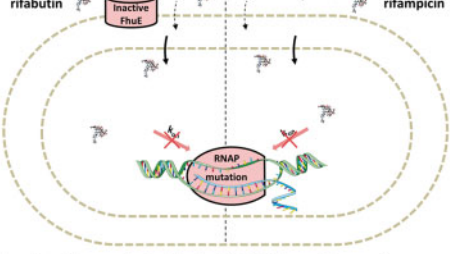
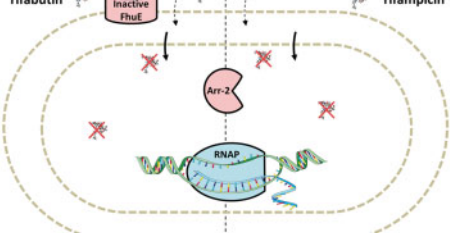
Schematic representation of drug mode of action		Mode of action description and phenotypic consequences	
Rifabutin	Rifampicin	Rifabutin	Rifampicin
No resistance mechanism			
		<ul style="list-style-type: none"> - FhuE-mediated active transport enables potent activity - MIC <0.125 mg/L - Pop1 	<ul style="list-style-type: none"> - Passive diffusion is not sufficient for potent activity - MIC 0.5 - 16 mg/L
Enzymatic inactivation of the drug due to Arr-2 expression			
		<ul style="list-style-type: none"> - FhuE mediated active transport overcomes enzymatic inactivation - MIC 0.125 - 8 mg/L - Pop2 	<ul style="list-style-type: none"> - Passive diffusion is not sufficient to overcome enzymatic inactivation - MIC >32 mg/L
Decreased RNA polymerase (RNAP) target affinity due to RpoB mutations			
		<ul style="list-style-type: none"> - FhuE mediated active transport overcomes RNAP decreased affinity - MIC <8 mg/L - Pop1 and Pop2 	<ul style="list-style-type: none"> - Passive diffusion is not sufficient to overcome RNAP decreased affinity - MIC >32 mg/L
Inactive drug uptake due to FhuE disruption and decreased RNAP target affinity due to RpoB mutations			
		<ul style="list-style-type: none"> - Passive diffusion is not sufficient to overcome RNAP decreased affinity - MIC >32 mg/L - Pop3 	<ul style="list-style-type: none"> - Passive diffusion is not sufficient to overcome RNAP decreased affinity - MIC >32 mg/L
Inactive drug uptake due to FhuE disruption and enzymatic inactivation of the drug due to Arr-2 expression			
		<ul style="list-style-type: none"> - Passive diffusion is not sufficient to overcome enzymatic inactivation - MIC >32 mg/L - Pop3 	<ul style="list-style-type: none"> - Passive diffusion is not sufficient to overcome enzymatic inactivation - MIC >32 mg/L

Figure 7. Model for rifabutin and rifampicin mode of action and resistance mechanisms in *A. baumannii*. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

antibiotics including colistin and cefiderocol. Notably, rifabutin maintained potency against *A. baumannii* subpopulations resistant to different classes of antibiotics, such as strains resistant to colistin, minocycline, tobramycin and cefiderocol. The absence of cross-resistance between rifabutin and the siderophore–drug conjugate cefiderocol suggests that these two drugs use different iron uptake systems for cellular influx. Cefiderocol transport systems have been identified in *E. coli* (CirA and Fiu) and in *Pseudomonas aeruginosa* (PiuA) but not in *A. baumannii*.¹⁹ Nevertheless, cefiderocol resistance in *A. baumannii* is mainly due to the expression of specific β -lactamases, justifying the absence of cross-resistance to rifabutin.²⁰

Rifabutin MICs determined in IC-MHA medium were consistent with MICs determined in IC-RPMI, in which the potent activity of rifabutin was originally observed.⁶ The rifabutin broth microdilution MIC method using IC-CA-MHB (CA-MHB supplemented with 0.1 mM PIH) medium sometimes led to skipped wells due to rifabutin uptake mutations. Interestingly, this phenomenon was observed only with highly susceptible strains (Pop1) and at rifabutin concentrations below 2 mg/L, which is in line with the low FoR observed at or above 2 mg/L. Skipped wells in the broth microdilution MIC method translates in agar dilution MIC tests to single colony growth, which, according to the CLSI, should be disregarded when reading agar dilution MIC endpoints.²¹ Therefore, the agar dilution MIC method using IC-MHA medium allows robust determination of rifabutin activity on all *A. baumannii* strains while the broth microdilution MIC method using IC-CA-MHB medium allows robust determination of rifabutin MICs only in Pop2 and Pop3 strains (MIC \geq 0.125 mg/L).

The trimodal MIC distribution and the dose-dependent FoR suggested that resistance to rifabutin is a multistep process. This was confirmed with the characterization of rifabutin resistance mechanisms in the Pop2 and Pop3 strains. All the strains with rifabutin MIC at or above 0.125 mg/L had disrupted rifabutin uptake (FhuE), a mutated rifabutin target (RpoB) or a rifabutin inactivation enzyme (Arr-2). Notably, we demonstrated that loss of rifabutin activity (MIC $>$ 32 mg/L) was conferred by a combination of at least two independent resistance mechanisms in clinical strains. In contrast, RpoB mutations or the presence of the Arr-2 inactivation enzyme led to loss of rifampicin activity (MIC $>$ 32 mg/L). It has been described that rifabutin retains activity in the case of specific RpoB mutations that confer rifampicin resistance in *Mycobacterium tuberculosis*.^{22–28} However, disruption of rifabutin uptake in *A. baumannii* Pop1 strains carrying RpoB mutations decreases rifabutin activity to the same extent as rifampicin. Moreover, Arr-2 expression has been shown to confer equal resistance to rifampicin and rifabutin.¹⁶ Altogether these data suggest that rifabutin overcomes RpoB mutation and Arr-2 expression resistance mechanisms because it is actively transported into *A. baumannii*. We propose a rifabutin mechanism of action in which, as in contrast to rifampicin passive diffusion, FhuE-mediated rifabutin active transport creates a high concentration of drug at the site of action, enabling activity against target mutation and enzymatic inactivation resistance mechanisms (Figure 7). The identification of one Pop3 strain only carrying the RpoB H535L mutation, which is also present in several Pop2 strains, suggests that rifabutin resistance mechanisms not identified in this study may exist in clinical isolates.

TonB-dependent transporter (TBDT)-mediated uptake of the rifamycin derivative CGP 4832 has previously been described.^{29–31} Rifamycin CGP 4832 is actively transported across the outer membrane of *E. coli* by the siderophore receptor FhuA, but we demonstrated that it is not transported by *A. baumannii* FhuE. Conversely, rifabutin seems not to be transported by any TBDT from *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa*, as rifabutin does not show potent activity against those Gram-negative species.⁶ In addition, none of the commercially available rifamycin antibiotics was transported by *A. baumannii* FhuE. These results are in line with TBDT high substrate specificity, as TBDT-mediated transport requires specific substrate binding to activate allosteric conformational transition of the transporter leading to the recruitment of the TonB energy transducing machinery through the so-called TonB box of the TBDT.³² It has been demonstrated that rifamycin CGP 4832 is transported in a TonB-dependent manner, and a crystallographic study suggested that the binding of CGP 4832 to FhuA induces an allosteric transition allowing physical interaction between TonB and the FhuA TonB box.³¹ In the same manner, we demonstrated that rifabutin is transported by FhuE in a TonB-dependent manner, suggesting that rifabutin binding to FhuE is required to induce active transport through the *A. baumannii* outer membrane.

Our *in vitro* data on rifabutin activity and mutational resistance frequency suggest that rifabutin concentrations must be at or above 2 mg/L to be effective against *A. baumannii* infections and to avoid rapid resistance development. Rifabutin is available on the market as an oral drug with a maximum recommended dose of 300 mg/day. However, pharmacokinetic studies have shown that maximum concentrations of rifabutin remained below 1 mg/L with a daily dose up to 600 mg.³³ Therefore, use of oral rifabutin in the treatment of *A. baumannii* infections will have only limited efficacy and would certainly lead to rapid development of resistance. In an attempt to overcome these limitations, BioVersys AG has developed an intravenous formulation of rifabutin (BV100) for the treatment of *A. baumannii* infections that have limited treatment options, including hospital- and ventilator-associated pneumonia and bloodstream infections, for patient populations where an intravenous formulation is required.

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Transparency declarations

All authors are employees and own shares or stock options of BioVersys AG.

Supplementary data

Figures S1 to S4 and Tables S1 to S10 are available as [Supplementary data](#) at JAC Online.

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