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Outer Membrane Permeability and Efflux Do Not Limit Antibacterial Activity of Sulbactam-Durlobactam in *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is a notorious Gram-negative nosocomial pathogen that is resistant to antibiotics due to its extremely low outer membrane permeability and inducible multi-drug efflux pumps that work in tandem to lower accumulation of several classes of antimicrobials. Sulbactam (SUL) is a penicillanic acid sulfone that inhibits the activity of class A β-lactamases with intrinsic activity against *A. baumannii* and very low frequency of resistance. But its activity is compromised by TEM-1 and Ambler Class D β-lactamases (also called oxacillinases (OXA)). Durlobactam (DUR) is a rationally designed DBO class non-β-lactam β-lactamase inhibitor with potent activity vs. clinically relevant class A, C and D β-lactamases. When combined with SUL, the resultant SUL-DUR combination has promising activity against multi-drug resistant clinical isolates of *A. baumannii*. The goal of this study was to evaluate if outer membrane permeability and efflux contribute to SUL-DUR susceptibility in *A. baumannii* using representative clinical isolates with a range of SUL-DUR MIC. SUL-DUR antibacterial activity was evaluated by broth microdilution using DUR at 4μg/mL with concomitant titration of SUL using CLSI guidelines. Outer membrane protein (OMP) composition was evaluated using SDS-Polyacrylamide gel electrophoresis and prominent bands were identified using protein mass spectrometry. Changes in the intrinsic efflux ability was quantified in the absence or presence of SUL-DUR using accumulation kinetics of the fluorescent dye, ethidium bromide (EtBr). OMP analysis of selected SUL-DUR strains did not identify protein bands that correlated with relative susceptibility. Additionally, the highly conserved OmpA protein, which is also the primary conduit for SUL and DUR was present across all strains. Efflux analysis revealed inherent differences in the ability of the strains to limit accumulation of EtBr but did not correlate with SUL-DUR susceptibility. EtBr accumulation was unaffected by exposure to SUL-DUR at a multiple of its antibacterial activity – a result that was also confirmed by molecular analysis of efflux pump gene expression under these conditions. In conclusion, alterations in outer membrane porin composition and efflux do not appear to contribute to resistance to SUL-DUR.

Introduction

Acinetobacter baumannii is a versatile nosocomial opportunistic pathogen that causes a variety of infections that include the skin, wounds and the respiratory and urinary tract in humans (Doi, 2015), particularly the elderly and immunocompromised (Gu H, 2018). Antibiotic resistance in *A. baumannii* is primarily attributable to its low outer membrane permeability due to the expression of low conductance porins (like OmpA and CarO) and inducible multi-drug efflux mechanisms - which work in concert to impede drug accumulation in this organism (Zgurskaya, 2015). Resistance to front-line therapies (e.g., carbapenems) is further enhanced by the presence of β-lactamases belonging to different classes (Rodriguez, 2018). Sulbactam is a penicillanic acid sulfone that inhibits the activity of Ambler class A β-lactamases with intrinsic activity against select Gram-negative organisms (Penwell, 2015), that fortunately includes *A. baumannii*. But its activity versus *A. baumannii* is compromised by the TEM-1 β-lactamase and Ambler Class D β-lactamases (also called oxacillinases or OXA) (Penwell, 2015). Durlobactam (ETX2514) is a novel, rationally designed DBO class (Coleman, 2011) non-β-lactam β-lactamase inhibitor with potent activity versus clinically relevant Ambler class A, C and D β-lactamases and penicillin-binding proteins (Durand-Reville, 2017).

When combined with sulbactam, the resultant SUL-DUR combination has promising activity against multi-drug resistant clinical strains of *A. baumannii* (McLeod, 2020). Spontaneous resistance to SUL-DUR occurred at a very low frequency and mapped to residues proximal to the active site of penicillin binding protein 3 (McLeod, 2018). In global surveillance studies, *A. baumannii* isolates with reduced susceptibility to SUL-DUR have been found to either encode for the metallo-β-lactamase NDM-1, which DUR does not inhibit, or mutations in PBP3, the primary target of SUL (McLeod, 2020). SUL-DUR is currently in Phase 3 clinical testing. The presence of these acquired resistance mechanisms has made it difficult to understand whether intrinsic mechanisms of resistance can contribute to elevated SUL-DUR MIC values.

Consequently, the goal of this study was to evaluate the contribution of non-β-lactamase-mediated mechanisms of resistance (*i.e.*, permeability and efflux) to SUL-DUR susceptibility. Specifically, we used Ethidium Bromide (EtBr) accumulation and compared outer membrane porin composition to evaluate the contribution of efflux and permeation to SUL-DUR susceptibility in clinical isolates. We also quantified changes in expression levels of the major efflux pumps in *A. baumannii* in response to exposure to SUL-DUR.

Experimental Procedures

(i) **Susceptibility testing:** The minimal inhibitory concentration (MIC) values were determined using broth microdilution according to the Clinical Laboratory Standards Institute (CLSI) methodologies. SUL-DUR MIC values were determined by titrating sulbactam in the presence of a fixed concentration of 4 μg/ml durlobactam.

(ii) **Outer membrane porin gel electrophoresis:** Outer membranes from log-phase cells were isolated using a previously published procedure (Iyer, 2018). Prominent bands of interest were excised and analyzed using fragmentation followed by mass spectrometry.

(iii) **Efflux Assays:** Overnight cultures of *A. baumannii* were grown in 2-ml MHBII at 35°C, diluted into fresh MHBII the next day and grown to an OD_{600nm} ~ 0.2. 2-ml aliquots were added to tubes containing saline (control) or SUL-DUR, SUL was added at a final concentration corresponding to 4X the MIC of the combination (μg/ml) and DUR was at 4μg/ml. The tubes were incubated at 35°C for 30 minutes with shaking at 240-rpm. At the end of the incubation, the cell suspension was centrifuged, and the cell pellet was resuspended in an appropriate volume of phosphate-buffered saline (PBS, pH 7.0) containing 8 μM ethidium bromide (EtBr) to get a final OD_{600nm} ~ 0.8. The kinetics of ethidium uptake into cells and subsequent increase in fluorescence was monitored at 530nm (excitation) and 600nm (emission) in 96-well microtiter plates.

(iv) **RNAseq assay to quantify efflux pump expression in response to SUL-DUR:** Overnight culture of *A. baumannii* ATCC 17978 was grown in 3-mL MHBII at 35°C with shaking at 240-rpm. Log-phase cultures were treated with SUL-DUR (2X MIC vs ATCC 17978) and incubated for 30 additional minutes at 35°C with shaking at 240-rpm. An additional flask with no drug added (untreated control) was also included. Based on the MIC, the concentration of the SUL-DUR added to the flask was 2 μg/mL, of each, SUL and DUR. After 30 min, cultures were treated with Qiagen RNAprotect reagent and incubated at room temperature for 5 min and cells pelleted at 3200xg for 10 minutes. The supernatant was discarded, and the cells pellets were frozen at -20°C. RNA purification was done using the Maxwell16 simplyRNA kit. Samples were treated with Dnase (Invitrogen Turbo DNA-free kit) and processed for RNAseq using the Epicentre ScriptSeq v2 RNA-seq Library Preparation kit. All samples were quantified by qPCR using the KAPA Library Quantification kit. Samples were diluted, pooled and processed for sequencing on an Illumina MiSeq using a 300-cycle v2 reagent kit. Data from the RNAseq run were processed in CLC Genomic Workbench version 20 using the RNA-seq module. Sequence reads were mapped to the ATCC 17978 reference genome.

A. baumannii Strains Used in this Study

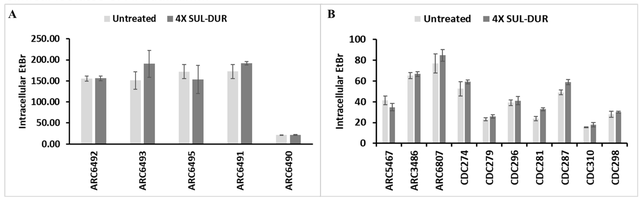
Strain ID	Description[a]	SUL-DUR[b] MIC (μg/ml)	IMI[c] MIC (μg/ml)
ARC6495	ADC-74-like; OXA-69; AdeI [D274E]; AdeR [V186I]; AdeS [Q163K]; **PBP3 [T526S]**	32	2
ARC6493	ADC-30-like; OXA-66; OXA-72; **PBP3 [A579T]**	16	>64
ARC6492	ADC-91-like; OXA-23; OXA-68; Partial AdeA; AdeRS not present; **PBP3 [T526S]**	32	64
ARC6491	ADC-91; OXA-23; OXA-68; Partial AdeA; AdeRS not present; **PBP3 [T526S]**	16	>64
ARC6490	ADC-30-like; OXA-66; OXA-72; AdeH [ΔE239-T245]; **PBP3 [H370Y T526S]**	32	64
CDC310	OXA-23; OXA-82	4	64
CDC279	ADC-30 [A245E]; TEM-1; OXA-23; OXA-66	4	64
CDC296	ADC-30; OXA-23; OXA-223	4	64
CDC298	ADC-30; TEM-1; OXA-66; OXA-237	4	16
CDC274	ADC-30; TEM-1; OXA-66; OXA-72; AdeS [A329S]	4	64
CDC287	ADC-30; OXA-66; OXA-72	2	>64
ARC3486	ADC-30; TEM-1; OXA-66; OXA-72	0.5	>32
ARC6807	ADC-74; OXA-23; OXA-69; OXA-72	2	>64
CDC281	ADC-30; TEM-1; OXA-82	1	8
ATCC 17978	ADC-26; OXA-259 (sequenced reference strain)	0.5	0.25
NCTC 13304	ADC-30; TEM-1: OXA-23; OXA-66 (SUL-DUR QC strain)	1	32

[a]Based on results from whole genome sequencing. [b]SUL-DUR; sulbactam-durlobactam. [c]IMI; imipenem.

The bacterial strains used in these experiments are part of the microbiological culture collection housed at Entasis Therapeutics, Waltham, MA, USA (labeled "ARC") or were acquired from the Centers for Disease Control and FDA Antibiotic Resistance Isolate Bank (labeled "CDC"). The *A. baumannii* clinical isolates used in this study were whole genome sequenced and their β-lactamase content was annotated. All strains used in this study are listed in the table above and only isolates that lacked genes encoding metallo-β-lactamases were chosen.

Efflux and SUL-DUR susceptibility

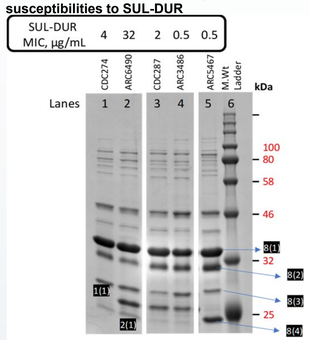
Effect of pre-exposure to SUL-DUR on the accumulation of EtBr in SUL-DUR sensitive or resistant clinical isolates of *A. baumannii*



The Y-axis represents final value of EtBr fluorescence at the end of 20 minutes for the SUL-DUR resistant (A) and sensitive (B) isolates as labeled (X-axis). Cell-associated EtBr fluorescence were determined for cells either incubated with a concentration of SUL-DUR (dark gray filled bars) corresponding to 4x the MIC for the strain or untreated (light gray bars). Error bars: s.d. around the mean (N=3).

Outer Membrane Composition: SUL-DUR sensitive & resistant clinical isolates

Outer membrane porin composition of *A. baumannii* clinical isolates with a range of susceptibilities to SUL-DUR



Tris-Glycine SDS PAGE analysis of purified outer membrane fractions from select *A. baumannii* clinical isolates.

Band Identities:
1(1) - CarO
2(1) - Putative porin
8(1) - OmpA family porin
8(2) - OmpA family porin
8(3) - CarO
8(4) - Putative porin

Evaluating major efflux pump RNA transcript levels in *A. baumannii* (ATCC 17978) in response to SUL-DUR treatment

Feature ID	RND-transporter Gene Names	Description & Localization		Experimental Fold Change	p-value (Wald test)
A1S_2735	*adeI*	MFP**	periplasm	-1.2	0.65
A1S_2736	*adeJ*	transmembrane pump	IM	-1.2	0.66
A1S_2737	*adeK*	outer membrane protein	OM	-1.1	0.84
A1S_2304	*adeF*	MFP	periplasm	-1.1	0.84
A1S_2305	*adeG*	transmembrane pump	IM	-1	0.92
A1S_2306	*adeH*	outer membrane protein	OM	1.2	0.69
A1S_1751	*adeA1* #	MFP	periplasm	1.3	0.42
A1S_1752	*adeA2* #	MFP	periplasm	1.3	0.36
A1S_1750	*adeB*	outer membrane protein	OM	1.1	0.53
N/A	*adeC* #	gene absent in ATCC 17978		-	-
A1S_1755	*adeT*	MFP	periplasm	1.1	0.88

**MFP – Membrane Fusion Protein
#Two adeA genes and the absence of adeC in ATCC 17978 has been described recently (Leus, 2018)

Conclusions

- Changes in permeability and induction of efflux mechanisms are major drivers of intrinsic antibiotic resistance in *A. baumannii*.
- Analysis of outer membrane composition did not reveal any correlation between specific proteins and relative SUL-DUR susceptibility, suggesting that outer membrane changes (i.e., permeation) is likely not a major contribution to SUL-DUR resistance.
- Efflux estimation using EtBr and global gene expression analysis suggest that the reduced susceptibility to SUL-DUR in *A. baumannii* (when observed) is primarily attributable to PBP3 mutations rather than increased efflux.
- We conclude that the two major non-β-lactamase mechanisms of resistance (efflux and outer membrane permeability) are unlikely to significantly contribute to reduced SUL-DUR susceptibility in clinical isolates of *A. baumannii*.

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