C1-1971

Single- and Multistep Resistance Selection with the Pleuromutilin Antibiotic BC-3781

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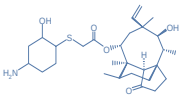
BC-3781



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ABSTRACT

Background: BC-3781, a novel pleuromutilin antibiotic in clinical development in patients, is characterized by excellent antibacterial activity against pathogens causing acute bacterial skin and skin structure infections and respiratory tract infections such as *Staphylococcus aureus*, group A and B *Streptococcus* spp. and *Streptococcus pneumoniae* among others. This study tested the ability to select for resistant mutants of BC-3781.

Methods: The *in vitro* emergence of resistance to BC-3781 was determined by measurement of the spontaneous mutation frequency of 2 *S. aureus* strains at 2-, 4- and 8-fold MIC and by serial passage of 5 *S. aureus* (1 MSSA, 1 CA-MRSA, 3 HA-MRSA including 1 VISA and 1 hVISA,), 2 *S. pyogenes* and 2 *S. pneumoniae* strains at sub-MIC levels for up to 50 passages by broth macrodilution. Daily passages were performed for at least 14 passages or until an 8-fold increase of the starting MIC was observed. Vancomycin, linezolid, azithromycin and moxifloxacin served as comparator antibiotics. Stable resistant mutants were characterized by sequencing of *rplC*, *rplD*, *rplV*, domains II and V of 23S rRNA. The identity of parent and mutant clones was confirmed by MLVF and PFGE.

Results: BC-3781 displayed very low spontaneous mutation frequencies ranging from <1.3x10^{-11} to <1.9x10^{-12} at all BC-3781 concentrations tested with no resistant clones selected. Resistance development to BC-3781 in *S. aureus* by multiple passages at sub-MIC appeared to be a slow process. Overall, the BC−3781 MIC increased 4- to 16-fold within 22-42 passages to 0.5−2 µg/ml (MICs after ten drug-free passages). Clones with elevated BC−3781 MIC values displayed mutations in the ribosomal protein L3 and L4 encoding genes *rplC* (D159G, deletion 153S, G152V, S158L) and *rplD* (G69R) and remained fully susceptible to linezolid. For *S. pneumoniae* and *S. pyogenes* no stable non-susceptible clones could be obtained. All resistant clones selected with the comparator antibiotics remained fully susceptible to BC−3781.

Conclusions: Data obtained in this study suggest a low potential of BC−3781 for the emergence of resistant staphylococcal and streptococcal isolates in the clinical setting.

INTRODUCTION

BC-3781 is a novel systemically available pleuromutilin antibiotic displaying potent antimicrobial properties against staphylococcal and streptococcal species and being suitable for treating many community-acquired infections, including those of the respiratory tract. Pleuromutilins inhibit protein biosynthesis by specific interaction with the peptidyl transferase center of the 50S ribosomal subunit.[1] The interaction is unique to this class of antibiotics and characterized by a specific interaction with the central part of domain V at 23S rRNA, subsequently preventing the correct positioning of the CCA-ends of tRNAs for peptide transfer.[2-4] Additionally, retapamulin, a topical pleuromutilin, was shown to inhibit the correct positioning of fmet-tRNA binding to the P-site *in vitro* suggesting that pleuromutilins also have an effect on translation initiation.[5]

Previous studies on the resistance development to pleuromutilins reported primarily mutations in 23S rRNA in *Mycoplasma gallisepticum* and *Mycbacterium smegmatis* whereas only mutations in *rplC* encoding the large ribosomal protein L3 have been reported in *S. aureus*, *S. pyogenes* and *E. coli*. For *Brachyspira hyodysenteriae* mutations in both, 23S rRNA and *rplC* have been described for pleuromutilin resistant isolates.[2,6-13]

The current study tested the ability to select for resistant mutants of BC-3781, in comparison with linezolid, vancomycin, azithromycin and moxifloxacin in *S. aureus*, *S. pneumoniae* and *S. pyogenes* using the multi-step resistance selection methodology. Additionally, spontaneous mutation frequencies are presented for *S. aureus*.

MATERIALS & METHODS

Bacterial strains used for the multi-passage resistance selection study included five *S. aureus* (1 MSSA, 1 CA-MRSA, 3 HA-MRSA including 1 VISA and 1 hVISA,) and 2 *S. pyogenes* (*erm*B and *mef* positive) and 2 *S. pneumoniae* (*erm*B and *erm*B+*mef* positive). The spontaneous mutation frequency was determined for 2 *S. aureus* (1 MSSA, 1 MRSA).

The MIC values of all strains were determined by broth microdilution according to CLSI (M7-A8 and M100-S20) using CAMHB for *S. aureus* and CAMHB supplemented with 5% lysed horse blood for *Streptococcus* spp.

The spontaneous mutation frequency was determined on Mueller-Hinton agar at 2-, 4- and 8-fold MIC for 10^{10} CFU. The *in vitro* emergence of resistance by serial passage was performed by broth macrodilution at 0.25- to 0.5-fold MIC.[5] Daily passages were performed for at least 14 passages or until an 8-fold increase of the starting MIC was observed. Passages were stopped at a maximum passage number of 50 or if an MIC of ≥32 µg/ml was reached. Selected clones were thereafter sub-cultured for 10 days on drug-free blood agar plates. The MIC values of clones and parents were determined by broth microdilution in order to test the stability of the acquired non-susceptibility phenotype. Vancomycin, linezolid, azithromycin and moxifloxacin served as comparator antibiotics.

Stable resistant mutants were characterized by sequencing of *rplC*, *rplD*, *rplV*, domains II and V of 23S rRNA.[10,11] The identity of parent and mutant clones was confirmed by MLVF and PFGE.[12,13]

RESULTS

- The spontaneous mutation frequency in *S. aureus* (MSSA and MRSA) at BC−3781 concentrations of 2-, 4- and 8-fold MIC was with < 1.3 x 10^{-11} to < 1.9 x 10^{-12} very low and no stable resistant clones were selected.
- Experimental induction of resistance to BC-3781, vancomycin, azithromycin, linezolid and moxifloxacin by multi-passages in five *S. aureus*, two *S. pyogenes* and two *S. pneumoniae* isolates is depicted in Figure 1.
- Overall, the *in vitro* resistance development for BC-3781 appeared to be a slow process. Multi-passage selection in the presence of sub-inhibitory concentrations yielded *S. aureus* clones with BC-3781 MIC values which rose from 0.06-0.25 µg/ml to 1-4 µg/ml (MIC values after ten drug-free passages 0.5−2 µg/ml) after 22-42 passages.
- The resistance development to BC-3781 in VISA and hVISA appeared to be as slow as for the other MSSA, CA-MRSA, and HA-MRSA strains tested.
- For *S. pneumoniae* and *S. pyogenes*, no stable non-susceptible clones could be generated within 20 and 50 passages, respectively.
- All selected clones were passaged 10 times in antibiotic-free medium and susceptibility tested against various antibacterial agents. The results for BC-3781 selected clones are shown in Table 1.

Figure 1. Resistance development in *S. aureus*

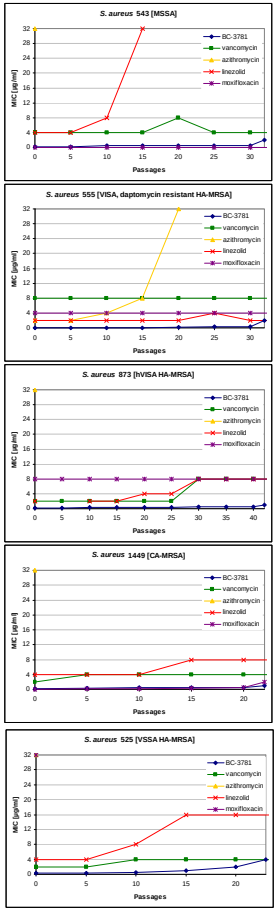

Table 1. MIC [µg/ml] of BC-3781 and comparators against *S. aureus* parent strains and clones selected in presence of BC-3781 (multi-passage) after 10 antibiotic-free subcultures

Strain	MIC [µg/ml] after 10 drug-free passages									
	MSSA SA543		VISA HA-MRSA SA555		hVISA HA-MRSA SA1449		VSSA HA-MRSA SA525		hVISA HA-MRSA SA873	
Antibiotic	Parent	Clone Pass. 32	Parent	Clone Pass. 32	Parent	Clone Pass. 22	Parent	Clone Pass. 23	Parent	Clone Pass. 42
BC-3781	0.12	**2**	0.06	**1**	0.12	**1**	0.25	**2**	0.12	**0.5**
Azithromycin	>32	>32	1	2	>32	>32	>32	>32	>32	>32
Chloramphenicol	8	8	8	4	8	4	8	8	8	8
Clindamycin	0.12	0.25	0.06	0.12	0.12	0.12	0.12	0.12	>8	>8
Linezolid	2	4	1	1	2	2	2	2	2	2
Moxifloxacin	0.03	0.06	4	4	0.06	0.12	>32	>32	8	8
Quinupristin/ dalfopristin	0.25	**1**	0.25	**1**	0.5	0.5	0.5	1	0.5	1
Retapamulin	0.12	**2**	0.06	**1**	0.06	**1**	0.12	**0.5**	0.06	0.12
Vancomycin	2	2	8	4	1	2	1	2	2	2

Significantly (≥ 4-fold) increased MIC values for clones compared to parent strains are marked bold and underlined.

Table 2. Potential resistance determinants in BC-3781 selected clones

S. aureus Strain	Parent/ clone	Initial MIC (µg/ml)	Selected BC−3781 Resistance		MICs [µg/ml] after 10 Antibiotic-free Subcultures of Mutants				Mutations associated with BC-3781 resistance		Mutations with other pleuromutilin derivatives reported for *S. aureus* in literature (Reference)
			MIC	Pass. #	BC-3781	LZD	RET	Q/D	L3	L4	
MSSA SA543	parent	0.12	-	-	0.12	2	0.12	0.25			
	clone	-	**2**	32	**2**	4	**2**	**1**	$D_{159}G$	-	L3 $D_{159}Y$ [3,5]
VISA HA-MRSA SA555	parent	0.12	-	-	0.06	1	0.06	0.25			
	clone	-	**2**	32	**1**	1	**1**	**1**	deletion $_{153}S$	-	L3 $S_{153}Y$ [5]
hVISA HA-MRSA SA1449	parent	0.12	-	-	0.12	2	0.06	0.5			
	clone	-	**1**	22	**1**	2	**1**	0.5	$G_{152}V$	$G_{69}R$	L3 $G_{152}D$ [3,5]
VSSA HA-MRSA SA525	parent	0.12	-	-	0.25	2	0.12	0.5			
	clone	-	**4**	23	**2**	2	**0.5**	1	$S_{158}L$	-	L3 $S_{158}L$ [3,5,9]
hVISA HA-MRSA SA873	parent	0.12	-	-	0.12	2	0.06	0.5			
	clone	-	**1**	42	**0.5**	2	0.12	1	-	-	-

Abbreviations: LZD, linezolid; RET, retapamulin; Q/D, quinupristin/dalfopristin;
Significantly (≥ 4-fold) increased MIC values for clones compared to parent strains are marked bold and underlined.

- Elevated MIC values were observed for retapamulin against four clones and for quinupristin/dalfopristin against two clones. However, BC-3781 showed no cross-resistance with linezolid, clindamycin, chloramphenicol, trimethoprim/sulfamethoxazole, antibiotics interacting with the peptidyl transferase center, nor with doxycycline, moxifloxacin and vancomycin (Table 1).
- Importantly, beside a very small and step-wise development of MIC increase, BC−3781 remained fully active against the linezolid-resistant clones selected using linezolid (data not shown in Tables).
- Genetical analysis of stable clones revealed changes in the deduced amino acid sequence of L3 protein in four of five *S. aureus* strains and in L4 protein in one clone. Substitutions in L3 at positions 152, 158, 159 and deletions at 153 have been reported earlier to be associated with pleuromutilin resistance albeit amino acid alterations were different in three out of four strains (Table 2).
- No changes were observed in domains II and V of 23S RNA or *rplV* (L22).

CONCLUSIONS

- BC-3781 displayed very low spontaneous mutation frequencies and the *in vitro* resistance development at sub-MIC levels appeared to be a slow process.
- Particularly for VISA and hVISA strains development of resistance to BC-3781 appeared to be as slow as for the other *S. aureus* (MSSA, CA-MRSA, HA-MRSA) tested with mutations in *rplC* and *rplD* being involved. Furthermore, no stable non-susceptible clones could be generated for *S. pyogenes* or *S. pneumoniae*.
- The low mutation frequency in combination with a very slow and step-wise resistance development of BC−3781 suggest the potential for a delayed emergence of resistance under selective pressure during clinical exposure. Further monitoring and characterization of non-susceptible isolates in surveillance studies and clinical trials are warranted.

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ACKNOWLEDGEMENTS

We thank Dianne Hoellman for her technical contributions to this study performed at Hershey Medical Center.