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Ability of CEM-102 (sodium fusidate), Linezolid, Daptomycin to Select Resistant *S. aureus* Mutants at Steady-state Plasma Levels

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Abstract (Revised)

Background: We tested the ability of CEM-102 (sodium fusidate), an established antistaphylococcal drug, to select for resistant clones of 8 methicillin-resistant and 1 methicillin-susceptible *S. aureus* strains of varying resistance phenotypes compared to linezolid and daptomycin using starting concentrations equivalent to each drug's steady-state plasma concentration and different conditions. **Methods:** The 8 MRSA were 3 CA-MRSA, 2 HA-MRSA, 1 hVISA, 1 VISA, 1 VRSA. In initial 12 h selections, each drug at steady-state concentration (CEM-102, 80 µg/mL; linezolid, 6.4 µg/mL; daptomycin, 6.7 µg/mL) was tested against 0.5 or 2.0 McFarland of inoculum in Mueller-Hinton broth (MHB) or MHB + 4 g/L human albumin and pH 6.5, with Ca^{2+} for daptomycin. Ten serial 12 h passages were done at MIC equal to steady-state concentration for each drug in MHB or MHB + 4 g/L human albumin, pH 6.5. Two-fold dilutions above and two below steady-state concentration were included. Combinations in all tests were in duplicate. **Results:** In the initial 12 h resistance selection in presence of steady-state concentration of each of the 3 agents, no mutants were selected by any drug on any medium. Serial passages at the steady-state plasma concentration on all *S. aureus* tested with CEM-102 did not yield mutants in MHB. Passage on CEM-102 in MHB with albumin (pH 6.5) yielded one, stable CEM-102 resistant mutant, originating from the VRSA strain in one of three independent experiments after six transfers. The resistance mechanism in this clone was a mutation (H457Y) in the EF-G protein encoded by *fusA*. Serial passages at steady-state concentrations of daptomycin and linezolid did not yield mutants in all medium/strains combinations tested. **Conclusions:** Under steady-state conditions, CEM-102, daptomycin, and linezolid did not (except for CEM-102 with a VRSA in 1 of three experiments) select for resistant mutants of *S. aureus* isolates. These data indicate that increased doses of CEM-102 prevent mutant selection by CEM-102.

Introduction

The problem of infections caused by MRSA has been exacerbated by emergence of strains which are non-susceptible to the glycopeptides. Skin and soft tissue infections, especially of the complicated variety, are most commonly caused by *Staphylococcus aureus* (often MRSA) and *Streptococcus pyogenes*.

Except for linezolid, there is currently no effective oral treatment for short or long term therapy of MRSA infections. Linezolid is effective oral therapy in principle, but is extremely expensive and toxic after long-term use (11).

CEM-102 (sodium fusidate) is an oral antibiotic with an established record of treating staphylococcal infections for many decades outside the United States, including those due to methicillin-resistant *S. aureus* (MRSA). It is effective against all staphylococci, including hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA).

We tested the ability of CEM-102 (sodium fusidate) to select for resistant clones of 8 methicillin-resistant and one methicillin-susceptible *S. aureus* strains of varying resistance phenotypes compared to linezolid and daptomycin using starting concentrations equivalent to each drug's steady-state plasma concentration and different media.

Materials and Methods

Bacteria and antimicrobials. Strains used in the study can be seen in Table 1. All strains were screened for the presence of *fusB* determinant (1, 12). CEM-102 was obtained from Cempra Pharmaceuticals, Inc. (Chapel Hill, NC) and daptomycin and linezolid were obtained from their respective manufacturers.

MIC determination. MICs of CEM-102, linezolid, and daptomycin were determined using a standard macrobroth dilution method according to the newest CLSI guidance (6). For daptomycin MIC determination, calcium was added and quality control strains were included in each run.

Initial 12 h resistance selection for *S. aureus* mutants in presence of antibiotic at steady-state plasma concentration. For resistance selective selection passage, 100 µl of bacterial suspension, with a density of 0.5 or 2.0 McFarland, was used as inoculum to 900 µl of Mueller-Hinton broth (BD Diagnostics, Sparks, MD).The Mueller-Hinton broth was supplemented with antimicrobials at their steady-state plasma concentration: CEM-102 at 80 µg/mL (14), linezolid at 6.4 µg/mL, or daptomycin at 6.7 µg/mL. In the second part of the study each antimicrobial at steady-state plasma concentration was tested against 0.5 or 2.0 McFarland of bacterial suspension in the presence of human albumin (concentration 4 g/L) at a pH of 6.5. Experiments were tested in duplicate. After 6 h incubation at 37°C, all mixtures were subcultured onto Muller-Hinton agar (MHA) plates supplemented with steady-state plasma concentrations of test antimicrobials and onto drug-free Blood Tryptcase Soy Agar (BTSA) plates (BD Diagnostics). A transfer of 100 µL of bacterial suspension after 6 h incubation was made to fresh selective medium and incubated for 6 hrs. After 6 h incubation (total incubation time of 12 hr), 100 µL of each suspension was subcultured onto MHA with steady-state plasma concentration of tested antimicrobial and onto drug-free BTSA plates. If visible growth was detected in broth, or on selection plates with steady-state plasma concentration of tested antimicrobial, bacteria were then subcultured onto drug-free BTSA plates (BD Diagnostics). Bacteria from pure cultures were subjected to macrobroth MIC determination by standard methodology (6) to confirm mutant presence and multiple-locus variable number tandem repeats typing (VNTR, formerly MLVA) (13) to confirm identity of resistant and parent clones.

Materials and Methods (cont.)

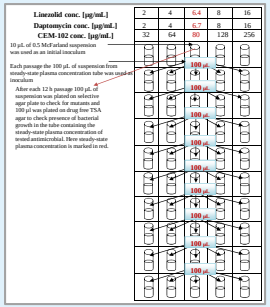
Table 1. Characterization of strains and MICs for all drugs used in the study.

STRAIN HMC#	PHENOTYPE	LOCATION	SOURCES	MIC (µg/mL)		
				CEM-102	DAPTOMYCIN	LINEZOLID
SA 510	MRSA, VRSA	Hershey, PA	Wound	0.125	1	1
SA 555	MRSA, VISA, DAPTO-NS	Hershey, PA	Blood (9)	0.25	4	4
SA 873	MRSA, hVISA	Hershey, PA	Respiratory specimen from cystic fibrosis-sputum (10)	0.25	2	4
SA 583	HA-MRSA, VSSA	Hershey, PA	Respiratory specimen from cystic fibrosis (10)	0.25	0.5	2
SA 703A	HA-MRSA, VSSA	Hershey, PA	Respiratory specimen (10)	0.5	0.5	1
SA 1475	CA-MRSA, VSSA	San Francisco, CA	Skin soft tissue specimen	1	1	2
SA 1506	CA-MRSA, VSSA	Philadelphia, PA	Blood	0.5	1	4
SA 1466	CA-MRSA, VSSA	Salt Lake City, UT	Wound (ulcer drainage)	0.25	2	2
ATCC 29213	MSSA, VSSA	www.atcc.org	Wound	0.5	1	2

Abbreviations: MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; VRSA, vancomycin-resistant *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; HA-MRSA, hospital-acquired MRSA, CA-MRSA, community-acquired MRSA; VSSA, vancomycin-susceptible *S. aureus*.

Serial passages at the steady-state plasma concentrations of tested antimicrobial agent. All MRSA and MSSA strains were tested by a multistep methodology (Fig. 1). Serial passages were performed every 12 h at MIC equal to the steady-state plasma concentration for each drug in Mueller-Hinton broth or Mueller-Hinton broth supplemented with 4 g/L human albumin and adjusted to a pH of 6.5, with calcium added for daptomycin selection. The two fold dilution above and two dilutions below steady-state plasma concentration were used with an initial inoculum of 10 µL of 0.5 McFarland. For each subsequent 12 h passage, 100 µL of inoculum was taken from the tube at the MIC equal to the steady-state plasma concentrations of each drug. Ten subsequent 12 h passages were performed. After each 12 h subsequent passage, 100 µL of each suspension was subcultured onto drug-free BTSA plates and incubated at 37°C for 20 hrs. If visible growth in broth was observed, bacteria from pure cultures were tested by MIC determination with standard methodology (6) and VNTR typing (13) to confirm identity of resistant and parent clones. Stability of the acquired resistance was determined by MIC determinations after 5 daily passages of the mutant in medium without antibiotics. A stable clone was defined as having the same MIC before and after 5 daily drug-free passages (± 1 doubling dilution). MICs of all finally passaged resistant clones were verified by CLSI methodology (6). Additionally, all selected resistant clones were tested for cross-resistance to other antimicrobials tested. All CEM-102 resistant and parental strains were tested for the presence of mutations in the *fusA* gene, which encodes the EF-G protein, as described previously (1, 12).

Figure 1. Diagram of the serial passages at the steady-state plasma concentrations



Results

Parental MICs (µg/mL) were : CEM-102, 0.125 to 1; daptomycin, 0.5 to 4; and linezolid, 1 to 4 (Table 1).

In the initial 12 h resistance selection in the presence of steady-state plasma concentrations of the tested antimicrobial agents, no mutants were selected on any antimicrobial in any type of medium. There was no growth present on selection Muller-Hinton agar plates supplemented with steady-state plasma concentrations of antibiotics. Bacterial growth on drug-free BTSA plates was present for CEM-102 and linezolid (thin biofilm) after 6 and 12 h of selection and for daptomycin >300 colonies were present after only 6 h incubation.

Serial passages at the steady-state plasma concentrations of CEM-102 in regular medium did not yield mutants. By contrast, passage with CEM-102 (sodium fusidate) in medium supplemented with albumin (pH 6.5) yielded a resistant clone originating from the one VRSA strain tested (Hershey VRSA [(4)]) in one of the three independent experiments. This resistant clone arose after the sixth passage in the second run in medium with albumin and low pH (pH 6.5). During the 7th passage, strain #510 (VRSA), developed resistance in cultures with 32, 64 and 80 µg/mL of CEM-102. The CEM-102 MIC increased up to 256 µg/mL [growth was observed in all five CEM-102 tested concentrations] and persisted throughout passages 7th-10th. Four organisms were stored and further analyzed: one from the 6th passage with a CEM-102 concentration of 80 µg/mL and three after the 10th passage with CEM-102 concentrations of 80, 128 and 256 µg/mL. Pure cultures of these isolates were tested by MIC determination (6) and VNTR typing (13) to confirm the identity of resistant and parent clones. All selected, resistant isolates had CEM-102 MICs of 256 µg/mL and VNTR profiles for all strains, including parental were identical. The CEM-102 MICs increased from 0.125 (parental MIC) to 256 µg/mL in selected clones.

Stability of the acquired resistance was determined by MIC determinations after 5 daily passages of the mutant in drug-free medium. The MICs of all finally passaged resistant clones were verified by CLSI methodology (6). Resistant clone was stable after 5 drug-free passages and CEM-102 MIC was > 256 µg/mL. Sequencing analysis of elongation factor G (EF-G), encoded by *fusA* gene in the resistant clones before and after drug-free passaging, showed a change in the deduced amino acid sequence of clones compared to the parent (Table 2). This change occurred in position 457 with Tyr substituted for His with MICs increasing from 0.125 to ≥ 256 µg/mL. The *fusB* determinant, also associated with fusidic acid resistance development, was not detected in any of *S. aureus* isolates used in our study.

Cross-resistance of CEM-102 to daptomycin was observed, with MICs increased 4 times, from 1 µg/mL to 4 µg/mL (Table 2). To investigate this phenomenon further, ten selected clones resistant to daptomycin and their parental strains, described in a previous study by Bogdanovich *et al*. (3), were tested for CEM-102 MICs by macrobroth dilutions (Table 3). We observed no cross-resistance in any of the isolates tested. The phenomenon of cross-resistance of CEM-102 with daptomycin in the single mutant that was isolated in this study requires confirmation; the mechanism is not known at the present time.

Table 2. Cross-reactivity of CEM-102 mutant after serial passage in steady-state plasma concentration of CEM-102 in medium supplemented with albumin, pH 6.5.

Strain	No. of passages	MICs (µg/mL)			*fusA* mutations
		CEM-102	Daptomycin	Linezolid	
SA 510	0	0.125	1	1	-
	6	256	Not tested	Not tested	Not tested
	10	256	4*	1	$H_{457}Y$
	15 including 5 drug-free	>256	4*	0.5	$H_{457}Y$

*cross-reactivity defined as ≥4 times increase in MICs

Table 3. CEM-102 MICs for ten selected clones resistant to daptomycin and their parental strains used in cross-resistant testing.

Strain No.	Phenotype	Daptomycin		CEM-102	
		Parental MICs*	Final MICs	Parental MICs	Final MICs
481	CA-MRSA	1	8	0.5	0.25
490	CA- MRSA	1	8	0.25	0.5
504	VISA	4	16	0.125	0.125
509	VRSA MI	1	8	0.125	0.125
510	VRSA PA	1	8	0.25	0.25
525	MRSA	1	8	0.25	0.5
547	MRSA	1	8	0.25	0.25
555	VISA, DAPTO R	16	32	0.06	0.06
540	MSSA	1	8	0.5	0.25
543	MSSA	1	8	0.25	0.25

*MICs in [µg/mL]

Results (cont.)

Bacterial growth on drug-free BTSA plates was absent after 3 subsequent passages on CEM-102 for all strains/medium combinations in all experiments performed. Serial passages at the steady-state plasma concentrations of daptomycin and linezolid did not yield mutants in any medium/strains combinations tested. Growth on drug-free BTSA plates was absent after 7 subsequent passages on daptomycin or linezolid.

Discussion

In our selection model, with constant active antibiotic pressure at their steady-state plasma concentration, we showed that if the levels of active CEM-102, daptomycin and linezolid are maintained the chance of developing nonsusceptible mutants is very low. Other multiselection studies were designed to have 18-24 h passages (see ref [3, 5] as an example), which lifted the antibiotic pressure during the selection process. In our study, no mutants were yielded for all strains/selection/medium combinations for daptomycin and linezolid. For 8 out of 9 strains tested, CEM-102 did not yield any mutants in all selection/medium combinations. In one strain, with a VRSA phenotype, in one out of three experiments repeated, a mutant emerged in multiselection study in the least favorable conditions (medium supplemented with albumin, pH 6.5) (2). Fusidic acid resistance in *S. aureus* results from point mutations within the chromosomal *fusA* gene encoding EF-G or by acquisition of a plasmid determinant (*fusB*) that encodes a poorly characterized resistance mechanism (1, 12). In our study we observed EF-G mutations at position $H_{457}Y$. Substitution $H_{457}Y$ has been associated with increased fusidic acid MICs (64- > 256 µg/mL) of selected mutants as described previously (1). None of the strains tested harbored *fusB* (1, 12).

The presence of albumin in the medium with an acidic pH of 6.5 may decrease activity of CEM-102, which is reflected in a 4-fold increase in MICs (2, 7). Such reduction in activity would result in decreasing the antibiotic pressure and increasing the chance of resistance selection. It is worth noting that the one selected CEM-102 mutant emerged from a very rare (only 9 known cases worldwide) VRSA isolate (8).

Bacterial growth present on drug-free agar medium reflects the lack of killing action of tested antibiotic. The presence of a thin film of bacteria was associated with CEM-102 and linezolid, which are bacteriostatic antimicrobials and growth of >300 colonies were characteristic for passaging on daptomycin, which is a known to be a bactericidal agent.

Conclusions

- Under steady-state plasma concentration conditions CEM-102 (sodium fusidate), daptomycin and linezolid did not (except for CEM-102 with one VRSA in one experiment on the 7th transfer) select for resistant mutants in *S. aureus* isolates tested.
- These data contradict previous assumptions on the selection by fusidic acid of resistant mutants.
- These results will require confirmation with additional *in vitro* and clinical studies.

Acknowledgments

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