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Antistaphylococcal Activity of Dihydrophthalazine Antifolates, a Family of Novel Antibacterial Drugs▽

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For a panel of 153 *Staphylococcus aureus* clinical isolates (including 13 vancomycin-intermediate or heterogeneous vancomycin-intermediate and 4 vancomycin-resistant strains), MIC_{50}s and MIC_{90}s of three novel dihydrophthalazine antifolates, BAL0030543, BAL0030544, and BAL0030545, were 0.03 and 0.25 μg/ml, respectively, for methicillin-susceptible strains and 0.03 and ≤0.25 μg/ml, respectively, for methicillin-resistant strains. For a panel of 160 coagulase-negative staphylococci (including 5 vancomycin-intermediate and heterogeneous vancomycin-intermediate strains and 7 linezolid-nonsusceptible strains), MIC_{50}s and MIC_{90}s were ≤0.03 and ≤0.06 μg/ml, respectively, for methicillin-susceptible strains and 0.06 and 0.5 μg/ml, respectively, for methicillin-resistant strains. Vancomycin was active against 93.0% of 313 staphylococci examined; linezolid was active against all *S. aureus* strains and 95.6% of coagulase-negative staphylococcus strains, whereas elevated MICs of clindamycin, minocycline, trimethoprim, and rifampin for some strains were observed. At 4× MIC, the dihydrophthalazines were bactericidal against 11 of 12 staphylococcal strains surveyed. The prolonged serial passage of some staphylococcal strains in the presence of subinhibitory concentrations of BAL0030543, BAL0030544, and BAL0030545 produced clones for which dihydrophthalazines showed high MICs (>128 μg/ml), although rates of endogenous resistance development were much lower for the dihydrophthalazines than for trimethoprim. Single-step platings of naïve staphylococci onto media containing dihydrophthalazine antifolates indicated considerable variability among strains with respect to preexistent subpopulations nonsusceptible to dihydrophthalazine antifolates.

The prevalence of multidrug-resistant staphylococci (2, 18) and the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) as a prominent cause of community-acquired skin and skin structure infections (28) are of concern to the medical community. Infections caused by coagulase-negative staphylococci (CoNS), once considered rare, are becoming increasingly frequent, particularly in patients with indwelling medical devices and those who are immunocompromised, and such infections are associated with significant morbidity and mortality (40). The adhesion of *Staphylococcus epidermidis* to endothelial cells is enhanced at temperatures encountered during a moderate fever (27), and the internalization of CoNS by host cells (1, 7, 27) probably contributes to antibiotic failure and infection persistence in some patients. Glycopeptides, especially vancomycin, are the current standard of care for the empirical treatment of suspected methicillin-resistant staphylococcal infections, but staphylococci with reduced susceptibility, tolerance, or resistance to vancomycin have appeared in clinical settings (2, 15, 19, 33), and their frequency is almost certainly underreported due to problems with identification and confusion over breakpoints (2, 39).

The spread of multidrug-resistant staphylococci mandates the development of novel therapeutic modalities. Of the antistaphylococcal drugs marketed during the past decade, only linezolid has therapeutically useful oral bioavailability; however, this oxazolidinone has been associated with serious toxicity (particularly during prolonged use), is not bactericidal toward staphylococci, and may support the emergence of endogenous resistance during long-term treatment (4, 16, 24, 26, 32, 34). Most staphylococci, particularly those isolated outside health care settings, remain susceptible to sulfamethoxazole-trimethoprim (38), though surely this will change as sulfamethoxazole-trimethoprim is used with increasing frequency to treat MRSA infections. Thus, an urgent need remains for new orally available compounds with activity against drug-resistant staphylococci and safety profiles compatible with long-term treatment.

Dihydrofolate reductase (DHFR) is an essential enzyme in most pathogenic bacteria, and the clinical success of trimethoprim has demonstrated that DHFR is an important chemotherapeutic target (41). BAL0030543, BAL0030544, and BAL0030545 (Fig. 1) are novel dihydrophthalazine derivatives of 2,4-diaminopyrimidine with potent activities against gram-positive pathogens, including staphylococci. The present study sought to determine (i) the MICs of BAL0030543, BAL0030544, BAL0030545, and comparators for 313 staphylococci, (ii) the bacteriostatic or bactericidal activities of the dihydrophthalazine antifolates and comparators against a dozen selected staphylococcal strains, (iii) the proclivity of the dihydrophthalazines and some comparators to select for endogenous resistance among 10 staphylococci with diverse resistotypes, and (iv) the frequency of preexistent resistance to the dihydrophthalazines in naïve populations of staphylococci.

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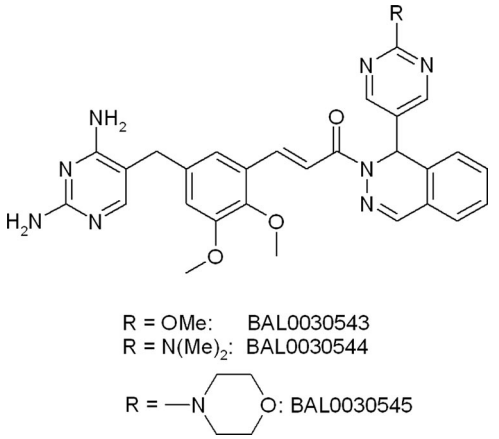

FIG. 1. Structures of dihydrophthalazine antifolates BAL0030543, BAL0030544, and BAL0030545. Me, methyl.

MATERIALS AND METHODS

Bacteria. The complete strain panel (313 strains) comprised 127 MRSA, 26 methicillin-susceptible *S. aureus* (MSSA), 135 methicillin-resistant CoNS (MRCoNS), and 25 methicillin-susceptible CoNS (MSCoNS) strains and included 2 Michigan vancomycin-resistant *S. aureus* (VRSA), 1 New York VRSA, 1 Pennsylvania (Hershey) VRSA, 10 vancomycin-intermediate *S. aureus* (VISA), 3 heterogeneous VISA (hVISA), 4 vancomycin-intermediate CoNS (VICoNS), 1 heterogeneous vancomycin-intermediate CoNS (hVICoNS), and 7 linezolid-nonsusceptible (Lzd^{NS}) CoNS strains. All VRSA strains and vancomycin-intermediate staphylococci not isolated at the Hershey Medical Center were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* through the agency of Eurofins Medinet, Inc. (Chantilly, VA). Most of the strains studied were isolated from patients during the past 8 years.

VISA and VICoNS strains were identified using Etest strips (AB Biodisk, Solna, Sweden), whereas hVISA and hVICoNS strains were identified by the macro Etest method (9), and the results were confirmed by population analyses. Where necessary, CoNS species were identified using API Staph galleries [bioMérieux (Suisse) SA, Geneva, Switzerland].

Antimicrobial agents and MIC testing. BAL0030543, BAL0030544, and BAL0030545, products of Basilea Pharmaceutica International AG (Basel, Switzerland), were prepared by the method of Guerry et al. (P. Guerry, S. Jolidon, R. Masciadri, H. Stalder, and R. Then, 30 May 1996, Switzerland international patent application WO 96/16046); other antimicrobial agents (clindamycin, linezolid, minocycline, rifampin, trimethoprim, and vancomycin) were obtained from commercial sources. With the exception of vancomycin, all of the comparators are used as oral monotherapies or as an oral component of combination therapy for the treatment of infections attributed to methicillin-resistant staphylococci (22, 30).

Agar dilution MICs were determined using Mueller-Hinton agar (MHA; Oxoid product no. CM0337) according to CLSI guidelines (12). *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were included with each set of MIC determinations; MICs for test strains were recorded only if MICs for quality control strains were within acceptable ranges (13). Vancomycin MICs were read after a full 24 h of incubation (13).

Time-kill studies. Time-kill profiles were obtained for six *S. aureus* strains (two MSSA and four MRSA strains) and six CoNS (two MSCoNS and four MRCoNS) (see Table 2) at drug concentrations of 1×, 2×, and 4× MIC. Glass tubes containing 5 ml of Mueller-Hinton broth (MHB; Oxoid product no. CM0405) with final inocula of 5×10^5 to 5×10^6 CFU/ml were incubated at 35°C in a water bath with shaking. Viable cells were quantified at 0, 3, 6, 12, and 24 h by spread plating 0.1-ml aliquots of 10-fold dilutions (in MHB) from each tube onto Trypticase soy agar–5% (vol/vol) defbrinated sheep blood (BBL) and incubating the plates for 24 to 48 h in ambient air at 35°C.

A given concentration of antimicrobial (expressed as a multiple of the MIC) was considered bactericidal if it reduced the inoculum size by ≥ 3 $\log_{10}$ CFU/ml within 24 h or bacteriostatic if the inoculum size was reduced by <3 $\log_{10}$ CFU/ml during the same period. With the sensitivity threshold and inocula employed, no problems in delineating 99.9% killing were encountered. Issues of antibiotic carryover were addressed by dilution, as described previously (31). Due to innate resistance by some strains to particular antibiotics, time-kill profiles were not obtained for seven strains with trimethoprim, five strains with clindamycin, and one strain each with linezolid, rifampin, and vancomycin.

Multipassage resistance selection studies. Five *S. aureus* (three MRSA, one VISA, one VRSA, and two Tmp^r) strains and five *S. epidermidis* (three methicillin-resistant, one vancomycin-intermediate, one Lzd^{NS}, and four Tmp^r) strains were subjected to serial passage in the presence of subinhibitory concentrations of antibiotics. Initial inocula ($\sim 1 \times 10^6$ CFU/ml) were prepared by suspending growth from an overnight MHA plate (BBL) in MHB (Oxoid). Glass tubes containing 1 ml of antibiotic-free or antibiotic-supplemented MHB were inoculated and incubated at 35°C for 24 h; antibiotic concentrations in the tubes ranged from 4 $\log_2$ steps above to 3 $\log_2$ steps below the MIC of each drug for each strain. Cultures were passaged daily for up to 50 days by using 10-μl inocula from the tubes with concentrations nearest the MIC (1 to 2 dilutions below the MIC) which had the same turbidity as antibiotic-free controls. Aliquots of inoculum were frozen at −70°C in double-strength skim milk. After 50 passages or when the MIC for a strain stabilized at >128 μg/ml during four successive passages, serial transfer in the presence of subinhibitory concentrations of antibiotic was discontinued and selected strains were subjected to 10 passages in antibiotic-free medium.

To confirm that resistant isolates obtained at the end of serial passaging derived from the parental strains, parental strains and clones obtained after the final passage were examined by pulsed-field gel electrophoresis using a CHEF-DR III apparatus (Bio-Rad, Hercules, CA) as described previously (25).

Chromosomal and plasmid DHFR genes from parental strains and from clones emerging during serial passage for which any of the dihydrophthalazine derivatives had elevated MICs were amplified and sequenced as described previously (10, 14). DHFR genes from clones for which the MICs of antifolates changed significantly during subsequent passage on antibiotic-free medium were also sequenced.

Single-step resistance selection studies. Bacterial cells were scraped from overnight plates, washed once with MHB (Oxoid), and resuspended in MHB at a final concentration of 10^{10} to 10^{11} CFU/ml. An aliquot (50 μl) of the bacterial suspension was spread onto MHA (Oxoid) containing the same antibiotics used for multipassage resistance selection at two, four, and eight times the agar dilution MIC. Plates were incubated aerobically at 35°C for 48 h. Randomly selected colonies growing on antibiotic-containing media were retested by agar dilution. Resistance in single-step studies was defined by an MIC ≥4 times higher than that for the parent strain. The resistance frequency at each MIC for each strain-antibiotic pair was calculated as the number of resistant colonies per inoculum (37).

RESULTS AND DISCUSSION

The dihydrophthalazine antifolates BAL0030543, BAL0030544, and BAL0030545 were identified at Basilea Pharmaceutica International AG as novel DHFR inhibitors with very encouraging in vitro antibacterial activities (36). The present study focused on the antistaphylococcal properties of the dihydrophthalazines toward a panel comprising predominantly recent clinical isolates, including vancomycin-intermediate and -resistant and Lzd^{NS} strains, as well as an abundance of methicillin-resistant strains.

MICs of BAL0030543, BAL0030544, BAL0030545, and comparators are summarized in Table 1. Among 153 *S. aureus* strains (including 10 VISA, 3 hVISA, and 4 VRSA strains), MIC_{90}s of the dihydrophthalazine derivatives were 0.25 and 0.125 to 0.25 μg/ml for MSSA and MRSA, respectively, and 0.03 to 0.06 and 0.5 μg/ml for Tmp^s and Tmp^r strains, respectively. Among 160 CoNS strains (including 4 VICoNS strains and 1 hVICoNS strain), MIC_{90}s were 0.03 to 0.06 and 0.5 μg/ml for MSCoNS and MRCoNS, respectively, and 0.03 to 0.06 μg/ml and 0.5 to 1 μg/ml, respectively, for Tmp^s and Tmp^r strains, respectively. MIC_{90}s of trimethoprim for the *S. aureus* and CoNS panels were >128 μg/ml. Vancomycin was active against all 313 staphylococci surveyed except the 22 strains (7.0%) for which MICs of this glycopeptide are known to be

TABLE 1. Summary of MICs for staphylococcal strains

Organism	Drug	MIC (μg/ml) for strains[a] with phenotype:							
		Methicillin susceptible		Methicillin resistant		Tmp^s		Tmp^r	
		Range	50%/90%	Range	50%/90%	Range	50%/90%	Range	50%/90%
S. aureus	BAL0030543	0.016–>32	0.03/0.25	≤0.008–>32	0.03/0.25	≤0.008–0.125	0.03/0.03	0.06–>32	0.25/0.5
	BAL0030544	≤0.008–>32	0.03/0.25	≤0.008–>32	0.03/0.125	≤0.008–0.06	0.03/0.03	0.03–>32	0.25/0.5
	BAL0030545	≤0.008–>32	0.03/0.25	≤0.008–>32	0.03/0.25	≤0.008–0.125	0.03/0.06	0.03–>32	0.25/0.5
	Trimethoprim	0.25–>128	0.5/>128	≤0.008–>128	0.5/>128	≤0.008–2	0.5/0.5	32–>128	>128/>128
	Vancomycin	0.5–8	1/2	0.5–>32	1/2	0.5–>32	1/2	0.5–>32	1/4
	Linezolid	1–2	2/2	0.25–2	2/2	0.25–2	2/2	1–2	1/2
	Clindamycin	≤0.06–>32	0.125/>32	≤0.06–>32	>32/>32	≤0.06–>32	>32/>32	≤0.06–>32	>32/>32
	Minocycline	0.016–2	0.125/0.25	0.03–16	0.125/2	0.03–16	0.125/0.25	0.016–8	0.25/8
	Rifampin	≤0.004–0.03	0.008/0.016	≤0.004–>16	0.016/0.016	≤0.004–>16	0.016/0.016	≤0.004–>16	0.008/>16
CoNS	BAL0030543	≤0.008–2	0.016/0.06	≤0.008–>32	0.06/0.5	≤0.008–0.125	0.016/0.03	0.016–>32	0.125/0.5
	BAL0030544	≤0.008–2	0.016/0.03	≤0.008–>32	0.06/0.5	≤0.008–0.125	0.016/0.03	0.016–>32	0.125/0.5
	BAL0030545	≤0.008–4	0.03/0.06	≤0.008–>32	0.06/0.5	≤0.008–0.125	0.03/0.06	0.03–>32	0.125/1
	Trimethoprim	0.06–128	0.25/64	0.03–>128	128/>128	0.03–4	0.25/2	16–>128	>128/>128
	Vancomycin	0.5–2	1/2	0.5–8	2/2	0.5–2	1/2	1–8	2/2
	Linezolid	1–2	1/2	1–>16	1/2	1–2	1/2	1–>16	1/2
	Clindamycin	0.125	0.125/0.125	≤0.06–>32	0.25/>32	≤0.06–>32	0.125/>32	0.125–>32	2/>32
	Minocycline	0.125–0.5	0.125/0.5	0.06–16	0.25/1	0.06–16	0.125/1	0.06–2	0.5/0.5
	Rifampin	≤0.004–0.016	≤0.004/0.016	≤0.004–>16	0.008/2	≤0.004–>16	0.008/0.016	≤0.004–>16	0.008/>16

[a] Numbers of strains were as follows: for *S. aureus*, 26 methicillin susceptible, 127 methicillin resistant, 125 Tmp^s, and 28 Tmp^r, and for CoNS, 25 methicillin susceptible, 135 methicillin resistant, 58 Tmp^s, and 102 Tmp^r.

compromised. Linezolid was active against all *S. aureus* strains and 153 (95.6%) of 160 CoNS strains examined. All MSCoNS strains were susceptible to clindamycin, and all methicillin-susceptible staphylococci were susceptible to minocycline and rifampin (Table 1).

Broth macrodilution MICs for the 12 staphylococcal strains examined by time-kill studies are presented in Table 2, and the time-kill profiles are summarized in Table 3. The bactericidal spectra of BAL0030543, BAL0030544, and BAL0030545 at 2× to 4× MIC encompassed 9 to 11 (75.0 to 91.7%) of the surveyed strains, whereas at 1× MIC the dihydrophthalazines were bactericidal toward only 3 to 6 (25.0 to 50.0%) of the strains. At 4× MIC, the dihydrophthalazine derivatives proved to be bactericidal against 11 of 12 staphylococcal strains; at this multiple of the MIC, BAL0030543 was bacteriostatic only toward strain CN197 [$\Delta(\log_{10}$ CFU/ml) = −2.7] and BAL0030544 and BAL0030545 were bacteriostatic only toward strain CN057 [$\Delta(\log_{10}$ CFU/ml) = −2.0 and −1.7, respectively]. Of the marketed antibiotics tested, trimethoprim and vancomycin had time-kill profiles resembling those of the dihydrophthalazine antifolates, though due to intrinsic resistance to trimethoprim (Table 1), a time-kill profile for this drug was obtained for only two CoNS strains and three *S. aureus* strains. At 4× MIC, kill rates for *S. aureus* during the first 6 h generally corresponded to the following order: BAL0030544, BAL0030545 > trimethoprim > BAL0030543 > vancomycin (where the comma indicates that the drugs have similar kill rates). At 4× MIC, kill rates for CoNS during the first 6 h generally corresponded to the following order: vancomycin >

TABLE 2. MICs for strains tested by time-kill studies

Strain[a]	MIC (μg/ml) of:								
	BAL0030543	BAL0030544	BAL0030545	Trimethoprim	Vancomycin	Linezolid	Clindamycin	Minocycline	Rifampin
S. aureus ATCC 29213*	0.03	0.125	0.06	1	1	4	0.125	0.125	0.016
SA505*	0.03	0.125	0.03	1	4	4	0.125	0.125	0.03
S. aureus ATCC 43300	0.03	0.06	0.03	1	1	2	>256	0.125	0.016
SA145	0.25	0.25	0.25	>128	1	4	>512	4	0.008
SA507	0.06	0.125	0.25	128	4	2	512	2	4
VRS1[b]	1	0.5	1	>128	>256	2	>512	1	>128
CN051*	0.125	0.125	0.25	128	2	2	0.125	0.125	0.016
CN057*	8	4	4	128	2	2	0.125	0.5	0.03
CN074	0.06	0.06	0.25	4	1	1	0.25	0.25	0.03
CN197	0.008	0.03	0.06	0.5	2	2	0.125	0.125	0.03
CN225	0.25	0.125	0.25	>128	8	2	>128	0.125	0.03
CN345	0.06	0.06	0.125	>128	2	64	1	0.25	0.016

[a] Designations beginning with SA indicate *S. aureus* strains, and designations beginning with CN indicate (coagulase-negative) *S. epidermidis* strains. Methicillin-susceptible strains are marked with an asterisk; all other strains are methicillin resistant.
[b] Michigan VRSA strain.

TABLE 3. Time-kill analyses for 12 staphylococci

Drug (no. of strains tested) and dose	No. of strains showing indicated % of killing at:											
	3 h			6 h			12 h			24 h		
	90	99	99.9	90	99	99.9	90	99	99.9	90	99	99.9
BAL0030543 (12 strains)												
4× MIC	4	0	0	12	6	0	12	12	5	12	12	11
2× MIC	4	0	0	12	6	0	12	12	4	12	12	11
1× MIC	3	0	0	12	4	0	12	8	3	8	6	6
BAL0030544 (12 strains)												
4× MIC	6	1	0	12	8	2	12	12	9	12	12	11
2× MIC	5	1	0	12	7	2	12	12	8	11	10	9
1× MIC	3	1	0	11	7	2	11	8	4	8	6	3
BAL0030545 (12 strains)												
4× MIC	5	0	0	12	7	1	12	12	7	12	11	11
2× MIC	3	0	0	12	8	2	12	12	6	11	11	10
1× MIC	3	0	0	10	4	1	10	8	4	9	6	5
Trimethoprim (5 strains)												
4× MIC	3	0	0	5	4	0	5	5	2	5	5	4
2× MIC	3	0	0	5	3	0	5	5	3	5	5	3
1× MIC	2	0	0	5	2	0	5	4	3	3	2	2
Vancomycin (11 strains)												
4× MIC	6	1	0	10	6	3	10	9	4	11	10	10
2× MIC	5	0	0	9	5	2	10	7	3	10	8	7
1× MIC	5	0	0	8	5	1	8	5	2	8	6	6
Linezolid (11 strains)												
4× MIC	1	0	0	1	0	0	5	0	0	8	2	0
2× MIC	0	0	0	1	0	0	4	0	0	8	1	0
1× MIC	0	0	0	0	0	0	2	0	0	2	0	0
Clindamycin (7 strains)												
4× MIC	1	0	0	0	0	0	3	0	0	7	1	0
2× MIC	0	0	0	0	0	0	3	0	0	7	3	0
1× MIC	0	0	0	0	0	0	2	0	0	5	0	0
Minocycline (12 strains)												
4× MIC	1	0	0	3	0	0	8	0	0	10	2	0
2× MIC	1	0	0	3	0	0	4	0	0	7	2	0
1× MIC	0	0	0	2	0	0	1	0	0	4	0	0
Rifampin (11 strains)												
4× MIC	7	3	1	9	3	2	11	6	1	11	9	5
2× MIC	5	3	1	9	3	1	9	5	1	10	8	2
1× MIC	4	0	0	8	1	0	9	2	0	5	3	1

BAL0030543, BAL030544, BAL0030545. At 4× MIC, rifampin was bactericidal toward 5 (SA145, CN051, CN057, CN074, and CN197) of 11 strains whereas clindamycin, linezolid, and minocycline were bacteriostatic toward all strains. While MIC and time-kill data for the comparators generally accord with published values (3, 8, 23), the 6-h kill rate for trimethoprim obtained in the present study was somewhat higher than that reported by Hackbarth et al. (21).

Representative resistance development profiles for dihydrophthalazine antifolates and comparators versus *S. aureus* ATCC 29213 are presented in Fig. 2, and the results of serial passages of selected *S. aureus* and *S. epidermidis* strains in the presence of subinhibitory antibiotic concentrations are shown in Table 4. Prolonged serial transfer in the presence of subinhibitory concentrations of BAL0030543, BAL0030544, or BAL0030545 revealed considerable strain variability in the development of endogenous resistance toward dihydrophthalazine antifolates and comparators. During ≤50 serial passages, two of five *S. aureus* and three of five *S. epidermidis* strains, including strains for which initial MICs of BAL0030543, BAL0030544, and BAL0030545 were ≤1 μg/ml, developed levels of resistance toward all three dihydrophthalazines equivalent to MICs of ≥64 μg/ml. Rates of development of endogenous resistance toward BAL0030543, BAL0030544, and BAL0030545 varied with the strain, being lowest for CN197

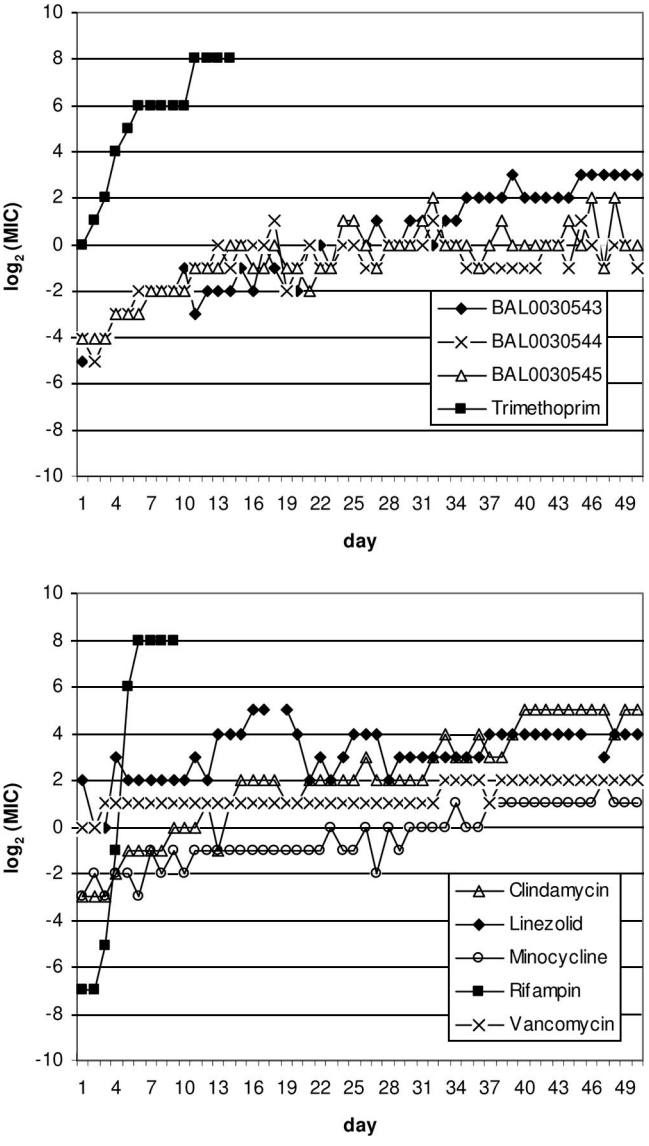

FIG. 2. Endogenous antibiotic resistance development during serial passage of *S. aureus* ATCC 29213.

TABLE 4. Results of multipassage resistance selection by the dihydrophthalazine antifolates and comparators

Strain (phenotype)[a]	Antibiotic	Initial MIC[b] (μg/ml)	No. of passages	Final MIC
S. aureus ATCC 29213 (MSSA)	BAL0030543	0.03	50	8
	BAL0030544	0.06	50	0.5
	BAL0030545	0.06	50	1
	Trimethoprim	1	14	>128
	Linezolid	4	50	16
	Minocycline	0.125	50	2
	Clindamycin	0.125	50	32
	Vancomycin	1	50	4
	Rifampin	0.008	9	>128
SA505 (MSSA, VISA)	BAL0030543	0.016	50	1
	BAL0030544	0.03	50	8
	BAL0030545	0.03	50	2
	Trimethoprim	1	14	>128
	Linezolid	4	50	16
	Minocycline	0.125	50	1
	Clindamycin	0.125	39	>128
	Vancomycin	4	50	8
	Rifampin	0.016	9	>128
S. aureus ATCC 43300 (MRSA)	BAL0030543	0.03	50	4
	BAL0030544	0.03	50	1
	BAL0030545	0.06	50	2
	Trimethoprim	1	20	>128
	Linezolid	2	50	64
	Minocycline	0.125	50	1
	Clindamycin	**>32**		
	Vancomycin	1	50	4
	Rifampin	0.004	9	>128
SA145 (MRSA)	BAL0030543	0.5	37	>128
	BAL0030544	0.25	47	>128
	BAL0030545	0.25	50	128
	Trimethoprim	**>128**		
	Linezolid	2	50	16
	Minocycline	4	50	8
	Clindamycin	**>32**		
	Vancomycin	1	50	2
	Rifampin	0.008	10	>128
VRS1 (MRSA, VRSA-MI)	BAL0030543	0.5	15	>128
	BAL0030544	0.25	20	>128
	BAL0030545	1	25	>128
	Trimethoprim	**>128**		
	Linezolid	2	50	32
	Minocycline	0.5	50	2
	Clindamycin	**>32**		
	Vancomycin	**>32**		
	Rifampin	**>32**		
CN051 (MSSE)	BAL0030543	0.25	50	64
	BAL0030544	0.125	38	>128
	BAL0030545	0.5	50	64
	Trimethoprim	**64**		
	Linezolid	2	50	128
	Minocycline	0.125	50	4
	Clindamycin	0.125	50	4
	Vancomycin	2	50	4
	Rifampin	0.008	9	>128
CN057 (MSSE)	BAL0030543	8	50	128
	BAL0030544	8	15	>128
	BAL0030545	8	23	>128
	Trimethoprim	**128**		
	Linezolid	2	50	64
	Minocycline	0.5	50	2
	Clindamycin	0.125	10	>128
	Vancomycin	2	50	4
	Rifampin	0.016	9	>128
CN197 (MRSE)	BAL0030543	0.03	50	1
	BAL0030544	0.06	50	0.125
	BAL0030545	0.06	50	1
	Trimethoprim	1	50	64
	Linezolid	2	50	32
	Minocycline	0.125	50	2
	Clindamycin	0.125	50	2
	Vancomycin	2	50	4
	Rifampin	0.008	12	>128
CN225 (MRSE, VISE)	BAL0030543	0.25	50	128
	BAL0030544	0.25	38	>128
	BAL0030545	0.25	36	>128
	Trimethoprim	**>128**		
	Linezolid	2	50	16
	Minocycline	0.125	50	1
	Clindamycin	**>32**		
	Vancomycin	8	50	16
	Rifampin	0.016	11	>128
CN345 (MRSE, Lzd^{NS})	BAL0030543	0.125	50	16
	BAL0030544	0.06	33	>128
	BAL0030545	0.06	50	4
	Trimethoprim	**>128**		
	Linezolid	32	50	128
	Minocycline	0.5	50	1
	Clindamycin	2	50	4
	Vancomycin	2	50	4
	Rifampin	0.008	9	>128

[a] Designations beginning with SA indicate *S. aureus* strains; VRS1 is a Michigan VRSA (VRSA-MI) strain. Designations beginning with CN indicate (coagulase-negative) *S. epidermidis* strains. MSSE, methicillin-susceptible *S. epidermidis*; MRSE, methicillin-resistant *S. epidermidis*; VISE, vancomycin-intermediate *S. epidermidis*.
[b] Values in bold correspond to antibiotics for which serial passages of the indicated strain were not performed.

and highest for SA145, VRS1, and CN225. MICs of the novel antifolates tended to increase smoothly, with the notable exception of those for CN345, for which a dramatic leap (8 to >128 μg/ml) in the MIC of BAL0030544 occurred between passages 29 and 30. The results suggest that, at least for a subset of staphylococci, BAL0030545 may have less proclivity to promote endogenous resistance development than either BAL0030543 or BAL0030544.

Three *S. aureus* strains passaged in the presence of trimethoprim gave rise to clones for which MICs of this drug were >128 μg/ml by ≤20 transfers, due to Phe98Tyr (ATCC 29213 and SA505) or Leu40Tyr (ATCC 43300) mutations in chromosomal DHFR genes; neither of these mutations had an impact on the strains' susceptibilities to the dihydrophthalazines. The trimethoprim MIC of 64 μg/ml achieved for a clone of *S. epidermidis* strain CN197 by 50 serial transfers may be related to a Gly43Arg mutation in the chromosomal DHFR gene (Basilea Pharmaceutica International AG, data on file).

For nine strains passaged in the presence of rifampin, MICs of this drug rose to >128 μg/ml by ≤12 transfers, consistent

TABLE 5. MIC changes as a consequence of serial passage

Strain	Antifolate	Change in MIC ($\Delta\log_2$)[a] resulting from passage:	
		With antibiotic	Without antibiotic
S. aureus ATCC 29213	BAL0030543	+8	ND
	BAL0030544	+3	ND
	BAL0030545	+4	ND
SA505	BAL0030543	+6	ND
	BAL0030544	+8	ND
	BAL0030545	+6	ND
S. aureus ATCC 43300	BAL0030543	+7	ND
	BAL0030544	+5	ND
	BAL0030545	+5	ND
SA145	BAL0030543	+9	−4
	BAL0030544	+10	0
	BAL0030545	+9	0
VRS1	BAL0030543	+9	−1
	BAL0030544	+10	0
	BAL0030545	+8	0
CN051	BAL0030543	+8	−2
	BAL0030544	+11	−6
	BAL0030545	+7	−6
CN057	BAL0030543	+4	0
	BAL0030544	+5	−1
	BAL0030545	+5	−1
CN197	BAL0030543	+5	ND
	BAL0030544	+1	ND
	BAL0030545	+4	ND
CN225	BAL0030543	+9	−4
	BAL0030544	+10	−7
	BAL0030545	+10	−7
CN345	BAL0030543	+7	−3
	BAL0030544	+12	−7
	BAL0030545	+6	−1

[a] MIC changes as a consequence of serial passage in the presence of subinhibitory concentrations of dihydrophthalazine antifolates and subsequent passage (10 times) in antibiotic-free medium are expressed as $\log_2$ dilution steps ($\Delta\log_2$). ND, not determined.

with reports that rifampin supports relatively rapid rates of endogenous resistance emergence (5). Mixed results were obtained for linezolid and clindamycin: after 50 passages in the presence of linezolid, MICs of the oxazolidinone ranged from 16 μg/ml (for 4 of 10 strains) to 128 μg/ml (for 2 of 10 strains), whereas after ≤50 passages in the presence of clindamycin, MICs of the lincosamide varied from 2 to 4 μg/ml (for 3 of 6 strains) to 32 to >128 μg/ml (for 3 of 6 strains). Following 50 serial passages in the presence of the respective antibiotic, none of the strains developed resistance toward minocycline or vancomycin according to CLSI breakpoints (13).

High MICs (≥128 μg/ml) of BAL0030543, BAL0030544, and BAL0030545 for clones of strains VRS1 and CN057 persisted following 10 passages in the absence of antibiotic, MIC drops of ≥2 $\log_2$ steps for clones of CN051 and CN225 in drug-free medium occurred, and mixed results were obtained for clones of SA145 and CN345 (Tables 5 and 6). A comparison of DHFR amino acid sequences corresponding to chromosomal and plasmid DHFR structural genes and the regions within 100 nucleotides upstream (encompassing the promoter region) and 100 nucleotides downstream of the DHFR structural genes of the six strains (SA145, VRS1, CN051, CN057, CN225, and CN345) for which one or more of the dihydrophthalazine antifolates displayed a conspicuous increase in MIC during serial passage in the presence of subinhibitory concentrations of these drugs revealed, in most cases, no sequence changes. Thus, the MIC variations may be attributable to regulatory elements affecting staphylococcal DHFR expression and/or the uptake or efflux of the drugs (6, 17).

After ≤50 serial passages in the presence of subinhibitory concentrations of BAL0030543, BAL0030544, or BAL0030545, amino acid substitutions Leu28Ser and/or Ile31Met in the plasmid-borne S1 isozyme [encoded by *dfr*(A)] were detected in clones of *S. aureus* strains SA145 and VRS1 for which dihydrophthalazines had elevated MICs. After ≤50 serial passages in the presence of dihydrophthalazines, amino acid substitutions Thr46Asn, Phe98Tyr, and Lys160Glu were observed consistently in the chromosomally encoded DHFRs of clones of *S. epidermidis* strain CN051, whereas amino acid substitutions Leu20Ile, Phe98Tyr, and His149Arg occurred consistently in dihydrophthalazine-nonsusceptible clones of *S. epidermidis* strain CN057. Additionally, an Leu24Ile mutation was detected in the chromosomal DHFR of a clone of *S. epidermidis* strain CN051 for which BAL0030543 had an MIC of 64 μg/ml (Table 6). Isoleucyl, isoleucyl, and asparaginyl residues occur at positions 20, 24, and 46, respectively, in the plasmid-encoded [*dfr*(G)] DHFR isozyme S3 (35), which is not inhibited by the dihydrophthalazines (11).

Single-step resistance frequencies for BAL0030543, BAL0030544, BAL0030545, and comparators were obtained for the 10 parental strains used in multipassage selection studies (Table 7). Frequency of resistance to the dihydrophthalazine antifolates, expressed as the number of resistant colonies per inoculum, ranged from 4.6×10^{-5} to 3.0×10^{-8} at 2× MIC to 3.2×10^{-6} to $<3.4 \times 10^{-11}$ at 8× MIC of BAL0030543, 8.0×10^{-5} to 4.5×10^{-9} at 2× MIC to 1.6×10^{-5} to $<4.0 \times 10^{-11}$ at 8× MIC of BAL0030544, and 1.7×10^{-4} to $<4.0 \times 10^{-11}$ at 2× MIC to 4.6×10^{-5} to $<3.3 \times 10^{-11}$ at 8× MIC of BAL0030545. These results point toward considerable variability among strains with respect to preexistent subpopulations nonsusceptible to the dihydrophthalazines. Single-step resistance frequencies of 10^{-10} to 10^{-11} for linezolid and vancomycin occurred across all strains surveyed at both 2× MIC and 8× MIC, indicating that the preexistent subpopulations nonsusceptible to these antibiotics were smaller than those nonsusceptible to the dihydrophthalazine antifolates.

At 2× MIC, the weakest selection pressure applied in the single-step analyses, the frequencies of resistance to clindamycin, linezolid, minocycline, and vancomycin tended to be lower than those to dihydrophthalazine antifolates. The highest resistance frequencies for BAL0030543, BAL0030544, and BAL0030545 were found for VRS1, CN051, and CN057, whereas the lowest resistance frequencies were found for SA505 and ATCC 43300. Similarly, by ≤50 serial passages, very high MICs of the dihydrophthalazines for VRS1, CN051, and CN057 emerged, whereas much lower MICs for SA505 and ATCC 43300 were obtained. At 8× MIC, the strongest selection pressure applied in the single-step analyses, dihy-

TABLE 6. MICs and DHFRs for selected strains

Strain[a]	Antifolate	Passage[b] (MIC [μg/ml])	Identity, phenotype, and/or mutation(s)[c] of:	
			Chromosomally encoded DHFR	Plasmid-encoded DHFR(s)
SA145	BAL0030543	0 (0.5)	wt	wt S1
		37 (>128)	wt	wt S1
		+10× −Abx (16)	wt	wt S1, I31M S1[d]
	BAL0030544	0 (0.25)	wt	wt S1
		47 (>128)	wt	L28S S1
		+10× −Abx (>128)	ND	ND
	BAL0030545	0 (0.25)	wt	wt S1
		50 (128)	wt	wt S1, I31M S1[d]
		+10× −Abx (128)	ND	ND
VRS1	BAL0030543	0 (0.5)	wt	wt S1
		15 (>128)	wt	L28V I31M S1
		+10× −Abx (128)	ND	ND
	BAL0030544	0 (0.25)	wt	wt S1
		20 (>128)	wt	L28V I31M S1
		+10× −Abx (>128)	ND	ND
	BAL0030545	0 (1)	wt	wt S1
		25 (>128)	wt	L28V I31M S1
		+10× −Abx (>128)	ND	ND
CN051	BAL0030543	0 (0.25)	T46N, F98Y, K160E	None
		50 (64)	L24I, T46N, F98Y, K160E	None
		+10× −Abx (16)	ND	ND
	BAL0030544	0 (0.125)	T46N, F98Y, K160E	None
		38 (>128)	T46N, F98Y, K160E	None
		+10× −Abx (4)	T46N, F98Y, K160E	None
	BAL0030545	0 (0.5)	T46N, F98Y, K160E	None
		50 (64)	T46N, F98Y, K160E	None
		+10× −Abx (1)	T46N, F98Y, K160E	None
CN057	BAL0030543	0 (8)	L20I, F98Y, H149R	None
		50 (128)	L20I, F98Y, H149R	None
		+10× −Abx (128)	ND	ND
	BAL0030544	0 (8)	L20I, F98Y, H149R	None
		15 (>128)	L20I, F98Y, H149R	None
		+10× −Abx (128)	ND	ND
	BAL0030545	0 (8)	L20I, F98Y, H149R	None
		23 (>128)	L20I, F98Y, H149R	None
		+10× −Abx (128)	ND	ND
CN225	BAL0030543	0 (0.25)	wt	wt S1
		50 (128)	wt	wt S1
		+10× −Abx (8)	wt	wt S1
	BAL0030544	0 (0.25)	wt	wt S1
		38 (>128)	wt	wt S1
		+10× −Abx (2)	wt	wt S1
	BAL0030545	0 (0.25)	wt	wt S1
		36 (>128)	wt	wt S1
		+10× −Abx (2)	wt	wt S1
CN345	BAL0030543	0 (0.125)	wt	None
		50 (16)	wt	None
		+10× −Abx (2)	wt	None
	BAL0030544	0 (0.06)	wt	None
		33 (>128)	wt	None
		+10× −Abx (2)	wt	None
	BAL0030545	0 (0.06)	wt	None
		50 (4)	wt	None
		+10× −Abx (2)	ND	ND

[a] Designations beginning with SA indicate *S. aureus* strains; designations beginning with CN indicate (coagulase-negative) *S. epidermidis* strains.
[b] +10× −Abx, subsequent passage of the strain from the preceding passage 10 times in MHB without antibiotic.
[c] wt, wild type; ND, not determined.
[d] Due possibly to the presence of a multicopy plasmid.

TABLE 7. Resistance frequencies of staphylococcal strains by single-step methodology

Strain[a]	Drug	Resistance frequency at:			Strain[a]	Drug	Resistance frequency at:		
		2× MIC	4× MIC	8× MIC			2× MIC	4× MIC	8× MIC
S. aureus ATCC 29213	BAL0030543	3.1×10^{-6}	4.6×10^{-8}	2.3×10^{-9}	CN051	BAL0030543	$>3.2 \times 10^{-5}$	3.2×10^{-5}	$<5.3 \times 10^{-8}$
	BAL0030544	4.5×10^{-9}	3.5×10^{-9}	$<5.0 \times 10^{-11}$		BAL0030544	7.1×10^{-5}	7.1×10^{-6}	2.4×10^{-7}
	BAL0030545	9.0×10^{-9}	$<3.3 \times 10^{-11}$	$<3.3 \times 10^{-11}$		BAL0030545	2.7×10^{-5}	1.8×10^{-7}	$<9.1 \times 10^{-8}$
	Trimethoprim	3.0×10^{-6}	1.5×10^{-7}	9.0×10^{-9}		Trimethoprim	Not tested	Not tested	Not tested
	Vancomycin	$<4.5 \times 10^{-11}$	$<4.5 \times 10^{-11}$	$<4.5 \times 10^{-11}$		Vancomycin	$<8.3 \times 10^{-11}$	$<8.3 \times 10^{-11}$	$<8.3 \times 10^{-11}$
	Linezolid	$<7.1 \times 10^{-11}$	$<7.1 \times 10^{-11}$	$<7.1 \times 10^{-11}$		Linezolid	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$
	Clindamycin	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$		Clindamycin	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Minocycline	$<4.5 \times 10^{-11}$	$<4.5 \times 10^{-11}$	$<4.5 \times 10^{-11}$		Minocycline	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$
	Rifampin	5.4×10^{-8}	4.6×10^{-8}	$<4.6 \times 10^{-8}$		Rifampin	1.0×10^{-7}	2.9×10^{-8}	2.1×10^{-8}
SA505	BAL0030543	3.0×10^{-8}	6.0×10^{-9}	$<5.0 \times 10^{-11}$	CN057	BAL0030543	4.0×10^{-5}	8.0×10^{-6}	3.2×10^{-6}
	BAL0030544	1.7×10^{-8}	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$		BAL0030544	8.0×10^{-5}	4.8×10^{-5}	1.6×10^{-5}
	BAL0030545	2.8×10^{-9}	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$		BAL0030545	1.7×10^{-4}	8.6×10^{-5}	4.6×10^{-5}
	Trimethoprim	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$		Trimethoprim	Not tested	Not tested	Not tested
	Vancomycin	$<4.5 \times 10^{-11}$	$<4.5 \times 10^{-11}$	$<4.5 \times 10^{-11}$		Vancomycin	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Linezolid	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$		Linezolid	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$
	Clindamycin	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$		Clindamycin	6.7×10^{-9}	1.7×10^{-10}	$<8.3 \times 10^{-11}$
	Minocycline	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$		Minocycline	$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$
	Rifampin	1.3×10^{-7}	9.3×10^{-8}	9.1×10^{-8}		Rifampin	$>4.0 \times 10^{-8}$	4.0×10^{-8}	2.7×10^{-8}
S. aureus ATCC 43300	BAL0030543	5.9×10^{-8}	1.2×10^{-8}	$<5.9 \times 10^{-11}$	CN197	BAL0030543	3.3×10^{-6}	3.3×10^{-7}	3.3×10^{-8}
	BAL0030544	2.7×10^{-8}	$<4.5 \times 10^{-11}$	$<4.5 \times 10^{-11}$		BAL0030544	3.3×10^{-6}	1.7×10^{-7}	8.3×10^{-8}
	BAL0030545	4.0×10^{-9}	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$		BAL0030545	3.7×10^{-7}	3.3×10^{-7}	2.2×10^{-9}
	Trimethoprim	3.0×10^{-7}	5.0×10^{-9}	3.0×10^{-9}		Trimethoprim	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$
	Vancomycin	$<5.3 \times 10^{-11}$	$<5.3 \times 10^{-11}$	$<5.3 \times 10^{-11}$		Vancomycin	$<6.3 \times 10^{-11}$	$<6.3 \times 10^{-11}$	$<6.3 \times 10^{-11}$
	Linezolid	$<1.4 \times 10^{-10}$	$<1.4 \times 10^{-10}$	$<1.4 \times 10^{-10}$		Linezolid	$<5.6 \times 10^{-11}$	$<5.6 \times 10^{-11}$	$<5.6 \times 10^{-11}$
	Clindamycin	Not tested	Not tested	Not tested		Clindamycin	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$
	Minocycline	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$	$<6.7 \times 10^{-11}$		Minocycline	1.7×10^{-9}	$<8.3 \times 10^{-11}$	$<8.3 \times 10^{-11}$
	Rifampin	1.7×10^{-7}	1.2×10^{-7}	$<1.2 \times 10^{-7}$		Rifampin	7.1×10^{-8}	1.4×10^{-8}	1.3×10^{-8}
SA145	BAL0030543	2.1×10^{-7}	6.9×10^{-8}	$<3.4 \times 10^{-11}$	CN225	BAL0030543	2.7×10^{-7}	7.8×10^{-9}	3.9×10^{-10}
	BAL0030544	2.2×10^{-6}	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$		BAL0030544	4.2×10^{-6}	3.7×10^{-8}	3.1×10^{-9}
	BAL0030545	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$	$<4.0 \times 10^{-11}$		BAL0030545	9.0×10^{-8}	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
	Trimethoprim	Not tested	Not tested	Not tested		Trimethoprim	Not tested	Not tested	Not tested
	Vancomycin	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$		Vancomycin	$<2.0 \times 10^{-10}$	$<2.0 \times 10^{-10}$	$<2.0 \times 10^{-10}$
	Linezolid	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$		Linezolid	$<1.8 \times 10^{-10}$	$<1.8 \times 10^{-10}$	$<1.8 \times 10^{-10}$
	Clindamycin	Not tested	Not tested	Not tested		Clindamycin	Not tested	Not tested	Not tested
	Minocycline	$<7.7 \times 10^{-11}$	$<7.7 \times 10^{-11}$	$<7.7 \times 10^{-11}$		Minocycline	4.0×10^{-9}	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$
	Rifampin	$>5.0 \times 10^{-8}$	5.0×10^{-8}	2.0×10^{-8}		Rifampin	$>2.7 \times 10^{-8}$	2.7×10^{-8}	2.0×10^{-8}
VRS1	BAL0030543	2.9×10^{-6}	4.3×10^{-9}	$<4.3 \times 10^{-9}$	CN345	BAL0030543	4.6×10^{-5}	$<7.7 \times 10^{-8}$	$<7.7 \times 10^{-8}$
	BAL0030544	6.8×10^{-9}	1.6×10^{-10}	$<5.3 \times 10^{-11}$		BAL0030544	3.3×10^{-7}	3.3×10^{-9}	1.7×10^{-9}
	BAL0030545	4.5×10^{-6}	9.1×10^{-10}	$<4.5 \times 10^{-11}$		BAL0030545	2.9×10^{-5}	5.9×10^{-7}	2.0×10^{-8}
	Trimethoprim	Not tested	Not tested	Not tested		Trimethoprim	Not tested	Not tested	Not tested
	Vancomycin	Not tested	Not tested	Not tested		Vancomycin	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Linezolid	$<1.4 \times 10^{-10}$	$<1.4 \times 10^{-10}$	$<1.4 \times 10^{-10}$		Linezolid	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$
	Clindamycin	Not tested	Not tested	Not tested		Clindamycin	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Minocycline	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$	$<5.0 \times 10^{-11}$		Minocycline	$<3.4 \times 10^{-10}$	$<3.4 \times 10^{-10}$	$<3.4 \times 10^{-10}$
	Rifampin	Not tested	Not tested	Not tested		Rifampin	4.6×10^{-8}	3.1×10^{-8}	2.3×10^{-8}

[a] For strain descriptions, see Table 4.

drophthalazine antifolate resistance frequencies clustered at lower values for *S. aureus* (10^{-9} to 10^{-11}) than for *S. epidermidis* (10^{-5} to 10^{-10}).

The results of this study suggest a possible role for dihydrophthalazine antifolates in the treatment of staphylococcal infections, including those caused by methicillin- and vancomycin-resistant strains and Lzd^{NS} strains. The dihydrophthalazines were bactericidal at low concentrations, and the application of good antibiotic stewardship (29) ought to preserve their antistaphylococcal activities and retard the emergence and dissemination of resistance toward these compounds. The MICs of BAL0030543, BAL0030544, and BAL0030545 were ≤0.5 μg/ml for 98.7% of the *S. aureus* strains surveyed, whereas the MICs of the three compounds were ≤1 μg/ml for 98.1% of the CoNS strains surveyed. These novel compounds inhibited not only wild-type and mutant chromosomally encoded DHFRs in *S. aureus* [*dfr*(B)] and *S. epidermidis* [*dfr*(C)] but also the plasmid-borne DHFR isozyme S1 encoded by *dfr*(A), which is highly resistant to trimethoprim. Unlike trimethoprim, the dihydrophthalazines were active against staphylococci harboring a chromosomal DHFR Phe98Tyr mutation (11). With their good oral bioavailabilities and favorable pharmacological/toxicological properties (20), dihydrophthalazine antifolates BAL0030543, BAL0030544, and BAL0030545 are potentially attractive therapies for multidrug-resistant staphylococoal infections, including those for which long-term (>2-week) therapy is warranted.

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