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In Vitro Activities of Three New Dihydrofolate Reductase Inhibitors against Clinical Isolates of Gram-Positive Bacteria▽

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BAL0030543, BAL0030544, and BAL0030545 are dihydrophthalazine inhibitors with in vitro potency against gram-positive pathogens. The MIC_{50}s for methicillin (meticillin)-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, hetero-vancomycin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Staphylococcus aureus* (VISA) range from 0.015 to 0.25 μg/ml (MIC_{90}s ≤ 0.5 μg/ml). MIC_{50}s for beta-hemolytic streptococci range from 0.03 to 0.06 μg/ml, MIC_{50}s for *Streptococcus pneumoniae* range from 0.06 to 0.12 μg/ml, MIC_{50}s for *Listeria monocytogenes* range from 0.015 to 0.06 μg/ml, and MIC_{50}s for *Streptococcus mitis* are ≤0.015 μg/ml. These three dihydrophthalazine antifolates have improved potency compared to that of trimethoprim and activity against gram-positive pathogens resistant to other drug classes.

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Dihydrofolate reductase (DHFR) is an enzyme with a pivotal role in the synthesis of intracellular tetrahydrofolic acid, which is essential in the synthesis of purines, some amino acids, and thymidine (6). DHFR is the sole source of tetrahydrofolic acid, and its inhibitors are employed in anti-infective and antitumor chemotherapy, most notably trimethoprim and methotrexate. Differences between mammalian and bacterial DHFR can be exploited in terms of the affinity of antibacterials for DHFR; for example, trimethoprim binds 5 $\log_{10}$ more tightly to bacterial DHFR than to vertebrate DHFR (7). Trimethoprim is the most widely used antimicrobial DHFR inhibitor in clinical practice and is employed as monotherapy to treat urinary tract infections, as therapy to treat tissue-based bacterial infections in the skin and chest in combination with sulfamethoxazole as co-trimoxazole, and as therapy to treat *Pneumocystis jirovecii* pneumonia.

In the last decade, new DHFR inhibitors have been developed and progressed to phase II and III clinical trials; most recently, iclaprim has completed phase III clinical studies in complicated skin and skin structure infections (9). A second class of DHFR inhibitors, the dihydrophthalazine antifolates, is currently in preclinical development. Three compounds, BAL0030543, BAL0030544, and BAL0030545, have demonstrated in vitro activity against multiresistant staphylococci and *Streptococcus pneumoniae* (2). The dihydrophthalazine substituent confers potent inhibitory activity against the more prevalent staphylococcal DHFR variants responsible for resistance to trimethoprim. Similar to other DHFR inhibitors, they can be administered both intravenously and orally (10).

In this study, we assessed the in vitro potency of three dihydrophthalazine antifolates, BAL0030543, BAL0030544, and BAL0030545, and a range of comparator agents against clinical isolates of gram-positive pathogens.

The antibacterial agents used in the study were obtained from the following sources: BAL0030543, BAL0030544, and BAL0030545, Basilea Pharmaceutica AG, Switzerland; daptomycin (lot no. 095703A), Cubist Pharmaceuticals, Inc., Lexington, MA; linezolid (lot no. 05003), Pfizer Ltd., Surrey, United Kingdom; moxifloxacin (lot no. BX01US1), Bayer plc, Berkshire, United Kingdom; vancomycin (lot no. 1671543B), Alpha Pharma, Devon, United Kingdom; minocycline (lot no. 014K1207) and trimethoprim (lot no. 68H140), Sigma Ltd., Dorset, United Kingdom. A total of 225 clinically significant gram-positive isolates from the collection held in the Department of Medical Microbiology, Southmead Hospital, Bristol, United Kingdom (1994 to 2008) were used (Table 1). Hetero-vancomycin-resistant *Staphylococcus aureus* (hVISA) and vancomycin-resistant *Staphylococcus aureus* (VISA) strains were identified by population analysis profiling (12). MICs were determined using the Clinical and Laboratory Standards Institute agar dilution method for staphylococcal and listerial strains (3), using Mueller-Hinton broth supplemented with 5% lysed horse blood for *S. pneumoniae*, *Streptococcus mitis*, and beta-hemolytic streptococci, and using *Corynebacteria* sp. Mueller-Hinton broth supplemented with 50 mg/liter calcium for daptomycin. *S. aureus* ATCC 29213 and *S. pneumoniae* ATCC 49619 were used as control strains. The percentage of susceptible strains using Clinical and Laboratory Standards Institute breakpoints was calculated (4).

The antibacterial activities of BAL0030543, BAL0030544, BAL0030545, and the comparator drugs are shown in Table 1. BAL0030543 and BAL0030544 had the lowest MIC_{50}s (0.03 μg/ml) of the four DHFR inhibitors tested against methicillin (meticillin)-sensitive *S. aureus* (MSSA) strains, and the maximum MIC for the BAL compounds was 0.25 μg/ml (BAL0030544 and BAL0030545). As expected, the MSSA strains were susceptible mainly to the comparator agents. BAL0030543 had the lowest MIC_{50} of the DHFR inhibitors

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TABLE 1. Antibacterial activities of BAL0030543, BAL0030544, and BAL0030545

Organism (no. of strains)	Compound	MIC (μg/ml)			% Susceptible[a]
		Range	50%	90%	
MSSA (25)	BAL0030543	0.015–0.06	0.03	0.03	100
	BAL0030544	0.015–0.25	0.03	0.03	100
	BAL0030545	0.015–0.25	0.12	0.25	100
	Trimethoprim	0.25–32	1	2	92
	Daptomycin	0.12–0.25	0.25	0.25	100
	Linezolid	2–2	2	2	100
	Minocycline	0.25–0.25	0.25	0.25	100
	Moxifloxacin	0.12–4	0.12	0.25	92
	Vancomycin	0.5–1	1	1	100
MRSA (25)	BAL0030543	0.015–0.06	0.015	0.06	100
	BAL0030544	0.03–0.25	0.06	0.25	100
	BAL0030545	0.06–0.06	0.06	0.06	100
	Trimethoprim	0.12–0.25	0.25	32	80
	Daptomycin	0.12–0.25	0.12	0.25	100
	Linezolid	2–2	2	2	100
	Minocycline	0.25–0.25	0.25	0.25	100
	Moxifloxacin	0.12–4	4	8	4
	Vancomycin	0.5–1	1	1	100
hVISA (25)	BAL0030543	0.015–0.06	0.03	0.06	100
	BAL0030544	0.03–0.25	0.25	0.25	100
	BAL0030545	0.03–0.06	0.06	0.06	100
	Trimethoprim	0.06–32	1	16	80
	Daptomycin	0.25–2	0.5	0.5	96
	Linezolid	1–1	1	2	100
	Minocycline	0.12–8	0.25	0.5	92
	Moxifloxacin	0.12–4	4	4	8
	Vancomycin	1–4	1	2	96
VISA (17)	BAL0030543	0.015–0.5	0.003	0.25	100
	BAL0030544	0.03–0.5	0.25	0.5	100
	BAL0030545	0.06–8	0.06	0.5	100
	Trimethoprim	0.12–32	1	32	71
	Daptomycin	0.25–2	1	2	71
	Linezolid	0.5–2	1	2	100
	Minocycline	0.12–8	1	4	96
	Moxifloxacin	8–8	8	8	0
	Vancomycin	1–4	2	4	52
Coagulase-negative staphylococci (29)	BAL0030543	0.015–16	0.06	8	86
	BAL0030544	0.03–32	0.06	8	86
	BAL0030545	0.03–16	0.06	4	86
	Trimethoprim	0.12–32	16	32	48
	Daptomycin	0.06–0.5	0.25	0.5	100
	Linezolid	0.25–2	1	2	100
	Minocycline	0.25–8	0.5	2	96
	Moxifloxacin	0.12–8	0.25	8	55
	Vancomycin	0.5–4	1	2	100
Group A streptococci (17)	BAL0030543	0.015–0.06	0.06	0.06	100
	BAL0030544	0.06–0.06	0.06	0.06	100
	BAL0030545	0.03–0.12	0.06	0.12	100
	Trimethoprim	0.12–0.5	0.5	1	N/A
	Daptomycin	0.015–0.03	0.015	0.03	100
	Linezolid	0.5–1	1	1	100
	Minocycline	0.12–0.25	0.12	0.25	100
	Moxifloxacin	0.25–0.5	0.25	0.5	N/A
	Vancomycin	0.12–0.25	0.25	0.25	100
Group B streptococci (17)	BAL0030543	0.015–0.06	0.03	0.06	100
	BAL0030544	0.06–0.12	0.06	0.12	100
	BAL0030545	0.06–0.12	0.06	0.12	100
	Trimethoprim	0.25–2	1	1	N/A
	Daptomycin	0.015–0.06	0.015	0.06	100

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TABLE 1—*Continued*

Organism (no. of strains)	Compound	MIC (μg/ml)			% Susceptible[a]
		Range	50%	90%	
	Linezolid	1–2	1	2	100
	Minocycline	0.12–32	16	32	29
	Moxifloxacin	0.25–0.5	0.25	0.5	N/A
	Vancomycin	0.12–0.25	0.25	0.25	100
Group C streptococci (13)	BAL0030543	<0.008–0.12	0.015	0.06	100
	BAL0030544	<0.008–0.25	0.06	0.06	100
	BAL0030545	0.03–0.25	0.06	0.12	100
	Trimethoprim	<0.008–8	0.5	1	N/A
	Daptomycin	0.015–0.12	0.03	0.12	100
	Linezolid	1–1	1	2	100
	Minocycline	0.12–16	0.25	4	84
	Moxifloxacin	0.25–0.5	0.25	0.5	N/A
	Vancomycin	0.12–0.5	0.12	0.5	100
Group G streptococci (12)	BAL0030543	0.015–0.06	0.03	0.06	100
	BAL0030544	0.06–0.25	0.06	0.06	100
	BAL0030545	0.03–0.12	0.03	0.06	100
	Trimethoprim	0.5–1	1	1	N/A
	Daptomycin	0.015–0.03	0.015	0.03	100
	Linezolid	1–2	2	2	100
	Minocycline	0.12–16	0.25	16	75
	Moxifloxacin	0.25–4	0.25	0.5	N/A
	Vancomycin	0.12–0.25	0.12	0.12	100
Streptococcus pneumoniae (15)	BAL0030543	0.015–0.12	0.06	0.12	100
	BAL0030544	0.03–0.25	0.12	0.25	100
	BAL0030545	0.03–0.25	0.12	0.12	100
	Trimethoprim	0.5–8	1	4	N/A
	Daptomycin	<0.008–0.5	0.06	0.12	N/A
	Linezolid	0.03–2	1	2	100
	Minocycline	0.12–8	0.25	0.25	93
	Moxifloxacin	0.12–0.5	0.25	0.25	100
	Vancomycin	0.06–0.5	0.12	0.25	100
Corynebacterium spp. (12)	BAL0030543	<0.008–8	0.06	4	83
	BAL0030544	0.015–16	0.12	4	83
	BAL0030545	<0.008–6	0.12	4	83
	Trimethoprim	0.12–16	16	16	N/A
	Daptomycin	0.03–0.12	0.06	0.12	N/A
	Linezolid	0.12–1	0.06	0.12	N/A
	Minocycline	0.06–32	2	16	N/A
	Moxifloxacin	0.25–16	1	8	N/A
	Vancomycin	0.12–0.5	0.25	0.5	N/A
Listeria monocytogenes (10)	BAL30543	0.015–0.015	0.015	0.015	100
	BAL30544	0.03–0.03	0.03	0.03	100
	BAL30545	0.06–0.06	0.06	0.06	100
	Trimethoprim	0.06–0.06	0.06	0.06	N/A
	Daptomycin	0.5–2	1	2	N/A
	Linezolid	2–2	2	2	N/A
	Minocycline	0.25–16	0.25	16	N/A
	Moxifloxacin	0.5–1	0.5	1	N/A
	Vancomycin	1–1	1	1	N/A
Streptococcus mitis (8)	BAL0030543	<0.008–0.06	<0.008		100
	BAL0030544	<0.008–0.06	0.03		100
	BAL0030545	<0.008–0.12	0.015		100
	Trimethoprim	<0.008–8	4		
	Daptomycin	0.006–0.5	0.25		
	Linezolid	<0.008–1	1		
	Minocycline	0.25–8	0.25		
	Moxifloxacin	0.12–1	0.25		
	Vancomycin	<0.008–1	1		

[a] N/A, breakpoint not available. A breakpoint of 0.5 mg/liter was used for BAL compounds for comparison.

against methicillin-resistant *S. aureus* (MRSA) strains, that is, 0.015 μg/ml, while BAL0030544 and BAL0030545 were equipotent (MIC_{50}, 0.06 mg/ml). Fluoroquinolone resistance was common among these MRSA isolates (moxifloxacin MIC_{50}, 4 μg/ml). BAL0030543, BAL0030544, and BAL0030545 had MIC_{50}s of 0.03, 0.25, and 0.06 μg/ml, respectively, against the hVISA and VISA strains, values similar to those of the MSSA and MRSA isolates. MIC_{90}s for the BAL compounds were 0.25 to 0.5 μg/ml against the VISA strains but lower against the hVISA strains, being in the range of 0.06 to 0.25 μg/ml. The MIC_{50} for all three BAL compounds was 0.06 μg/ml against coagulase-negative staphylococci; the MIC_{90}s ranged from 4 to 8 μg/ml. In contrast, the trimethoprim MIC_{90} was 32 μg/ml.

The BAL0030543, BAL0030544, and BAL0030545 MIC_{50} values for beta-hemolytic streptococci (Lancefield groups A, B, C, and G) ranged from 0.015 to 0.06 μg/ml, and no isolates had a MIC of >0.25 μg/ml for the BAL compounds. These strains were also susceptible to the comparator agents tested, with the exception of minocycline against group B streptococci. The BAL compounds were markedly more potent against *S. pneumoniae* strains than trimethoprim. BAL0030543, BAL0030544, and BAL0030545 MIC_{50}s for *Corynebacteria* spp. were 0.06, 0.12, and 0.12 μg/ml, respectively, with the MIC_{90} being 4 μg/ml for all three drugs. This is significantly more potent than the trimethoprim MIC_{50}/MIC_{90} at 16/16 μg/ml. All five DHFR inhibitors had excellent activity against *Listeria monocytogenes*, with MIC_{50}s of ≤0.06 μg/ml and MIC_{90}s of ≤0.06 μg/ml. BAL0030543, BAL0030544, and BAL0030545 had lower MIC_{50}s of ≤0.03 μg/ml against *S. mitis* than trimethoprim (MIC_{50}, 4 μg/ml).

Five MRSA and two MSSA strains were resistant to trimethoprim, but none had a MIC of >0.5 mg/liter to the BAL compounds. Six of the VISA strains were trimethoprim resistant, of which five strains had BAL MICs of <0.5 mg/liter. One VISA strain had a raised MIC to the BAL compounds (≥8 mg/liter). Similarly, of the four hVISA strains that were trimethoprim resistant, all had BAL MICs of <0.5 mg/liter. Of the 15 coagulase-negative staphylococcus strains that were trimethoprim resistant, four strains had BAL MICs of >0.5 mg/liter.

The present study confirms the in vitro potency of BAL0030543, BAL0030544, and BAL0030545 against *S. aureus* isolates. Previously, it has been shown that the MIC_{50}s of all three compounds were 0.03 μg/ml against MSSA strains, MRSA strains, and strains with reduced vancomycin susceptibility (5). Our data indicate that these compounds had MIC_{50}s in the range of 0.015 to 0.25 μg/ml, depending on the compound and the *S. aureus* resistance phenotype. All the MSSA, MRSA, and hVISA isolates had MICs of ≤0.25 μg/ml. VISA strains had higher MIC_{90}s than other *S. aureus* stains, which has been described before and is probably related to the higher trimethoprim MICs in this group in general. BAL0030543, BAL0030544, and BAL0030545 are about fourfold less active against trimethoprim-resistant *S. aureus* than susceptible strains (5). BAL0030543, BAL0030544, and BAL0030545 have been shown in time-kill experiments to produce a 3-log reduction in viable counts of most *S. aureus* isolates and are more bactericidal against *S. aureus* than minocycline, linezolid, or clindamycin (8).

Our data extends the data available on the BAL DHFR inhibitors against beta-hemolytic streptococci, indicating that all three compounds are highly active against Lancefield group A, B, C, and G streptococci. The previously reported geometric mean MIC for *Streptococcus pyogenes* was 0.25 μg/ml, fourfold higher than the MIC_{50}s obtained for our strains (11). Some *Corynebacteria* sp. isolates had MICs above 1 μg/ml, while all three compounds had MICs of ≤0.25 μg/ml against *S. pneumoniae*, ≤0.06 μg/ml against *L. monocytogenes*, and ≤0.12 μg/ml against *S. mitis*.

In conclusion, the improved in vitro potency of BAL0030543, BAL0030544, and BAL0030545 against gram-positive bacterial organisms compared to that of other DHFR inhibitors and their activity against isolates resistant to other drug classes justify further assessment of their utility in the therapy of gram-positive bacterial infection. The ability of dihydrophthalazine antifolates to be administered orally and parentally is an additional therapeutic benefit.

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