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SB 205952, a Novel Semisynthetic Monic Acid Analog with at Least Two Modes of Action

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The biological properties of SB 205952, a nitrofuryl oxazole derivative of monic acid, differ from those of the closely related antibacterial agent mupirocin. Compared with mupirocin, SB 205952 has increased antimicrobial potency, an extended spectrum including mupirocin-resistant staphylococci, and rapid bactericidal activity. SB 205952, like mupirocin, is a potent inhibitor of bacterial isoleucyl-tRNA synthetase (IRS) in mupirocin-susceptible organisms but does not inhibit IRS from mupirocin-resistant staphylococci, indicating that SB 205952 has more than one mechanism of action. SB 205952 rapidly inhibits protein, RNA, and DNA syntheses in mupirocin-susceptible and mupirocin-resistant staphylococci. In each case, the effect on RNA synthesis is relaxed by treatment with chloramphenicol, indicating that inhibition of RNA synthesis is probably a secondary consequence of stringent control. It is proposed that SB 205952 possesses one or more mechanisms of action in addition to IRS inhibition, probably mediated by its nitrofuryl component.

Mupirocin (pseudomonic acid A) is produced by *Pseudomonas fluorescens* NCIB 10586 (15) and inhibits bacterial isoleucyl-tRNA synthetase (IRS) (21), which charges its cognate tRNA species with isoleucine. Mupirocin inhibits the formation of isoleucyl adenylate, the first step of this aminoacylation reaction, leading to depletion of charged $tRNA^{Ile}$. The resulting amino acid starvation not only leads to bacteriostasis through inhibition of protein synthesis but also has widespread effects on cellular metabolism through induction of the stringent response (7, 22, 36). Mupirocin has potent antibacterial (39) and antimycoplasmal activities, with a predominant spectrum of activity against gram-positive organisms. The molecule contains an ester linkage which is vulnerable to hydrolysis in vivo, resulting in the formation of antimicrobially inactive monic acid (29). Mupirocin is therefore restricted to topical use, but it has been widely applied in the treatment of staphylococcal and streptococcal skin infections and eradication of staphylococci from skin and nasal sites (6).

Resistance to mupirocin is mediated predominantly by two mechanisms: failure to penetrate the cell envelope to reach its target site or possession of a resistant IRS enzyme. The first mechanism occurs in *Escherichia coli*, other members of the family *Enterobacteriaceae*, and *Pseudomonas aeruginosa* and is responsible for intrinsic resistance, in which the compound fails to penetrate the bacterial outer membrane to interact with IRS. This has been demonstrated by the increased susceptibilities of permeability mutants (1, 42) or the sensitivities of the IRS enzyme from organisms for which the MIC is high (22, 38a). The second mechanism occurs in the producing organism *P. fluorescens* NCIB 10586, in which IRS has a greatly reduced affinity for mupirocin (23), and in resistant variants of normally susceptible staphylococcal species. Staphylococcal resistance falls into two categories: high (MIC, >1,000 μg/ml) or moderate (MIC, 4 to 1,000 μg/ml). High-level resistance is associated with the possession of two IRS enzymes (17), the normal chromosomal enzyme and a second mupirocin-resistant IRS which is usually plasmid encoded (11, 25). The sequence of the gene encoding the resistant enzyme differs substantially from the native *ileS* gene, indicating acquisition from an external and unidentified source (19). Staphylococcal strains exhibiting moderate-level resistance do not hybridize with a probe to the gene encoding high-level resistance and possess only a single IRS with an altered affinity for mupirocin (14, 17).

Semisynthetic derivatives of monic acid have been prepared (5, 43) with the objective of improving their spectra of activity and potencies over those of mupirocin, particularly with respect to their activities against mupirocin-resistant staphylococci. SB 205952 was one of a series of C-1 oxazoles (5) which fulfilled these criteria. The properties of SB 205952 (5-[5-nitrofur-2-yl]-2-[1-normon-2-yl] oxazole A; Fig. 1) were noted to be exceptional in several respects. This compound not only exhibited an increased antibacterial potency and spectrum of activity but was also bactericidal against staphylococci. Because nitrofuran compounds represent another class of antimicrobial agents, some of which are used therapeutically (nitrofurantoin, nitrofurazone, furazolidone) (12), it was considered probable that the additional properties of SB 205952 could be related to the possession of the terminal nitrofuryl moiety.

The studies presented here describe the microbiological

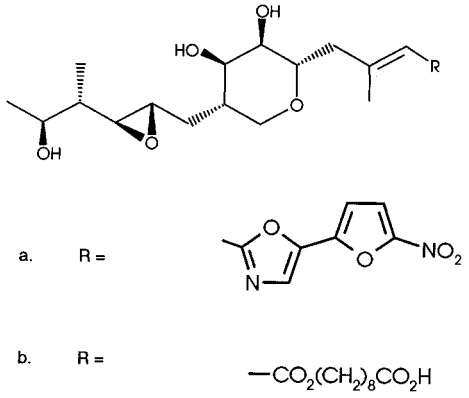

FIG. 1. Chemical structures of SB 205952 (5-[5-nitrofur-2-yl]-2-[1-normon-2-yl] oxazole A) (a) and mupirocin (b).

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TABLE 1. Descriptions and origins of bacterial strains

Organism	Strain	Description	Reference
S. aureus	NCTC 6571 (ATCC 9144)	Antibiotic assay strain	18
	8325-4	NCTC 8325 cured of phages 11, 12, and 13	30
	RN4220	Wild type, plasmid host	13
	RN4220(pMZ1)	RN4220 carrying high-level mupirocin resistance gene on shuttle vector pOX300	11
	C7	Highly mupirocin-resistant clinical isolate, New York, N.Y.	
E. coli	DC0 (UB1005)	F^- *nalA37 metB1*	35
	GC 4415	*sfiA*::*lacZ*	24
	WP2	Wild type	4
	WP100	*uvrA155 lexA102*	4
	CM611	*uvrA155 recA1*	4

properties of SB 205952 and investigations to characterize its mechanisms of action.

MATERIALS AND METHODS

Organisms. The bacterial strains used in the study are listed in Table 1. Additional organisms used for susceptibility testing and enzyme inhibition studies were clinical isolates maintained in the SmithKline Beecham culture collection. All organisms were maintained in liquid nitrogen storage. MIC data for the strains used in the viable count, SOS, and macromolecular synthesis studies are given in Table 2.

Antibiotics and chemicals. Mupirocin, lithium salt, was obtained as a laboratory standard from SmithKline Beecham, Worthing, United Kingdom. SB 205952 (31) and polymyxin B nonapeptide (PMBN) were prepared by the SmithKline Beecham Medicinal Chemistry Department. Cerulenin, chloramphenicol, ciprofloxacin, nitrofurantoin, rifampin, and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) were obtained from Sigma Chemical Co. (Poole, United Kingdom). The following radiolabelled chemicals were purchased from Amersham Life Science (Amersham, United Kingdom): [*methyl*-^{3}H]thymidine (70 to 85 Ci/mmol), [5-^{3}H]uridine (25 to 30 Ci/mmol), [U-^{14}C]glycine (>100 mCi/mmol), L-[3,4-^{3}H]glutamine (20 to 50 Ci/mmol), and [U-^{14}C]isoleucine (>300 mCi/mmol). All other chemicals were purchased from standard commercial sources.

In vitro susceptibility testing, viable counts, and lytic activity. The activities of the various antibiotics were determined by standard agar dilution procedures. Blood agar base (Oxoid, Basingstoke, United Kingdom) was used for nonfastidious organisms. This medium was supplemented with 5% chocolated defibrinated horse blood (TCS Microbiology Ltd., Buckingham, United Kingdom) for growth of fastidious organisms. Anaerobic bacteria were tested on Wilkins-Chalgren Agar (Oxoid) supplemented with 5% defibrinated horse blood lysed with 10% saponin. *Legionella* strains were tested on yeast extract agar (Difco Laboratories Ltd., West Molesey, United Kingdom) containing Difco Legionella supplement and 5% defibrinated horse blood (lysed by the addition of 30% distilled water). The inocula were 1-μl spots of an overnight broth culture containing 10^5 to 10^6 CFU. Anaerobes were incubated at 37°C in an anaerobic jar (BBL, Becton-Dickinson UK Ltd., Oxford, United Kingdom) in a CO_2-H_2 atmosphere (Oxoid Gas Generating Kit, Anaerobic System). The MIC was defined as the lowest concentration of compound completely inhibiting visible growth after 18 to 24 h of incubation. MIC determinations were also carried out in Tryptone Soy Broth (TSB; Oxoid) or brain heart infusion broth (BHI; Oxoid) by a standard microtiter procedure. Antichlamydia activity was determined as the lowest concentration preventing normal inclusion development, as determined by the method of Beale and Upshon (3).

Studies to determine bactericidal activity were carried out on exponential-phase cultures grown in TSB or BHI. Samples of 1 ml were serially diluted in phosphate-buffered saline (PBS) and were plated onto the corresponding agar. Colonies were counted after incubation at 37°C for 18 to 24 h. The detection limit was 10^2 CFU/ml.

Lytic activity was determined by measuring the A_{675} in a Philips PU8620 spectrophotometer.

Isoleucine antagonism studies were carried out in microtiter plates with M9 minimal medium (2) supplemented with thiamine (0.03 mM), nicotinic acid (0.03 mM), biotin (0.01 mM), and 19 amino acids (0.1 mM) excluding isoleucine. The MICs determined in this medium and in the same medium to which 1 mM isoleucine was added were compared. The final inoculum was 1/20 of an overnight broth culture grown in the isoleucine-free medium.

IRS inhibition studies. *Staphylococcus aureus* strains were grown to the late stationary phase in shake flasks (240 rpm) containing Nutrient Broth No. 2. For extraction of IRS, cells were harvested by centrifugation at 5,000 × *g* and were washed several times in cold PBS. Bacterial synthetases were extracted by sonication (six 15-s bursts at 15 μm on ice; MSE Soniprep; MSE Scientific Instruments, Crawley, United Kingdom) in the presence of lysostaphin (150 μg/ml) in the same buffer; this was followed by treatment with DNase (10 μg/ml), overnight dialysis, and ultracentrifugation at 200,000 × *g* for 1 h. All enzymes were stored at −20°C in the presence of 30% glycerol.

IRS activity was assayed as the charging of $tRNA^{Ile}$ with [U-^{14}C]isoleucine (10) under conditions in which counts were approximately proportional to time and enzyme concentration. Assay mixtures contained 30 mM Tris, 2 mM dithiothreitol, 10 mM $MgCl_2$, 70 mM KCl, 1.56 mg of *E. coli* tRNA (Boehringer Mannheim, Lewes, United Kingdom) per ml, 5 mg of equine ATP per ml, and [U-^{14}C]isoleucine at 4.8 μM. The concentrations of inhibitor resulting in 50% inhibition of [U-^{14}C]isoleucine charging (IC_{50}s) were determined after preincubation of increasing concentrations of compound with IRS for 5 min; this was followed by the addition of substrates and cofactors and reaction for 10 min at 37°C.

Induction of the SOS response. The method used to induce the SOS response was a modification of the α-complementation assay described by Phillips et al. (33) with the *E. coli sfiA*::*lacZ* fusion strain (GC 4415) of Huisman and D'Ari (24). The organism was incorporated into Difco LB agar (250 μl of overnight broth culture in 20 ml) to which 1 mg of PMBN and 10 mg of X-gal had been added. Test solutions (50 μl) at 100× the MIC were placed in wells cut in the

TABLE 2. Susceptibilities of the bacterial strains used in the study to antimicrobial agents

Organism and strain	MIC (μg/ml)							
	SB 205952	Mupirocin	Nitrofurantoin	Chloramphenicol	Ciprofloxacin	Vancomycin	Cerulenin	Rifampin
S. aureus								
NCTC 6571	0.13	0.13	8	2	0.19	2	32	0.06
8325-4	0.19	0.6	16	8	2	16	100	0.2
C7	16	>2,048	32	8.8	1.1	2.2	128	0.13
RN4220	0.06	0.16	ND[a]	ND	ND	ND	ND	ND
RN4220(pMZ1)	8	>2,048	ND	ND	ND	ND	ND	ND
E. coli								
DC0	16	64	16	4	0.03	ND	ND	8
GC4415	4	128	8	4	0.1	ND	ND	ND

[a] ND, not determined.

TABLE 3. Antimicrobial spectrum and potency of SB 205952 in comparison with those of mupirocin

Organism (no. of strains)	Antibiotic	MIC (μg/ml)	
		Range	90%[a]
Staphylococcus aureus[b]			
Mup^s (63)	SB 205952	0.03–0.5	0.13
	Mupirocin	0.13–1	0.5
Mup^{mr} (21)	SB 205952	1–4	4
	Mupirocin	2–64	64
Mup^{hr} (12)	SB 205952	2–32	32
	Mupirocin	>512	>512
Coagulase-negative staphylococci (23)	SB 205952	0.015–0.25	0.13
	Mupirocin	0.06–1	0.25
Streptococcus pyogenes (16)	SB 205952	<0.015–0.13	0.13
	Mupirocin	0.06–0.25	0.25
Streptococcus pneumoniae (50)	SB 205952	<0.015–1	1
	Mupirocin	0.06–4	1
Enterococci (10)	SB 205952	4–64	64
	Mupirocin	4–128	128
Enterobacteriaceae (12)[c]	SB 205952	4–128	128
	Mupirocin	64–>128	>128
Pseudomonas aeruginosa (4)	SB 205952	>128	NA[d]
	Mupirocin	>128	NA
Legionella pneumophila (10)	SB 205952	0.013–0.5	0.5
	Mupirocin	≤0.015–0.06	0.06
Chlamydia trachomatis (2)	SB 205952	8–16	NA
	Mupirocin	>128	NA
Bacteroides spp. (13)	SB 205952	4–8	8
	Mupirocin	>128	>128
Mycoplasma pneumoniae (5)	SB 205952	0.1–0.25	NA
	Mupirocin	1–5	NA
Clostridium spp. (7)	SB 205952	2–4	NA
	Mupirocin	>128	>128

[a] 90%, MICs inhibiting 90% of strains tested.
[b] *S. aureus* phenotypes: Mup^s, mupirocin susceptible; Mup^{mr}, moderately mupirocin resistant; Mup^{hr}, highly mupirocin resistant.
[c] *E. coli*, and *Klebsiella*, *Proteus*, *Morganella*, *Enterobacter*, and *Serratia* spp.
[d] NA, not applicable.

agar. Plates were observed for induction of β-galactosidase (as indicated by a blue coloration in a halo around the well) after 18 h of incubation at 37°C. Ciprofloxacin was included as a positive control, and PBS was included as a negative control.

Measurement of macromolecular synthesis in staphylococci. DNA, RNA, and protein syntheses were monitored in mid-exponential-phase staphylococcal cultures (10^7 to 10^8 CFU/ml in BHI) by the incorporation of the radiolabelled precursors [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, and L-[3,4-^{3}H]-glutamine or L-[U-^{14}C]isoleucine, respectively, into trichloracetic acid (TCA)-precipitable material. Precursors were added to the cell suspension 3 min before the addition of antibiotics, the linearity of incorporation having been previously established. Final concentrations were 1 μCi/ml for the ^{3}H-labelled compounds and 0.1 μCi/ml for the ^{14}C-labelled compound. Final antibiotic concentrations were 4× the MICs. Samples were removed at intervals, and incorporation was terminated by the addition of 1 ml of 10% (wt/vol) TCA and cooling on ice (8). The precipitated material was collected on Whatman GF/C glass fiber filters, washed with TCA and ethanol, dried, and counted in a Beckmann LS 7800 scintillation counter. Lipid synthesis was determined by the addition of [^{3}H]acetic acid and then the extraction of the cells with chloroform and methanol (9). Peptidoglycan synthesis was determined by the addition of [U-^{14}C]glycine and then by fraction-

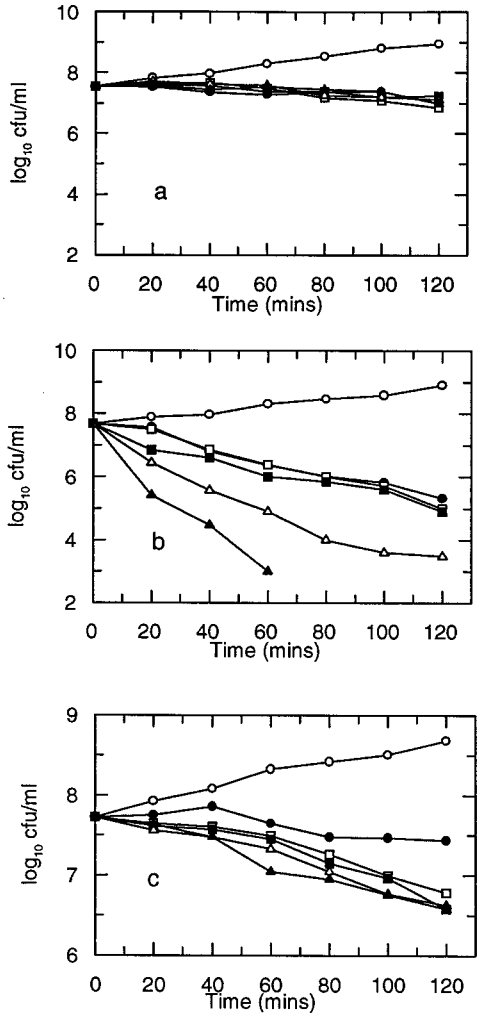

FIG. 2. Effects of SB 205952 and mupirocin on survival of staphylococcal strains during exposure to antibiotic for 2 h. (a) Mupirocin versus *S. aureus* 8354-4; (b) SB 205952 versus *S. aureus* 8325-4; (c) SB 205952 versus *S. aureus* C7. ○, control; ●, 1× the MIC; □, 2× the MIC; ■, 4× the MIC; △, 8× the MIC; ▲, 16× the MIC.

ation by TCA precipitation and protease digestion (32). When chloramphenicol (500 μg/ml) was included to relax the stringent response, chloramphenicol was added to one set of cultures simultaneously with the test compounds. Samples were removed after 40 min. The percent inhibition was calculated relative to inhibition of a control containing chloramphenicol (500 μg/ml) alone or inhibition of an antibiotic-free control. In all experiments, known inhibitors were included as positive controls.

RESULTS

In vitro antimicrobial activity. The activities of SB 205952 and mupirocin against a range of human pathogens are given in Table 3. The overall level of potency of the nitrofuryl oxazole against mupirocin-susceptible species was approximately twofold greater than that of mupirocin, making it a highly potent agent against staphylococci, streptococci, and other community-acquired respiratory pathogens including *Legionella pneumophila* and *Mycoplasma pneumoniae*. The activity of SB 205952 against mupirocin-resistant organisms is a notable feature of the compound (Table 3). SB 205952 displayed an increased breadth of spectrum against intrinsically mupirocin-resistant species (*Bacteroides*, *Clostridium*, and *Chlamydia* species, *E. coli*, *Enterobacter* species) and resistant variants of normally susceptible species, i.e., staphylococci expressing moderate-level and high-level resistance to mupirocin. However, these mupirocin-resistant staphylococcal strains did show some level of cross-resistance to mupirocin (Table 3), and some mupirocin-resistant species showed little or no increase in susceptibility to SB 205952 compared with that to mupirocin. These included enterococci; *Klebsiella*, *Proteus*, *Morganella*, and *Serratia* species; and *P. aeruginosa* (Table 3).

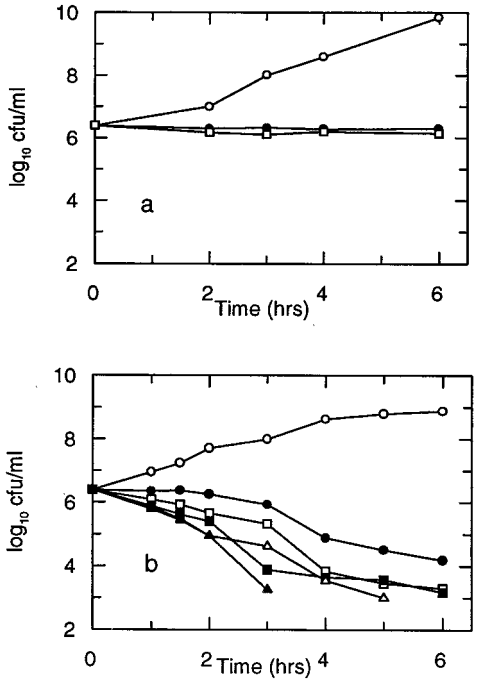

FIG. 3. Effects of SB 205952 and mupirocin on survival of staphylococcal strains during exposure to antibiotic for 6 h. (a) Mupirocin versus *S. aureus* 8325-4; (b) SB 205952 versus *S. aureus* C7. ○, control; ●, 1× the MIC; □, 2× the MIC; ■, 4× the MIC; △, 8× the MIC; ▲, 16× the MIC.

Bactericidal and lytic activities. Mupirocin was slowly bactericidal (0.5 log killing in 2 h) against *S. aureus* 8325-4 (Fig. 2) at 1 to 16× the MIC, whereas SB 205952 reduced counts by 1.5 logs in 2 h at 2× MIC and >4 logs in 1 h at 16× the MIC. The killing action of SB 205952 was notably slower against the highly mupirocin-resistant staphylococcal clinical isolate C7 (Fig. 2), although prolonged exposure to this antibiotic indi-

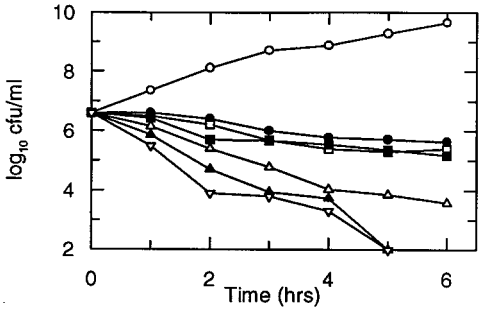

FIG. 4. Effects of SB 205952 and mupirocin on survival of *E. coli* DC0 during exposure to antibiotic for 6 h. ○, control; ●, mupirocin at 2× the MIC; □, mupirocin at 16× the MIC; ■, SB 205952 at 2× the MIC; △, SB 205952 at 4× the MIC; ▲, SB 205952 at 8× the MIC; ▽, SB 205952 at 16× the MIC.

TABLE 4. Inhibition of IRS by SB 205952 and mupirocin

Source of enzyme	SB 205952		Mupirocin	
	IC50 (ng/ml)	MIC (μg/ml)	IC50 (ng/ml)	MIC (μg/ml)
S. aureus NCTC 6571	2.4	0.13	0.8	0.13
S. aureus 8325-4	2.4	0.06	1.5	0.06
S. aureus 1083	19	4	12	4
S. aureus C35	35	4	8	32
S. aureus C7	400,000	16	20,000	>2,048
S. aureus RN4220(pMZ1)	1,000,000	4	9,600	>2,048

cated that the bactericidal action was delayed rather than prevented and that the rate of killing subsequently increased (Fig. 3b). Mupirocin remained predominantly bacteriostatic (Fig. 3a) even against the susceptible strain 8325-4. The moderating effect of high-level mupirocin resistance on the lethal action of SB 205952 was confirmed in the isogenic staphylococcal strains RN4220 and RN4220(pMZ1) (data not shown). SB 205952, unlike mupirocin, was also bactericidal against *E. coli* (Fig. 4). In this case, the rate of killing was intermediate between that seen with the mupirocin-susceptible and the mupirocin-resistant strains of staphylococci. Spectrophotometric absorbance measurements at 675 nm carried out on *S. aureus* and *E. coli* strains in parallel with the susceptibility studies indicated that the loss of viability was not accompanied by cell lysis (data not shown).

IRS inhibition. SB 205952 and mupirocin were both potent inhibitors of IRS activity from the mupirocin-susceptible staphylococcal strains NCTC 6571 and 8325-4 (Table 4). The compounds also inhibited the enzyme from moderately mupirocin-resistant strains, but despite superior activity relative to that of mupirocin in terms of MICs, a higher concentration of SB 205952 was consistently required to inhibit enzyme activity by 50%. For the IRSs from two highly mupirocin-resistant *S. aureus* strains, C7 and RN4220pMZ1, 20,000 and 9,600 ng of mupirocin per ml, respectively, was required to achieve 50% inhibition, confirming the resistant nature of the target enzyme. Antibacterially, SB 205952 was active at 4 to 16 μg/ml against these strains. This was not reflected in the IC_{50}s of SB 205952 (400,000 to 1,000,000 ng/ml), which indicated a very low affinity for the enzyme.

Effect of SB 205952 on induction of the SOS response. Nitrofurans are known to produce DNA damage through activation to highly reactive electrophilic intermediates by bacterial nitroreductases or interaction with molecular oxygen leading to free radical formation (12). Such damage induces the SOS response (28). To determine whether the nitrofuryl group in SB 205952 produced similar DNA damage, the compound was tested for its ability to induce the SOS response by the *E. coli* GC 4415 *sfiA*::*lacZ* reporter gene assay. The result obtained for β-galactosidase production was negative, as was that with mupirocin, suggesting that neither compound invoked the SOS response. Nitrofurantoin gave a strong positive reaction in this test at the same multiple of the MIC. The 4-quinolone ciprofloxacin was used as the positive control (33) and gave a strong positive reaction. Mupirocin and nitrofurantoin were also tested in combination and were added to the test well either simultaneously or consecutively. Nitrofurantoin induced the SOS response irrespective of the presence of mupirocin.

Activities of SB 205952 and control compounds against DNA repair-deficient mutants of *E. coli*. The DNA-damaging properties of nitroheterocyclic compounds cause repair-deficient mutants to be hypersusceptible to their actions (20, 26). Two DNA repair-deficient strains of *E. coli* exhibited hypersusceptibility to nitrofurantoin compared with the susceptibility of the corresponding repair-proficient strain WP2 (Table 5). In each case, the strains were double mutants with a mutation in *uvrA* plus an additional mutation in *recA* or *lexA*. Only one of these strains (WP 100 *uvrA recA*) showed increased susceptibility to SB 205952 and, in addition, was unusually susceptible to mupirocin and ciprofloxacin. In this strain the increase in susceptibility to SB 205952 and mupirocin was of similar magnitude, 8- to 16-fold, compared with an increase of >100-fold in susceptibility to nitrofurantoin. These differences, together with the lack of a correlation between hypersusceptibility to nitrofurantoin and SB 205952 in the *uvrA lexA* strain, suggest that the increased susceptibility of WP 100 to SB 205952 was related to the permeability of the strain to monic acid analogs rather than any similarity to the nitrofurantoin mode of action.

Isoleucine antagonism. Growth inhibition by mupirocin as a consequence of IRS inhibition can be overcome by the addition of isoleucine to the growth medium (22). The effect of a

TABLE 5. Activities of SB 205952 and other agents against DNA repair-deficient mutants of *E. coli*

Strain	MIC (μg/ml)			
	SB 205952	Mupirocin	Nitrofurantoin	Ciprofloxacin
DCO[a]	16	64	8	0.03
WP2[a]	16	64	4	0.03
CM 611 *uvrA155 lexA102*	8	64	0.5	0.004
WP 100 *uvrA155 recA1*	1	8	0.03	≤0.002

[a] DNA repair proficient.

TABLE 6. Effect of exogenous isoleucine on the activities of SB 205952 and mupirocin in minimal medium

Organism	MIC (μg/ml)			
	SB 205952		Mupirocin	
	MM[a]	MM + 1 mM Ile	MM	MM + 1 mM Ile
S. aureus				
Russell	0.06	1	0.06	2
K227	4	32	16	64
Clarke	8	32	8	32
C18	8	32	8	64
L2	16	64	>512	>512
F89	16	32	>512	>512
C12	8	128	>512	>512
E. coli ESS	0.13	2	0.13	2

[a] MM, minimal medium.

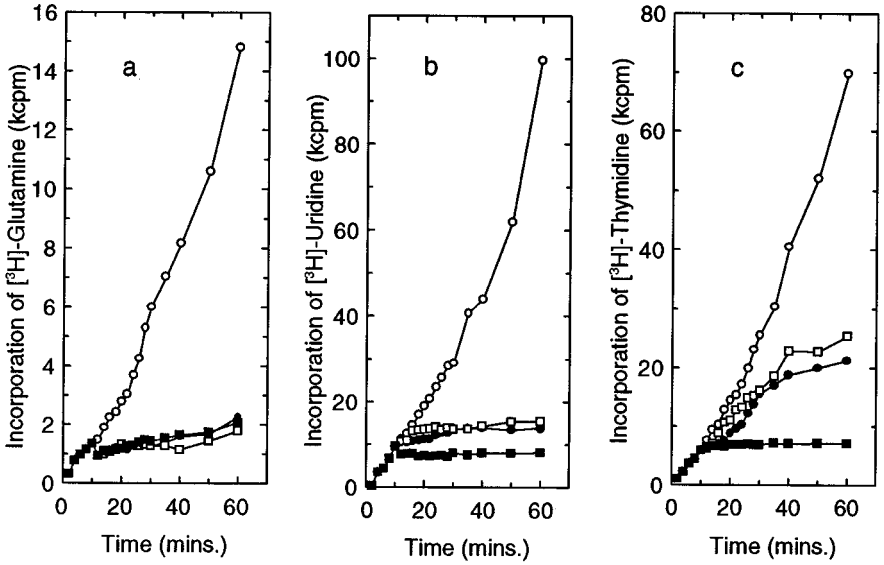

FIG. 5. Effects of SB 205952, mupirocin, and other agents at 4× the MIC on macromolecular synthesis in *S. aureus* 8325-4. (a) Protein synthesis, determined by incorporation of [^{3}H]glutamine. ○, control; ●, mupirocin; □, SB 205952; ■, chloramphenicol. (b) RNA synthesis, determined by incorporation of [^{3}H]uridine. ○, control; ●, mupirocin; □, SB 205952; ■, rifampin. (c) DNA synthesis, determined by incorporation of [^{3}H]thymidine. ○, control; ●, mupirocin; □, SB 205952; ■, ciprofloxacin.

high concentration of isoleucine in the growth medium on the activity of SB 205952 was determined against strains of *S. aureus* and *E. coli* in minimal medium (Table 6). The addition of isoleucine did not affect the growth rates or growth yields of the organisms used (data not shown). The MICs of SB 205952 were consistently higher in medium containing isoleucine, as were those of mupirocin (Table 3). The antagonistic effect of isoleucine on SB 205952 activity occurred with all staphylococcal strains, irrespective of their susceptibility to mupirocin.

Effect of SB 205952 on macromolecular synthesis in staphylococci. The effect of SB 205952 on macromolecular synthesis in staphylococci was examined in *S. aureus* 8325-4 (Mup^s) and C7 (highly mupirocin resistant). In each case a specific inhibitor with a known mechanism of action was included as a positive control. In *S. aureus* 8325-4 the effect of SB 205952 was compared with that of mupirocin at the same multiples of the MIC. Figure 5 shows the effect of SB 205952 on the incorporation of radiolabelled precursors into protein, RNA, and DNA in *S. aureus* 8325-4 (Mup^s). Within 2 min of addition, incorporation of [^{3}H]glutamine into protein ceased. RNA synthesis was also completely inhibited within 6 min of compound addition. The effect on DNA synthesis was more gradual. Incorporation of [^{3}H]thymidine continued for approximately 30 min after the addition of SB 205952, but at a progressively

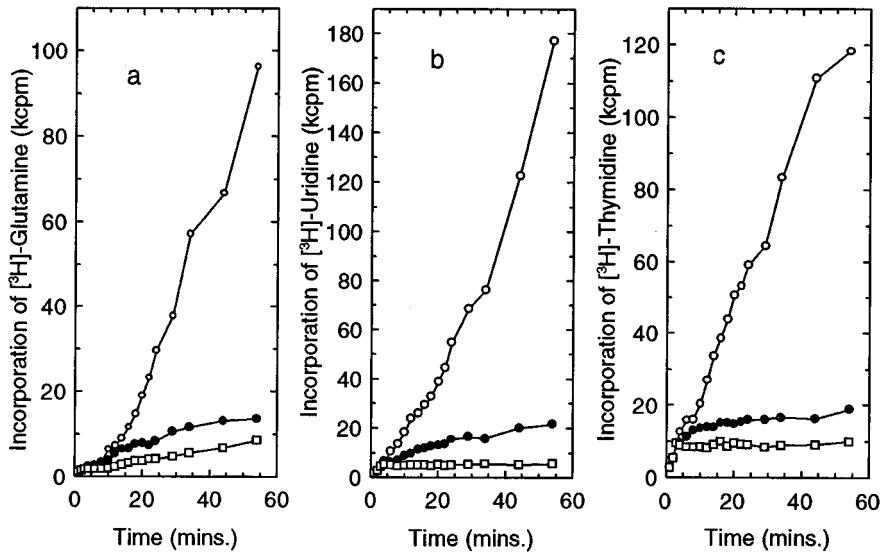

FIG. 6. Effects of SB 205952 and other agents at 4× the MIC on macromolecular synthesis in *S. aureus* C7. (a) Protein synthesis, determined by incorporation of [^{3}H]glutamine. ○, control; □, SB 205952; ●, chloramphenicol. (b) RNA synthesis, determined by incorporation of [^{3}H]uridine. ○, control; □, SB 205952; ●, rifampin. (c) DNA synthesis, determined by incorporation of [^{3}H]thymidine. ○, control; □, SB 205952; ●, ciprofloxacin.

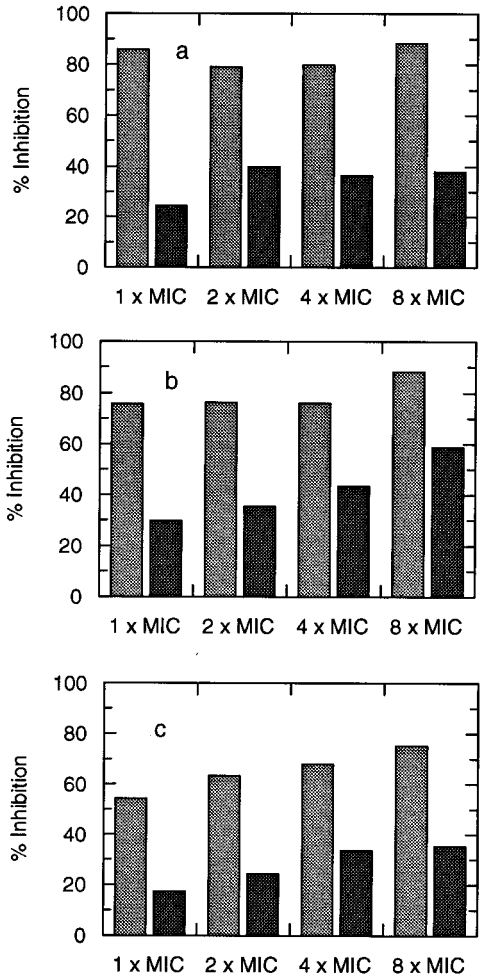

FIG. 7. Inhibition of RNA synthesis in staphylococci by SB 205952 and mupirocin at 1, 2, 4, and 8× the MIC, with (lightly shaded bars) and without (heavily shaded bars) the simultaneous addition of chloramphenicol (500 μg/ml) to relax the stringent response. (a) Mupirocin versus *S. aureus* 8325-4; (b) SB 205952 versus *S. aureus* 8325-4; (c) SB 205952 versus *S. aureus* C7.

declining rate relative to that for the control. The effect of mupirocin in this organism was almost identical to that of SB 205952 (Fig. 5).

The effect of SB 205952 on macromolecular synthesis in *S. aureus* C7 (highly mupirocin resistant) (Fig. 6) indicated that the compound was equally inhibitory to protein, RNA, and DNA syntheses. The effect of SB 205952 on DNA synthesis was more marked than that in the mupirocin-susceptible strain 8325-4. In order to determine whether effects on RNA synthesis were the result of direct inhibition by the compound or a secondary consequence of inducing stringency, the stringent response was relaxed by the simultaneous treatment of the cells with the protein synthesis inhibitor chloramphenicol (16). Inhibition of RNA synthesis by SB 205952 was greatly reduced in cultures of *S. aureus* 8325-4 and C7 in which the stringent response had been relaxed in this way (Fig. 7). Relaxation of stringent control had the same effect on inhibition of RNA synthesis by mupirocin in the susceptible strain.

The effects of SB 205952 and mupirocin on the incorporation of precursors into lipid and peptidoglycan were also determined in the same strains (data not shown). Some inhibition was observed after prolonged exposure to the antibiotics, but the effects were minor compared with the inhibition of protein, RNA, and DNA syntheses. In each case, the specific inhibitors used as control compounds (chloramphenicol, rifampin, ciprofloxacin, cerulenin, vancomycin), also at 4× the MIC, rapidly inhibited the incorporation of precursors into the appropriate macromolecule.

DISCUSSION

SB 205952, the nitrofuryl oxazole of monic acid, exhibits antimicrobial properties that differ from those of the closely related antibiotic mupirocin. These include increased potency against mupirocin-susceptible organisms, an extended antimicrobial spectrum, and increased bactericidal activity. An interesting feature of SB 205952 is its activity against clinical mupirocin-resistant variants of staphylococci, including those exhibiting high-level target-based resistance. Cell-free enzyme inhibition studies indicate that SB 205952 is a potent inhibitor of IRS from mupirocin-susceptible staphylococci and, like mupirocin, inhibits the enzyme from moderately mupirocin-resistant strains at higher concentrations. In either case, the results are consistent with the inhibition of IRS being a target in these organisms. However, SB 205952 exhibits a very low affinity for the IRS from highly mupirocin-resistant staphylococci (IC_{50}s, 400,000 to 1,000,000 ng/ml). This is not consistent with the level of antibacterial activity shown by SB 205952. It must therefore be concluded that the compound has one or more additional mechanisms of action. The bactericidal action of SB 205952 is consistent with this hypothesis, whereby SB 205952 exerts an additional bactericidal mechanism(s) of action even when the primary target is IRS. It is interesting that the initiation of bactericidal activity was delayed in highly mupirocin-resistant staphylococcal strains. A synergistic interaction between IRS inhibition and the additional mechanism(s) of action may be necessary for rapid cell death.

The unusual features of SB 205952 are not readily attributable to possession of the nitrofuran moiety. For example, SB 205952 failed to induce the SOS response or to show consistent hyperactivity against DNA repair-defective mutants, two features which are characteristic of DNA-damaging agents such as nitrofurans. However, the compound has been shown to act as a substrate for the activating nitroreductase enzymes from *E. coli* in vitro (34).

Further studies to elucidate the additional mechanism(s) of action of SB 205952 were carried out on a mupirocin-susceptible strain of *S. aureus* (8325-4) and a highly mupirocin-resistant strain (C7) in which all events were expected to be the consequence of the non-IRS-based action mechanism(s). SB 205952 rapidly inhibited DNA, RNA, and protein syntheses in mupirocin-susceptible organisms, while peptidoglycan and lipid syntheses were affected to a lesser extent. The pattern of inhibition displayed by mupirocin was consistent with previously published data (21). Surprisingly, SB 205952 also rapidly inhibited DNA, RNA, and protein syntheses in *S. aureus* C7. In the case of mupirocin, inhibition of protein synthesis is assumed to result directly from the action on IRS that restricts the availability of charged $tRNA^{Ile}$, whereas the effects on the synthesis of RNA, DNA, and other macromolecules are secondary consequences of inducing the stringent response (22).

Stringency is invoked by an increase in the ratio of uncharged to charged tRNA (36), which causes a ribosome-bound enzyme, ppGpp synthetase I, to synthesize the regulatory nucleotide ppGpp (guanosine 5′,3′-bispyrophosphate). Accumulation of this nutritional stress alarmone confers altered promoter specificity on RNA polymerase (37), leading to pleiotropic changes in gene transcription. Chloramphenicol

binds to the A site of the ribosomal peptidyltransferase center, reducing the rate of ppGpp synthesis and relaxing the stringent response (16). Under relaxed conditions, mupirocin has relatively little effect on RNA synthesis. The presence of ppGpp has recently been confirmed in staphylococci (7a), and the results obtained here demonstrating the restoration of RNA synthesis during cotreatment with chloramphenicol and mupirocin in staphylococci are consistent with the relaxation of stringency. SB 205952 also appears to induce stringency not only in *S. aureus* 8325-4 (the expected consequence of IRS inhibition) but also in *S. aureus* C7. SB 205952 therefore appears to have a second mechanism of action which induces stringency but which is independent of IRS inhibition. Nitrofurantoin and related compounds (27, 38) are among the agents known to invoke ppGpp accumulation and stringency, and oxidative inactivation of dihydroxyacid dehydratase has been implicated (38). This enzyme is a key component of the isoleucine biosynthetic pathway in which it functions to convert α,β-dihydroxy-β-methylvalerate to α-keto-β-methylvalerate (41). The results obtained with SB 205952 could therefore be rationalized on the basis of the stringency induced by the nitrofuryl moiety acting on an enzyme in the isoleucine biosynthetic pathway rather than the monic acid component inhibiting IRS. This hypothesis is consistent with the observation that exogenous isoleucine antagonizes the antimicrobial activity of SB 205952 (Table 6).

It is unlikely that the bactericidal action of SB 205952 can be attributed to induction of stringency because this regulatory mechanism promotes the formation of a stable dormant state and is known to reduce lethal events such as autolysis (40). The bactericidal actions of nitrofuran compounds are complex, incompletely understood, and likely to involve multiple mechanisms and target sites. A similar situation may exist with SB 205952.

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