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RNA Polymerase Inhibitors with Activity against Rifampin-Resistant Mutants of *Staphylococcus aureus*

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A collection of rifampin-resistant mutants of *Staphylococcus aureus* with characterized RNA polymerase β-subunit (*rpoB*) gene mutations was cross-screened against a number of other RNA polymerase inhibitors to correlate susceptibility with specific *rpoB* genotypes. The *rpoB* mutants were cross-resistant to streptolydigin and sorangicin A. In contrast, thiolutin, holomycin, corallopyronin A, and ripostatin A retained activity against the *rpoB* mutants. The second group of inhibitors may be of interest as drug development candidates.

Bacterial DNA-dependent RNA polymerase is an attractive drug target because RNA chain elongation is essential for bacterial growth (6, 16). Among those antibiotics discovered in the last 50 years, there are several known, or suspected, inhibitors of bacterial DNA-dependent RNA polymerase that have not been developed and could be candidates for new drugs. These agents include thiolutin (18), holomycin (B. Oliva, A. O'Neill, J. M. Wilson, P. J. O'Hanlon and I. Chopra, submitted for publication), streptolydigin (6, 16), the ripostatins, corallopyronins and sorangicins (10–12, 23) (Fig. 1). However, bacterial resistance has already developed to the rifamycins, the only class of RNA polymerase inhibitor that is in use clinically (21). Therefore, before considering whether other RNA polymerase inhibitors might be developed, it is important to establish whether resistance to rifamycins, such as rifampin, also confers cross-resistance to the other agents. Some attempts to address this issue have been made (9, 12, 13, 15, 24). However, the data are incomplete and the genetic basis of resistance to rifamycins in those strains used for cross-screening has rarely been determined. Furthermore, some data are contradictory; e.g., cross-resistance between rifampin and streptolydigin has been observed by some authors (13) but not by others (9, 15).

To assist the evaluation of these older agents we cross-screened them against a collection of rifampin-resistant mutants of *Staphylococcus aureus*, generated in an isogenic background, with defined RNA polymerase β-subunit (*rpoB*) gene mutations. These *S. aureus* strains, which provide a model for *rpoB* mutations occurring in naturally occurring isolates of staphylococci and other organisms (1, 7, 8, 15, 22, 28, 29), have allowed us to correlate susceptibility with specific *rpoB* genotypes.

The antibiotics used here were either purchased from Sigma (rifampin and streptolydigin) or were gifts from H. Reichenbach, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany (corallopyronin A, ripostatin A, and sorangicin A); P. O'Hanlon, SmithKline Beecham Pharmaceuticals, Harlow, United Kingdom (holomycin and thiolutin); and Pharmacia & Upjohn (rifabutin). Spontaneous rifampin-resistant mutants of *S. aureus* 8325-4 (20) were isolated by plating approximately 10^8 CFU onto Iso-Sensitest agar (Oxoid, Basingstoke, United Kingdom) containing 0.032 μg of rifampin/ml (four times the MIC). A number of rifampin-resistant mutants were picked at random, and their MICs of rifampin were determined by agar dilution in Iso-Sensitest agar using an inoculum of 10^6 CFU/spot (2). This resulted in the identification of a series of mutants for which the MICs of rifampin were in the range 0.25 to 1024 μg/ml.

The *rpoB* gene mutations were determined in three low-level-resistant mutants (MIC, 0.25 μg/ml), three intermediate-level-resistant mutants (MIC, 8 to 16 μg/ml), and three high-level-resistant mutants (MIC, >500 μg/ml). Total DNA was prepared (25) from the mutants and the parental strain 8325-4 and was subjected to PCR amplification of *rpoB* using the primers F3 and F4 (1) (Table 1). The amplification products were visualised by agarose gel electrophoresis (25) and then extracted from gels by solubilization in QG buffer (Qiagen, Crawley, United Kingdom). DNA was purified using the QIAquick PCR purification kit (Qiagen) and then sequenced from both F3 and F4 using an Applied Biosystems 377 DNA sequencer. This procedure resulted in the identification of mutations in all strains apart from Rif21, Rif22, and Rif26. Additional primers (rif1 and rif6) (Table 1) were used to amplify the whole of *rpoB* in these mutants and all primers (Table 1) used for sequencing of the amplified products.

Nine mutational changes were found in the rifampin-resistant mutants occurring at seven positions from amino acid 137 to 486 (Table 2). With the exception of the mutation at amino acid 137, the other mutations were all located in cluster I of *rpoB* (15, 16) and are either identical to those previously reported for rifampin resistance in *S. aureus* (1, 28) or involve different amino acid substitutions (e.g., Asp471→Glu and His481→Asp [at sites 471 and 481]) where other mutational changes are already known to confer rifampin resistance (1, 28). The mutation at position 137 (Gln137→Leu) in mutant Rif21 has not previously been reported in *S. aureus*. Furthermore, it does not appear to represent a mutational site which exactly corresponds to those found in the *rpoB* genes of other organisms (16). However, we observed an identical mutation in two other independent mutants (Rif22 and Rif26) that also

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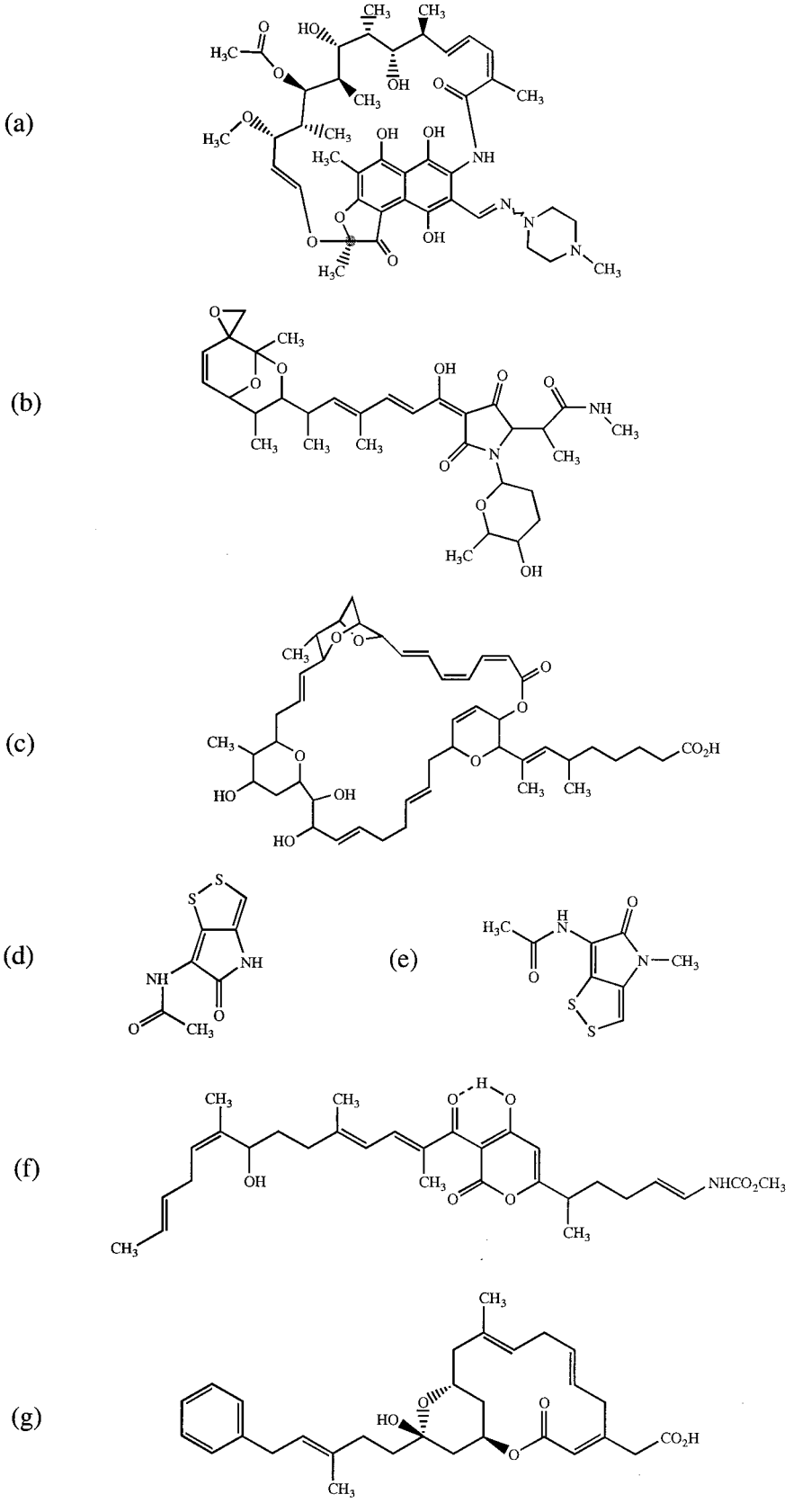

FIG. 1. Structures of rifampin (a), streptolydigin (b), sorangicin A (c), holomycin (d), thiolutin (e), corallopyronin A (f), and ripostatin A (g).

TABLE 1. Primers used for PCR amplification and sequencing of regions of *rpoB* from rifampin-resistant mutants of *S. aureus* 8325-4

Primer[a]	Nucleotide sequence (5′-3′)	Position (bp) in *rpoB* (direction)
F3	AGTCTATCACACCTCAACAA	1325–1344 (sense)
F4	TAATAGCCGCACCAGAATCA	2026–2007 (antisense)
rif1	ATCTGTTTGGCAGGTCAAGTTGTC	1–24 (sense)
rif2	ACAGATGCTAAAGATGTTGTATAC	526–549 (sense)
rif3	TCAATTAAAGTATATGTTCCTAAC	1042–1065 (sense)
rif4	ACCAATATAAACGATACCACGATC	2505–2482 (antisense)
rif5	ATCACGAGCCATACCAGCTTCTTC	3045–3022 (antisense)
rif6	AAATTGCGTATTAATCAGTAACTC	3569–3546 (antisense)

[a] Primers rif1 to rif6 were based on existing *rpoB* sequence data (GenBank accession no. X64172).

displayed low-level resistance to rifampin, and mutations conferring rifampin resistance in *Escherichia coli* (19) and *Rickettsia typhi* (27) have been reported at the amino terminus of the β-subunit, corresponding to positions 135 and 125 in *S. aureus*. The *S. aureus* rifampin-resistant mutants studied here displayed cross-resistance to streptolydigin and sorangicin A (Table 2). However, cross-resistance was not observed with thiolutin, holomycin, corralopyronin A, or ripostatin A (Table 2). For control purposes we also screened the set of *rpoB* mutants for cross-resistance to another member of the rifamycin class, rifabutin. In all cases cross-resistance was observed (data not shown).

The emergence of bacterial resistance to antimicrobial agents is a serious threat to human health (3–5). One approach to the problem is the discovery and development of new antibacterial agents with novel targets that will be effective against organisms resistant to current agents (3, 4). However, since this process is complex and lengthy (3, 4) an alternative strategy is the reevaluation of older unexploited antibiotic classes that have not so far been developed for human use (4, 30).

When choosing earlier agents for development it is desirable to select compounds that are structurally unrelated to current agents so that problems of cross-resistance mediated by existing mechanisms are minimized (4). Apart from the fact that the structures of rifampin, ripostatin A, and sorangicin A all contain cyclic frameworks (25, 14, and 30 membered, respectively) (Fig. 1), there is essentially very little structural similarity between rifampin and the earlier RNA polymerase inhibitors. This is particularly the case when comparing the macrocyclic naphthol-based structure in rifampin with the tetramic acid structure of streptolydigin and with the structurally simple thiolutin and holomycin systems (Fig. 1). Thus, none of the earlier RNA polymerase inhibitors described here has any obvious pharmacophoric similarity to rifampin. In view of their structural novelty these agents might therefore be expected to retain activity against rifampin-resistant isolates. However, there have been reports of cross-resistance between some of the older compounds described here and rifampin (13, 24). We examined this in more detail by establishing whether the nonrifamycin-type RNA polymerase inhibitors were active against genetically defined rifampicin-resistant (*rpoB*) mutants of *S. aureus*.

On the basis of the staphylococcal cross-resistance patterns we conclude that streptolydigin and sorangicin A are not potential drug candidates. In contrast, holomycin, thiolutin, corallopyronin A, and ripostatin A are worthy of further study since they overcome rifampin-resistant genotypes. There are also no reports that these agents exhibit cross-resistance with other antibiotic resistance determinants. Our data suggest that this second group of inhibitors could have an application in the treatment of infections caused by rifampin-resistant and -sensitive staphylococci. However, since these agents have a spectrum of activity that also encompasses other bacterial species (10, 12, 17, 24, 26; Oliva et al., submitted for publication), they may have applications which could extend beyond their use simply as antistaphylococcal agents. With the exception of corallopyronin A, the other three agents are less active as antimicrobial agents (MICs, 8 to 4 μg/ml) (Table 2). Therefore, it will probably be necessary to derive more-active analogs of these agents for administration as antibacterial drugs.

Other aspects of these compounds, e.g., their toxicity, will need to be addressed before they can be considered as development candidates. Preliminary toxicity studies have been performed on some of these agents. Corallopyronin and ripostatin are only weakly active against eukaryotic cells in vitro (10, 23), and corallopyronin is reported to show no toxicity for mice when administered subcutaneously (10). Thiolutin appears to possess activity against eukaryotic cells since it has antifungal activity (14, 26) and is reported to be moderately toxic in mice (26). Holomycin appears to lack antifungal activity (Oliva et al., submitted for publication), but there are no published animal toxicity data for this compound. Some of the toxicity studies on these compounds were conducted more than 50 years ago (26), and clearly more-detailed studies are required to determine the specificities of these agents for prokaryotic organisms.

Although studies on cross-resistance between rifampin, and holomycin, thiolutin, and corallopyronins have not been reported, the lack of cross-resistance between rifampin and ripostatin A that we observed is consistent with earlier findings (12). Similarly, we have confirmed an earlier report of cross-resistance between sorangicins and rifampin (24). Our results with streptolydigin are only in partial agreement with earlier reports. Iwakura et al. (13) demonstrated cross-resistance in *E. coli* between rifampin and streptolydigin at the level of *rpoB*. However, although mutations conferring resistance to streptolydigin are known to occur in *rpoB* (between clusters I and II in *E. coli*) (9, 15) others have reported no relationship between these mutations and resistance to rifampin (9). It has therefore been concluded that each drug has a separate binding site in the β-subunit of RNA polymerase (9, 15). We suggest, however, that the streptolydigin binding site, at least in *S. aureus*, may extend into cluster I or is influenced by the nature of the amino acids present in this region.

TABLE 2. Susceptibility of *S. aureus* 8325-4 *rpoB* mutants to various antibiotics

Strain	Mutation in RpoB	MIC (μg/ml) of[a]:						
		RIF	STL	SOR A	HOL	THL	COR A	RIP A
8325-4		0.008	16	0.032	8	8	0.5	8
Rif21	Gln137→Leu	0.25	32	0.016	8	8	0.5	8
Rif37	Asp471→Glu	0.25	32	1	8	8	0.5	8
Rif39	Leu466→Ser	0.25	32	1	8	8	0.5	8
Rif28	His481→Asn	8	64	16	8	4	0.5	8
Rif34	Asp471→Tyr	8	64	2	8	8	0.5	8
Rif35	Ser464→Pro	16	64	1	4	4	1	8
Rif38	Ala477→Asp	512	64	2	8	4	1	8
Rif23	Ser486→Leu	1,024	64	32	8	8	0.5	4
Rif40	His481→Asp	1,024	>128	>64	4	8	0.5	4

[a] Abbreviations: COR A, corallopyronin A; HOL, holomycin; RIF, rifampin; RIP A, ripostatin A; SOR A, sorangicin A; STL, streptolydigin; THL, thiolutin.

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