

Activity of and Development of Resistance to Corallopyronin A, an Inhibitor of RNA Polymerase[∇]

Katherine Mariner,¹ Martin McPhillie,² Rachel Trowbridge,¹ Catriona Smith,¹
Alex J. O'Neill,¹ Colin W. G. Fishwick,² and Ian Chopra^{1*}

Antimicrobial Research Centre and Institute of Molecular and Cellular Biology, University of Leeds,
Leeds LS2 9JT, United Kingdom,¹ and Antimicrobial Research Centre and School of Chemistry,
University of Leeds, Leeds LS2 9JT, UK²

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We explored the properties of corallopyronin A (CorA), a poorly characterized inhibitor of bacterial RNA polymerase (RNAP). It displayed a 50% inhibitory concentration of 0.73 μ M against RNAP, compared with 11.5 nM for rifampin. The antibacterial activity of CorA was also inferior to rifampin, and resistant mutants of *Staphylococcus aureus* were easily selected. The mutations conferring resistance resided in the *rpoB* and *rpoC* subunits of RNAP. We conclude that CorA is not a promising antibacterial drug candidate.

Antibiotics of the rifamycin class (e.g., rifampin) are currently the only inhibitors of bacterial RNA polymerase (RNAP) that are approved for clinical use (3). Bacterial RNAP therefore remains an attractive target for the discovery and development of new antibacterial agents (3). Corallopyronin A (CorA) and myxopyronin B (MyxB) (Fig. 1) are structurally related α -pyrone antibiotics first described around 30 years ago (8, 9). Both antibiotics are active against Gram-positive bacteria and inhibit bacterial RNAP *in vitro* (8, 9). Furthermore, MyxB has recently been cocrystallized with the beta-prime subunit of RNAP (2, 12). However, whether these antibiotics have the potential to be developed as clinical candidates requires further information. In this study, we examined the potency of CorA against RNAP, its mechanism of action, and its antibacterial spectrum. We also determined the frequency of selection of spontaneous resistance to CorA in *Staphylococcus aureus* and the nature of the mutations conferring resistance.

CorA was provided by G. Hofle, Helmholtz Centre for Infection Research, Germany. MyxB was a gift from T. Moy, Cubist Pharmaceuticals, Lexington, MA. Rifampin was obtained from Sigma-Aldrich (Poole, United Kingdom). Yeast Pol II was a kind gift from D. Bushnell, Stanford University, Stanford, CA.

The activities of CorA and rifampin against *Escherichia coli* RNAP *in vitro* were compared using the Kool NC-45 universal RNAP template (Epicentre, Madison, WI) (11). Although CorA inhibited *E. coli* RNAP, rifampin was a more potent inhibitor of the enzyme (Table 1). The activity of CorA against *E. coli* RNAP that we observed (50% inhibitory concentration [IC_{50}] of 0.73 μ M) is similar to the previously published value of 2 μ g/ml (3.8 μ M) (9). The activities of CorA and rifampin against yeast Pol II *in vitro* were compared in a modified nonspecific transcription assay (D. Bushnell personal commu-

nication) using Polyribose C template (Sigma, Poole, United Kingdom) and SYBR Green I (Invitrogen, Paisley, United Kingdom) detection. The addition of CorA at 100 μ M inhibited yeast Pol II activity by 10.1% \pm 3.2%, compared with 37.3% \pm 2.6% for rifampin. The lack of activity of CorA against eukaryotic cells correlates with previous studies showing only 7% inhibition of wheat germ Pol II by a 40 μ g/ml (75.8 μ M) concentration of the antibiotic (9).

CorA is reported primarily to inhibit the growth of Gram-positive bacteria (9). We determined its activity against several species (Table 1) by microdilution in Mueller-Hinton broth according to British Society for Antimicrobial Chemotherapy guidelines (1). In agreement with previously published work (9), the antibacterial activity of CorA was limited to the Gram-positive bacteria tested, i.e., *S. aureus* SH1000 (6) and *Bacillus subtilis* (17). Both wild-type and AcrAB efflux pump-deficient strains of *E. coli* (14) proved insusceptible to CorA (Table 1), the latter result suggesting that CorA is not a substrate for the AcrAB-TolC efflux system. However, an *E. coli* *tolC* knockout mutant (16) was >32-fold more susceptible to CorA than the wild type (Table 1), indicating that CorA may be a substrate for

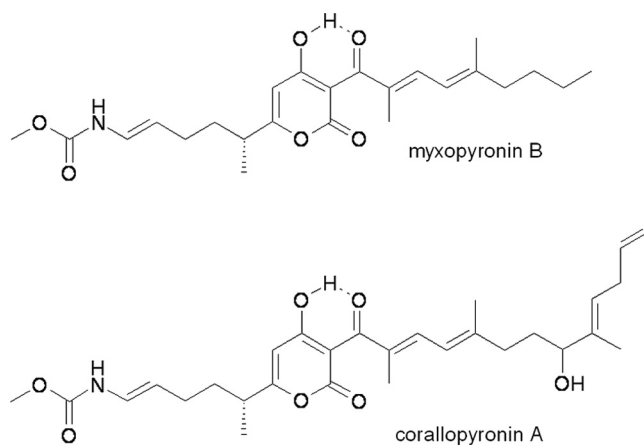


FIG. 1. Structures of CorA and MyxB.

* Corresponding author. Mailing address: Antimicrobial Research Centre and Institute of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom. Phone: 44-113-343-5604. Fax: 44-113-343-3167. E-mail: i.chopra@leeds.ac.uk.

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TABLE 1. Inhibition of RNAP by rifampin and CorA *in vitro*, the susceptibility of bacteria to these antibiotics, and their effects on the cytoplasmic membrane of *S. aureus*

Inhibitor	Avg IC ₅₀ for RNAP ± SD	MIC (μg/ml)						Avg % membrane integrity ± SD ^c
		<i>S. aureus</i> SH1000	<i>E. coli</i> 1411	<i>E. coli</i> SM1411 (<i>acrAB</i>) ^a	<i>E. coli</i> AB734	<i>E. coli</i> ES100 (<i>tolC</i>) ^b	<i>B. subtilis</i> 1534	
Rifampin	11.5 ± 1.1 nM	0.015	4	4	16	0.5	128	80.8 ± 6.1
CorA	0.73 ± 0.2 μM	2	>128	>128	>128	4	32	97.9 ± 10.4

^a *E. coli* 1411 Δ*acrAB*::Tn903Kan^r (14).

^b *E. coli* AB734 *tolC*::Tn10 (16).

^c Compared to the drug-free control (100%) and measured by the BacLight assay following exposure of *S. aureus* SH1000 to 4× MIC of compounds for 10 min.

TolC efflux systems that are linked to membrane fusion proteins and cytoplasmic membrane transporters other than AcrAB.

Although CorA inhibits RNAP, it is not known whether this alone accounts for its antibacterial activity. To examine the mechanism of action of CorA, we utilized *B. subtilis* antibiotic biosensors containing promoter-luciferase reporter constructs which are induced by conditions of antibiotic stress (10, 11, 17). Rifampin and CorA induced the biosensor (*yvgS*) responsive to inhibition of RNA synthesis (10). Although rifampin only induced *yvgS* (data not shown), CorA also induced the *fabHB* biosensor that is responsive to inhibition of fatty acid biosynthesis (10). However, induction was only just above the published threshold for this biosensor (17) and 3-fold lower than that exhibited by triclosan, a known inhibitor of fatty acid biosynthesis (17). Therefore, although CorA might possess an additional mechanism of action involving inhibition of fatty acid synthesis, further studies are required to confirm this suggestion. Nevertheless, inhibition of fatty acid biosynthesis has been observed with other antimicrobial agents that contain an α-pyrone moiety (5).

With the notable exception of daptomycin, inhibitors that damage the bacterial cytoplasmic membrane tend to be unsuitable for further development as drug candidates (13). Consequently, the ability of CorA to damage the membrane of *S. aureus* was investigated using the BacLight assay (11). Neither CorA nor rifampin caused membrane damage, as the cells essentially maintained 100% membrane integrity after exposure for 10 min to the antibiotics at 4× MIC (Table 1).

Mutations conferring resistance to CorA in *E. coli* have been selected by random mutagenesis of the genes encoding RNAP (12). However, the frequency of spontaneous mutation to

CorA resistance and the nature of mutants arising by this route have not been determined. The mutation frequency of resistance to CorA in *S. aureus* SH1000 at a selective concentration of 4× MIC was $7.2 \times 10^{-8} \pm 2.4 \times 10^{-8}$ (number of mutants per total number of viable bacteria), which was comparable to that of rifampin at $1.4 \times 10^{-7} \pm 2.2 \times 10^{-7}$. Eight independent mutants of *S. aureus* SH1000 displaying resistance to CorA (Table 2) were selected for analysis of cross-resistance to a panel of agents representing the major antibiotic classes, including rifampin. Cross-resistance did not occur (data not shown). However, cross-resistance to the related α-pyrone antibiotic MyxB was observed (Table 2), suggesting, by analogy to the random mutagenesis study (12), that the spontaneous CorA-resistant mutants probably possessed changes in RNAP. Accordingly, the genes encoding the RNAP α, β, and β' subunits (*rpoA*, *rpoB*, and *rpoC*, respectively) in the CorA mutants were subjected to PCR amplification and DNA sequencing as previously described (15). Mutations were found in either *rpoB* or *rpoC* (Table 2). Some resistant mutants contained identical mutations (e.g., Cor2/Cor8 and Cor3/Cor4) or mutations affecting the same amino acid residue (e.g., Cor1, Cor3, Cor4). The remaining two mutants (Cor5, Cor6) contained mutations that were independent of each other and the mutants described above (Table 2). The introduction of mutations in *E. coli* RNAP corresponding to those in Cor1, Cor3, Cor4, Cor5, and Cor7 (i.e., at S₁₃₂₂, E₁₂₇₉, and L₁₃₂₆ of wild-type *E. coli rpoB*) and Cor6 (i.e., K₃₄₅ of wild-type *E. coli rpoC*) have previously been reported to confer resistance to both Myx and CorA in *E. coli* (12).

The *S. aureus* Cor mutants described here exhibited only a slight loss of fitness compared to strain SH1000 when mean generation times were measured (7) (Table 2). This suggests

TABLE 2. Properties and nature of CorA-resistant mutants of *S. aureus* SH1000 isolated in this study

Strain	CorA MIC (μg/ml)	MyxB MIC (μg/ml)	Avg generation time (min) ± SD	Amino acid substitution (codon change) ^a	
				β subunit	β' subunit
SH1000	2	2	37.2 ± 3.1	–	–
SH1000-Cor1	>128	>128	45.3 ± 3.1	S ₁₁₂₇ P (TCA-CCT)	None
SH1000-Cor2	64	>128	46.9 ± 3.6	None	L ₁₁₆₅ R (CTT-CGT)
SH1000-Cor3	>128	>128	41.1 ± 4.9	S ₁₁₂₇ L (TCA-TTA)	None
SH1000-Cor4	>128	>128	42.7 ± 2.2	S ₁₁₂₇ L (TCA-TTA)	None
SH1000-Cor5	64	>128	36.7 ± 3.7	E ₁₀₈₄ K (GAA-AAA)	None
SH1000-Cor6	>128	>128	38.1 ± 4.4	None	K ₃₃₄ N (AAA-AAC)
SH1000-Cor7	128	128	44.5 ± 8.0	L ₁₁₃₁ F (TTG-TTT)	None
SH1000-Cor8	64	>128	47.8 ± 1.1	None	L ₁₁₆₅ R (CTT-CGT)

^a *S. aureus* numbering.

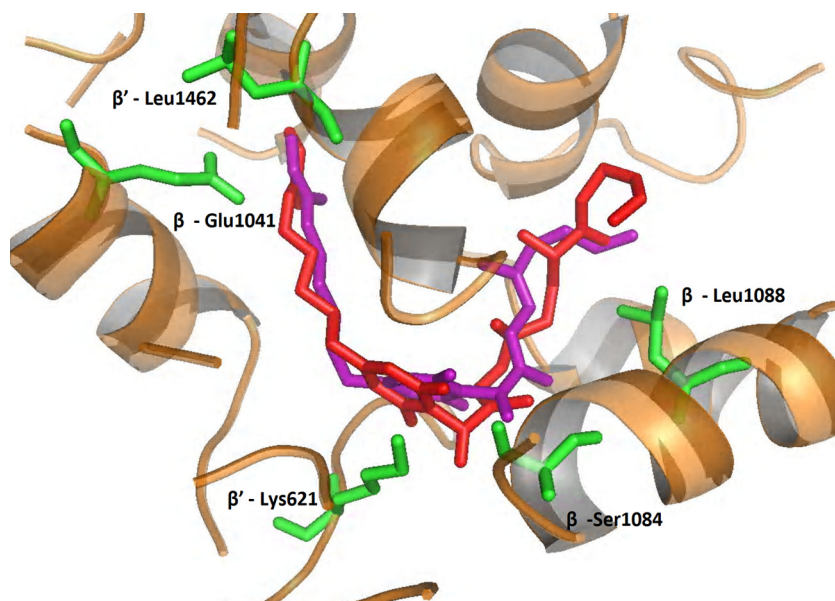


FIG. 2. Model of myxopyronin (purple) and CorA (red) bound to the beta subunits of RNAP, showing the proximity to the mutated residues in the RNAP of *S. aureus* strains SH1000-Cor1 to -Cor8. The residue numbers are those of *T. thermophilus* RNAP (4).

that if such mutants arose in the clinical setting they might not suffer a competitive disadvantage compared to CorA-sensitive strains; i.e., CorA-resistant mutants could persist in natural populations even in the absence of CorA selection pressure.

Docking of CorA in the MyxB binding site of *Thermus thermophilus* RNAP was performed using eHITS (18). This involved exhaustive docking of CorA in the MyxB binding site and selection of a binding conformation using a number of criteria. These included satisfying key hydrogen bonding interactions (i.e., with G₃₄₄, W₁₂₇₆, and E₁₂₇₉), a good predicted binding affinity (calculated via a combination of hydrogen bonding, van der Waals interactions, hydrophobic contacts, and space-filling properties), and a visual assessment of the overall fit within the site. The altered residues in the *S. aureus* Cor mutants, by aligning the *S. aureus* and *T. thermophilus* RNAP subunit sequences (4), were all located directly within the myxopyronin binding site as previously described (Fig. 2) (2, 12). The occurrence of spontaneous mutations at this site (the switch region) strongly supports the hypothesis that CorA also binds to RNAP, inhibiting transcription.

In summary, we have shown that CorA inhibits RNA biosynthesis at the level of RNAP and does not damage the bacterial cell membrane. Its activity both as an RNAP inhibitor and as an antibacterial agent is inferior to that of rifampin. CorA has a limited spectrum of antibacterial activity and, like rifampin, a high propensity for selection of resistance. Therefore, CorA is of limited interest for development as a future drug candidate, especially for monotherapy. However, if derivatives of CorA with improved features can be developed, these could be future antibiotic candidates. Indeed, close inspection of the myxopyronin-RNA cocrystal structure (2, 12) indicates that it may be possible to produce synthetic hybrid Myx-CorA variants with increased potency.

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