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Furanyl-Rhodanines Are Unattractive Drug Candidates for Development as Inhibitors of Bacterial RNA Polymerase▽§

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Previous studies suggest that furanyl-rhodanines might specifically inhibit bacterial RNA polymerase (RNAP). We further explored three compounds from this class. Although they inhibited RNAP, each compound also inhibited malate dehydrogenase and chymotrypsin. Using biosensors responsive to inhibition of macromolecular synthesis and membrane damaging assays, we concluded that in bacteria, one compound inhibited DNA synthesis and another caused membrane damage. The third rhodanine lacked antibacterial activity. We consider furanyl-rhodanines to be unattractive RNAP inhibitor drug candidates.

Bacterial RNA polymerase (RNAP) is an attractive drug target (2, 8, 14). However, it is underexploited, since the rifamycins are the only RNAP inhibitors that have been developed for clinical use (2, 8, 14). Recently several furanyl-rhodanines that apparently possess antibacterial activity and inhibit *Escherichia coli* (RNAP) were described (15). This series had been expanded from a compound considered to be a specific RNAP inhibitor on the basis of cross-screening against unrelated enzymes and failure to inhibit in an *in vitro* transcription-translation assay using a reticulocyte lysate (15). In view of our interest in RNAP inhibitors (1, 2, 10), we decided to examine in more detail compound 1 (Table 1), the most potent of the recently identified furanyl-rhodanine RNAP inhibitors (15). We also considered whether other furanyl-rhodanines (compounds 2 and 3 in Table 1) reported to possess antibacterial activity (4, 16) might also be inhibitors of RNAP.

Compound 1 was purchased from Cheshire Sciences Ltd. (United Kingdom). Compounds 2 and 3 were purchased from Chembridge Corporation and the Sigma-Aldrich rare chemical library, respectively. The purity of each sample was >95%, and the published structure for each compound (Table 1) was confirmed by full spectroscopic analysis (see the supplemental material).

We determined the abilities of compounds 1 to 3 to inhibit RNAP in an *in vitro* assay using the Kool NC-45 universal RNA polymerase template (Epicentre, Madison, WI). Compounds 1 to 3 demonstrated similar 50% inhibitory concentrations (IC_{50}s) for *E. coli* RNAP (Table 1). However, rifampin, rifamycin SV, and corallopyronin A (obtained from G. Hofle, Helmholtz Centre for Infection Research, Germany), which are all well-documented inhibitors of RNAP (2, 7, 10), were more potent inhibitors of RNAP than the three furanyl-rhodanines examined here (Table 1). Compounds 2 and 3 have also been reported, respectively, to inhibit a class C β-lactamase (4) and penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus* (16). We therefore considered the possibility that compounds 1 to 3 might be nonspecific enzyme inhibitors. The inhibitory activities of compounds 1 to 3 at 100 μM (i.e., approximately 3 to 6 times their IC_{50}s against RNAP) were determined for bovine chymotrypsin and porcine malate dehydrogenase, commonly used specificity screens (12). Assays were run as previously described (12). Compounds 1 to 3 exhibited substantial inhibition of both enzymes (Table 2). In contrast, rifampin, at approximately 4 times its IC_{50} for RNAP (47.2 nM), exhibited only negligible inhibition (<10%) of chymotrypsin and malate dehydrogenase activities (Table 2).

Compounds 1 to 3 are reported to possess antibacterial activity (4, 15, 16). We therefore sought to examine their modes of action and in particular to elucidate whether inhibition of bacterial growth might be attributed to inhibition of RNAP activity within the whole cell. A variety of bacterial strains were used for these studies (Table 3). Initially we evaluated the antibacterial activities of the three furanyl-rhodanines (compounds 1 to 3 in Table 4) by microdilution in Mueller-Hinton broth according to British Society for Antimicrobial Chemotherapy guidelines (11). In contrast to published data for compound 1 (15), we were unable to detect antibacterial activity against *Staphylococcus epidermidis* (MIC > 256 μg/ml against *S. epidermidis* strain ATCC 11047) or *E. coli* (Table 4), including strains treated with the outer membrane permeabilizing agent polymyxin B nonapeptide (PMBN) (3) or deficient in the AcrAB efflux pump (9). Compound 1 also lacked activity against *Staphylococcus aureus* and *Bacillus subtilis* (Table 4). We have no obvious explanation for the discrepancy between our data for compound 1 and the previously published results (15), especially since the structure of compound 1 (Table 1) was confirmed by full spectroscopic analysis (see the supplemental material) to be the same as that previously examined (15). In contrast to the results for compound 1, we confirmed that the

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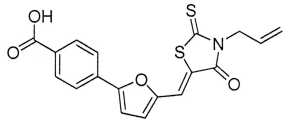
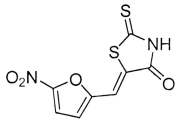
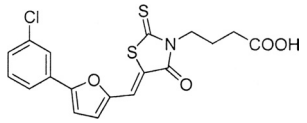
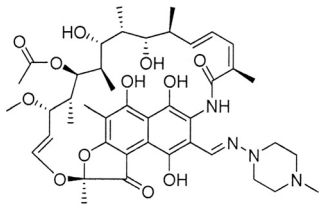
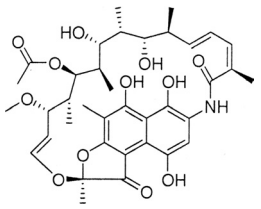
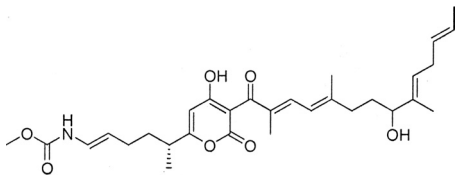
TABLE 1. Inhibition of *E. coli* RNAP by various compounds

Compound	Structure	IC_{50} for RNAP (μM)
1		37.28 ± 3.94
2		32.21 ± 6.80
3		16.26 ± 2.47
Rifampin		0.0115 ± 0.0011
Rifamycin SV		0.0086 ± 0.0014
Corallopyronin A		0.7261 ± 0.243

other two furanyl-rhodanines (compounds 2 and 3) reported to possess antibacterial activity (4, 16) did indeed prevent the growth of *S. aureus*, *B. subtilis*, and *E. coli* (Table 4). Compounds 2 and 3 displayed moderate activity against *E. coli* 1411 which was not enhanced in the AcrAB deletion mutant, strain SM1411 (Table 4). Therefore, these compounds appear not to be substrates for the AcrAB efflux pump. However, treatment of *E. coli* 1411 with the outer membrane permeabilizing agent

TABLE 2. Inhibition of malate dehydrogenase and chymotrypsin by various compounds

Compound	Concn (μM)	% inhibition ± SD of mean	
		Malate dehydrogenase	Chymotrypsin
1	100	96.3 ± 1.3	67.4 ± 16.6
2	100	99.7 ± 0.1	49.4 ± 0.8
3	100	88.1 ± 0.8	96.7 ± 1.2
Rifampin	0.0472	8.3 ± 2.2	7.8 ± 4.8

TABLE 3. Bacterial strains

Strain	Relevant description/genotype	Reference/ source
S. aureus SH1000	*rbsU*$^+$ derivative of *S. aureus* 8325-4	6
E. coli 1411	Parent of SM1411	9
E. coli SM1411	*E. coli* 1411 DacrAB:Tn*903* Kan^r	9
S. epidermidis ATCC 14990	ATCC type strain	American Type Culture Collection
B. subtilis 1S34	Parental strain of biosensors	13
B. subtilis 1S34 *yorB*	Biosensor responsive to inhibition of DNA synthesis	13
B. subtilis 1S34 *yvgS*	Biosensor responsive to inhibition of RNA synthesis	13
B. subtilis 1S34 *yheI*	Biosensor responsive to inhibition of protein synthesis	13
B. subtilis 1S34 *ypuA*	Biosensor responsive to inhibition of cell envelope synthesis	13
B. subtilis 1S34 *fabHB*	Biosensor responsive to inhibition of fatty acid synthesis	13

TABLE 4. Susceptibilities of bacteria to rhodanine compounds 1 to 3 and their effects on *B. subtilis* antibiotic biosensors and the cytoplasmic membrane of *S. aureus*

Compound	MIC[a] (μg/ml) for:					Induction of *B. subtilis* antibiotic biosensor[a,c]				Membrane disruption	
	S. aureus SH1000	*S. epidermidis* 11047	*E. coli* 1411	*E. coli* 1411 + PMBN[b]	*E. coli* SM1411	*B. subtilis* 1S34	DNA	RNA	Protein	Cell envelope	Fatty acid	% membrane integrity[a,d]	% membrane potential[a,e]
1	>256	>256	>256	>256	>256	>256	ND	ND	ND	ND	ND	ND	ND
2	4	ND	16	2	16	1	8.0 ± 0.9	1.3 ± 0.1	1.3 ± 0.3	1.0 ± 0.1	1.4 ± 0.2	82.8 ± 14.9	104.8 ± 6.9
3	4	ND	>128	16	>128	1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	27.3 ± 4.2	43.1 ± 8.0

[a] ND, not determined.
[b] PMBN (4 μg/ml) added together with compound 1, 2, or 3.
[c] Maximum reporter signal induced in the respective biosensor, expressed as a ratio of the signal in noninduced control cultures.
[d] Compared to drug-free control (100%) and measured by the *Bac*Light assay following exposure of *S. aureus* SH1000 to 4× MIC of compound for 10 min.
[e] Compared to drug-free control (100%) and determined using the fluorescent dye $DiSC_3(5)$ following exposure of *S. aureus* SH1000 to 4× MIC of compound for 10 min.

PMBN enhanced the activities of both compounds (Table 4), suggesting that their uptake across the intact outer membrane is poor.

Biosensors that contain promoter-reporter constructs which are induced by conditions of antibiotic-induced stress can be used for whole-cell-based screening and characterization of inhibitors (13). Based upon a reference compendium of antibiotic-triggered microarray experiments, promoters which are induced in response to treatment of *Bacillus subtilis* with antibiotics of similar modes of action have been discovered and manipulated to genetically engineer five promoter-luciferase reporter fusion strains (13). These biosensor strains (Table 3) signal the presence of inhibitors of fatty acid (*fabHB* promoter), DNA (*yorB*), cell envelope (*ypuA*), RNA (*yvgS*), and protein (*yheI*) biosynthesis and therefore represent an excellent tool for screening of antibacterial agents and potential identification of their mechanisms of action. Induction thresholds for detection of inhibitors by these biosensors have been experimentally defined as 2.5-fold for *yorB*, 2-fold for *yvgS*, *yheI*, and *fabHB*, and 1.7-fold for *ypuA* (13). Rifampin, rifamycin SV, and corallopyronin A consistently caused at least a 2-fold induction in the biosensor (*yvgS*) that signals inhibition of RNA synthesis (data not shown), but compounds 2 and 3 failed to induce the *yvgS* biosensor, displaying induction ratios of <2.0 (Table 4). The lack of antibacterial activity exhibited by compound 1 (see above) prevented its assessment in this assay.

Further studies were conducted with compounds 2 and 3 to try to establish the basis of their antibacterial activities. Compound 2 triggered the biosensor sensitive to DNA synthesis, whereas compound 3 had no effect on any of the biosensors (Table 4). Compound 3 appears to promote membrane damage, since it caused a loss of membrane integrity and membrane potential in *S. aureus* as measured by the *Bac*Light and $DISC_3(5)$ fluorescence assays (5) (Table 4).

In summary, we propose that due to a broad range of identified targets and whole-cell membrane damaging activity, furanyl-rhodanines should be considered nonspecific inhibitors. Consequently, we do not consider them attractive for development as bacterial RNAP inhibitors. We consider this warning to be timely since the suggestion that furanyl-rhodanines are specific inhibitors of bacterial RNAP is being perpetuated in the review literature (2, 8, 14).

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