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New Insights into the Bacterial RNA Polymerase Inhibitor CBR703 as a Starting Point for Optimization as an Anti-Infective Agent

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CBR703 was reported to inhibit bacterial RNA polymerase (RNAP) and biofilm formation, considering it to be a good candidate for further optimization. While synthesized derivatives of CBR703 did not result in more-active RNAP inhibitors, we observed promising antibacterial activities. These again correlated with a significant cytotoxicity toward mammalian cells. Furthermore, we suspect the promising effects on biofilm formation to be artifacts. Consequently, this class of compounds can be considered unattractive as antibacterial agents.

Bacterial RNA polymerase (RNAP) is essential for bacterial growth and survival and is thus an attractive target for drug development (1, 2). Along with the recently FDA-approved fidaxomycin (3), the rifamycins, applied as first-line antituberculosis drugs, are the only RNAP inhibitors that are in clinical use (2). However, similarly to other anti-infectives, the use of rifamycins resulted in the occurrence of resistant bacterial strains (1, 4–7), which represents a remarkable threat to public health (8, 9). Consequently, there is a need to focus on novel promising inhibitors. Recently, interesting peptidic and peptidomimetic (10–12) as well as nonpeptidic (13–18) small-molecule RNAP inhibitors have been described. Another example is CBR703 (Fig. 1), whose mechanism of action is reported to be different from that of the rifamycins (19, 20). This compound has been identified in a high-throughput screening searching for small-molecule inhibitors of RNAP (19). Two more-potent analogs in that report reveal the potential of optimizing CBR703 by structural enlargement. Furthermore, pursuing the hypothesis that RNAP is of particular importance for bacterial survival in biofilms, Villain-Guillot et al. showed CBR703 to significantly reduce *Staphylococcus epidermidis* biofilm mass (21). We therefore considered CBR703 to be a promising starting point for drug development. Consequently, we focused on CBR703 to perform systematic modifications on its core structure, aiming to obtain a more appropriate starting point for further structural optimization.

Detailed information concerning the materials and methods used in synthesis and biology can be found in the supplemental material.

In total, 30 final compounds and 24 intermediates were obtained and tested for *Escherichia coli* RNAP inhibition and their ability to inhibit the growth of *E. coli* TolC (see Table S1 to S3 in the supplemental material). According to their structures, the synthesized derivatives can be divided into three groups with modifications in part A, B, or C (Fig. 1). Compounds 1 to 25 (see Scheme S1 in the supplemental material) with introduction of substituents into the aromatic moieties (part A or B) were prepared by condensation of an intermediate amide with hydroxylamine (22, 23). In order to ensure an appropriate coverage of lipophilic and electronic properties, the substituents were chosen rationally from all quadrants of a Craig plot (e.g., Hansch-Fujita π versus σ constant) (24). The results (see Table S1) showed that compounds 1 to 25 display decreased RNAP inhibition compared to CBR703, with the exception of two compounds (18 and 19) with similar activities (50% inhibitory concentrations [IC_{50}s] in the range of 20 μM). As previously reported (19), there were two more-potent CBR703 analogs with a larger size, one of which was optimized by replacing the linker amidoxime with a pyrazole system. To investigate this structural modification, compounds 26 to 30 with a different linking part (part C) have been synthesized (see Table S2). Remarkably, in our case, replacement of the amidoxime moiety by other functional groups, including N-heterocycles, led to a decrease in or complete loss of activity. Additionally, all amide intermediates turned out to be inactive against RNAP (see Table S3). Surprisingly, 11 compounds, including intermediates with little or even no RNAP inhibition, showed stronger antibacterial potency in *E. coli* TolC than CBR703. Compound 3a with a MIC of 2 μg/ml was even more potent than rifampin. The fact that no correlation between RNAP inhibition and antibacterial activity (see Table S1 to S3) could be observed led us to the conclusion that additional mechanisms besides RNAP inhibition must be responsible for the antibacterial activity.

To obtain further information about the antibacterial profiles, four compounds (Fig. 1) were selected based on the results of the previous experiments (see Table S1 to S3 in the supplemental material) and compared with reference compounds. In a first step, the effects of these compounds on the growth of *E. coli* K-12, *Pseudomonas aeruginosa* PAO1, *Bacillus subtilis*, and *Staphylococcus aureus* were investigated (Table 1). Notably, compounds 7 (the best compound against *E. coli* TolC bearing an amidoxime group) and 19 (the most active RNAP inhibitor) showed only moderate activity against *B. subtilis*. Compound 3a (the most active compound against *E. coli* TolC) exhibited rather potent activities against *B. subtilis* and *S. aureus*. For compound 26 (the only com-

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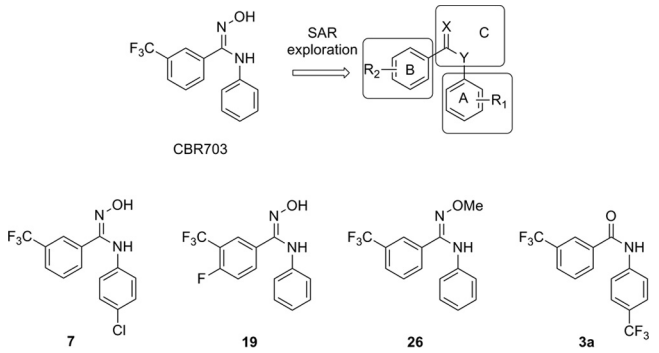

FIG 1 CBR703 and the most potent compounds in different classes: compound 7, best compound against *E. coli* TolC bearing an amidoxime group; compound 19, most RNAP-inhibitory derivative; compound 26, the only RNAP inhibitor after replacement of the amidoxime linker; compound 3a, most active against *E. coli* TolC. SAR, structure activity relationship.

pound with RNAP inhibition after replacement of the amidoxime linker), we observed no detectable activities against Gram-positive species. None of the compounds inhibited the growth of Gram-negative strains K-12 and PAO1. In addition, the toxicity of the inhibitors toward mammalian cells was tested using the human embryonic kidney 293 (HEK293) cell line. Interestingly, the most active compound in the MIC experiment, compound 3a, showed significant cytotoxicity and the other tested compounds were also at least moderately toxic (Table 2). As it is known that lipophilic compounds bind to serum proteins, which were also present in our MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay as a component of fetal calf serum (FCS), we added the same amount of FCS (10%) to the bacterial growth medium and performed the MIC determinations in *E. coli* TolC. Surprisingly, the antibacterial activity of the tested compounds was abolished or drastically reduced (see Table S4). This finding led to the assumption that the cytotoxicities of our compounds are even more pronounced in the absence of serum.

As it had been shown that CBR703 efficiently eradicated biofilm-embedded bacteria (21), we considered that this effect could be due to Fe(III) chelation (25, 26). The fact that the amidoxime moiety plays a prominent role in the activity in our compounds and the well-known property of amidoxime functional groups to form Fe(III) complexes gave rise to the presumption that the amidoximes display their antibacterial effect due to such a complexation (27, 28). Consequently, we examined this hypothesis. First, the ability of CBR703 to form Fe(III) complexes was confirmed by a color change reaction (29). After addition of a CBR703 solution, the brown red $FeCl_3$ solution turned to blue whereas this change was not observed after addition of compound 26 (see Table S5 in the supplemental material). In a following step, the complex stability constants were determined by potentiometric titration. Thereby, it was uncovered that formation of $Fe(OH)_3$ was observed even under acidic conditions (pH = 4). This means that, under physiological conditions, CBR703 cannot form stable Fe(III) complexes. These results were supported by biological tests which were performed in parallel. Indeed, addition of Fe(III) had an effect on the anti-TolC activity of the positive control deferoxamine mesylate (DFO)—a known iron chelator with antibacterial activity (30)—but not on that of CBR703, leading to our conclusion that the antibacterial effects of CBR703 are not attributable to iron complexation (see Fig. S1). Interestingly, each of the three most antibacterial compounds (compounds 3a, 10a, and 21a) possesses two strong electron-withdrawing (leading to a polarity decrease) and highly lipophilic CF_3 groups which might be the reason for their antibacterial potency. Such properties could facilitate cell penetration and could furthermore result in nonspecific inhibition of a variety of other enzymes.

During the determination of MIC values, we found that CBR703 showed slight precipitation at 100 μg/ml whereas its MIC was determined to be 100 μg/ml in the literature (21). Beyond that, a significant effect on *Staphylococcus epidermidis* biofilm was reported at concentrations between 100 and 400 μg/ml. At these concentrations, we observed strong and concentration-dependent precipitation of CBR703 and selected derivatives in Mueller-

TABLE 2 Investigation of cytotoxicity in HEK293 cells[a]

Compound	LD_{50} at 24 h (μM)	LD_{50} at 72 h (μM)
CBR703	58	52
7	43	40
19	34	33
26	25	38
3a	15	13
Rifampin	>100	81
Doxorubicin	5	0.3

[a] The most potent antibacterial compound, compound 3a, was also the most toxic one. Rifampin, negative control; doxorubicin, positive control; LD_{50}, 50% lethal dose.

TABLE 1 RNAP inhibition and antibacterial profile of selected compounds[a]

Compound	% inhibition of *E. coli* RNAP (at 50 μM) or IC_{50} value	MIC (μg/ml)[b]				
		Gram-negative bacteria			Gram-positive bacteria	
		E. coli TolC	*E. coli* K-12	*P. aeruginosa* PAO1	*B. subtilis*	*S. aureus*
CBR703	18 μM[c]	14	>25	>25	>25	>25
7	35	9	>25	>25	23	>25
19	19 μM[c]	21	>50	>50	43	>50
26	29	24	>25	>25	>25	>25
3a	n.i	2	>25	>25	5	11
Rifampin	24 nM[c]	6	7	13	5	0.02

[a] No correlation between RNAP inhibition and antibacterial activities was observed, suggesting that the antibacterial activity was due to a mechanism other than RNAP inhibition. The standard deviations (SD) in these experiments were <25% (in most cases, <15%). n.i, no inhibition (<10% inhibition).
[b] >25 and >50, MIC determinations were limited due to insufficient solubility of the compound.
[c] IC_{50} value.

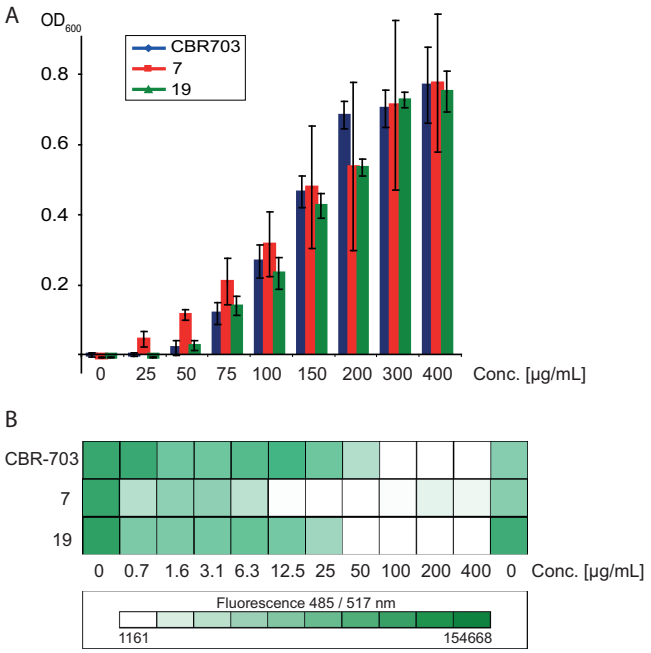

FIG 2 Correlation between precipitation and biofilm mass. (A) Concentration-dependent precipitation of CBR703 and compounds 7 and 19 in MHB. (B) Quantification of the washed biofilm mass. A complete biofilm reduction can be observed only at concentrations at which precipitates have formed. The most prominent effect can be observed for compound 7.

Hinton broth (MHB) (Fig. 2A; see also Fig. S2 in the supplemental material), the medium used in the literature (21). Nevertheless, we evaluated all compounds on *S. aureus* biofilms with concentrations in a soluble range but without observing an effect. At higher concentrations (100 to 400 μg/ml), CBR703 and its derivatives (e.g., compounds 7 and 19) showed a clear reduction in biofilm formation (Fig. 2B), indicating a correlation between antibiofilm activity and precipitation.

In this work, we designed and synthesized derivatives of CBR703 as a follow-up to a published paper (19), aiming to optimize their promising biological effects by modifying the core structure. However, no compound showed enhanced RNAP inhibition. Nevertheless, in some cases we observed promising antibacterial activities. These again turned out to correlate with a significant cytotoxicity toward HEK293 cells. Furthermore, the reported effects on biofilm formation, which were among the main reasons for choosing CBR703 as a starting point, were suspected to be artifacts due to compound precipitation. This finding should be a reminder to the scientific community to be cautious with published data, as they could include such artifacts (31). Consequently, we rank this class of compounds as unattractive for development as antibacterial agents.

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