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New insights into the bacterial RNA polymerase inhibitor CBR703 as a starting point for optimization as an anti-infective agent

Weixing Zhu,[1] Jörg Haupenthal,[1] Matthias Groh,[1] Michelle Fountain[3] and Rolf W. Hartmann[1, 2]*

[1] *Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Department of Drug Design and Optimization and* [2] *Pharmaceutical and Medicinal Chemistry, Saarland University, Campus C2.3, D-66123 Saarbrücken, Germany.* [3] *Helmholtz Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany.*

** Corresponding author. Fax: (+49) 681 302 70308 E-mail: rolf.hartmann@helmholtz-hzi.de*

ABSTRACT

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 towards 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 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, similar 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 need to focus on novel promising inhibitors. Recently, interesting peptidic and peptidomimetic (10 – 12) as well as non peptidic small molecule RNAP inhibitors (13 – 18) 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 this 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 the *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 *E. coli* RNAP inhibition and their ability to inhibit the growth of *E. coli TolC* (Supplemental Material Table S1 – 3). According to their structures, the synthesized derivatives can be divided into three groups with modifications in A, B or C (Fig. 1). **1** – **25** (Scheme S1) with introduction of substituents into the aromatic moieties (A or B) were prepared by condensation of an intermediate amide with hydroxylamine (22, 23). In order to assure an appropriate coverage of lipophilic and electronical properties, the substituents were chosen rationally from all quadrants of a Craig Plot (e.g. Hansch-Fujita π versus σ constant) (24). The results (Table S1) showed that compounds **1** – **25** display a decreased RNAP inhibition compared to **CBR703** with the exception of two compounds (**18** and **19**) with similar activity (IC_{50}s in the range of 20 µM). As reported (19), there were two more potent **CBR703** analogs with larger size, one of which was optimized by replacing the linker amidoxime with a pyrazole system. To investigate this structural modification, **26** – **30** with a different linking part (C) have been synthesized (Table S2).

Remarkably, in our case, replacement of the amidoxime moiety by other functional groups including N-heterocycles led to a decrease or complete loss of activity. Additionally, all amide intermediates turned out to be inactive against RNAP (Table S3). Surprisingly, 11 compounds including intermediates with little or even no RNAP inhibition showed a stronger antibacterial potency in *E.coli TolC* than **CBR703**. **3a** with a MIC of 2 µg/mL was even more potent than rifampicin. The fact that no correlation between RNAP inhibition and antibacterial activity (Table S1 – 3) 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 (Table S1 – 3), and compared with reference compounds. In a first step, the effects of these compounds on the growth of *E. coli K12*, *Pseudomonas aeruginosa PAO1* (*PAO1*), *Bacillus subtilis* (*B. subtilis*) and *Staphylococcus aureus* (*S. aureus*) were investigated (Table 1). Notably, **7** (best compound against *E.coli TolC* bearing an amidoxime group) and **19** (most active RNAP inhibitor) only showed moderate activity against *B. subtilis.* **3a** (most active against *E.coli TolC*) exhibited rather potent activities against *B. subtilis* and *S. aureus.* For **26** (the only compound with RNAP inhibition after replacement of the amidoxime linker), we observed no detectable activities against Gram-positives. None of the compounds inhibited the growth of the Gram-negative strains *K12* and *PAO1*. In addition, the toxicity of the inhibitors towards mammalian cells was tested using the Human Embryonic Kidney (HEK) 293 cell line. Interestingly, the most active compound **3a** in the MIC experiment showed a significant cytotoxicity and also the other tested compounds were at least moderately toxic (Table 2). As it is known that lipophilic compounds bind to serum proteins, which are also present in our MTT 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 (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 for the activity in our compounds and the well-known property of amidoxime functional groups to complex Fe(III) gave rise to the presumption that the amidoximes display their antibacterial effect due to such a complexation (27, 28). Consequently,

we examined this hypothesis. Firstly, 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 while this change was not observed after addition of **26** (Table S5). In a following step, the complex stability constants were determined by potentiometric titration. Thereby it was uncovered that only under acidic conditions (pH = 4) formation of $Fe(OH)_3$ was observed. 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 **CBR703**, leading to our conclusion that the antibacterial effects of **CBR703** are not attributed to iron complexation (Fig. S1). Interestingly, each of the three most antibacterial compounds (**3a**, **10a**, **21a**) possesses two strong electron withdrawing- (leads to polarity decrease) and highly lipophilic CF_3 groups which might be the reason for their antibacterial potency. Such properties could facilitate cell penetration and furthermore result in non-specific inhibition of a variety of other enzymes.

During the determination of MIC values we found that **CBR703** showed a slight precipitation at 100 µg/mL while in the literature its MIC was determined to be 100 µg/mL (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 a strong and concentration-dependent precipitation of **CBR703** and selected derivatives in Mueller Hinton Broth (MHB) (Fig. 2A and Fig. S2), the medium used in 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 – 400 µg/mL) **CBR703** and its derivatives (e.g. **7** and **19**) showed a clear reduction in biofilm formation (Fig. 2B), indicating a correlation between anti-biofilm activity and precipitation.

In this work we designed and synthesized derivatives of **CBR703** as follow up work to a published paper (19) aiming to optimize their promising biological effects by modifying the core structure. However, no compound showed an enhanced RNAP inhibition. Nevertheless, in some cases we observed promising antibacterial activities. These again turned out to correlate with a significant cytotoxicity towards HEK 293 cells. Furthermore, the reported effects on biofilm formation, which were one of 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 be artifacts (31). Consequently, we rank this class of compounds as unattractive for the development as antibacterial agents.

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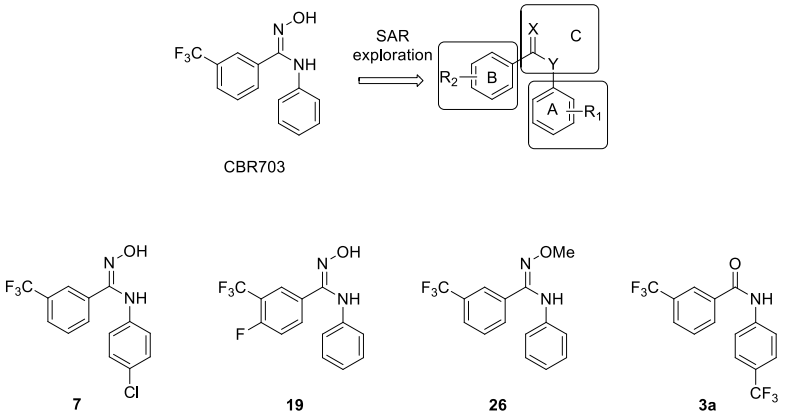

Fig. 1 CBR703 and the most potent compounds in different classes: **7**, best compound against *E.coli TolC* bearing an amidoxime group; **19**, most RNAP inhibitory derivative; **26**, the only RNAP inhibitor after replacement of the amidoxime linker; **3a**, most active against *E. coli TolC*.

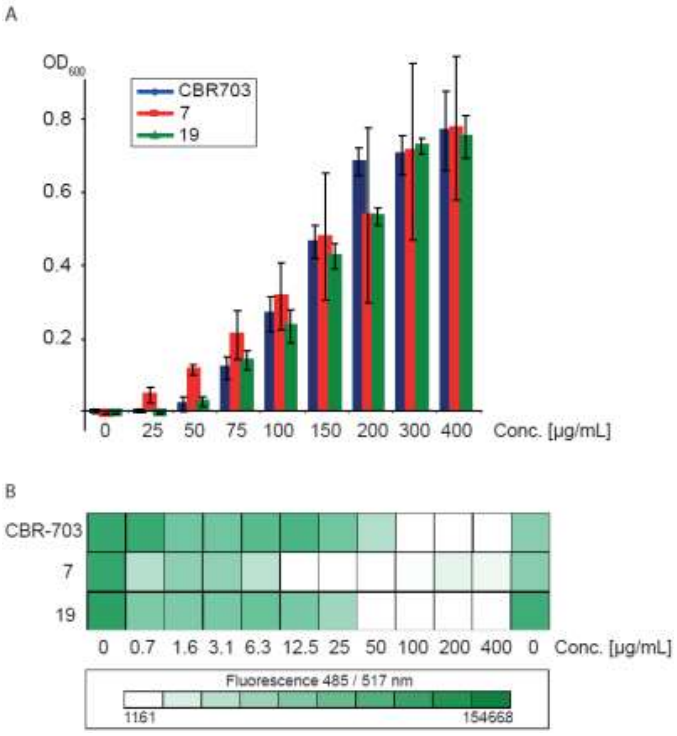

Fig. 2 Correlation between precipitation and biofilm mass. (A) Concentration dependent precipitation of **CBR703**, **7** and **19** in MHB. (B) Quantification of the washed biofilm mass. A complete biofilm reduction can only be observed at concentrations at which precipitates have formed. The most prominent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

256 **TABLE 1** RNAP inhibition and antibacterial profile of selected compounds

compound	% Inhibition of *E. coli* RNAP (at 50 µM)	MIC (µg/mL)[a]				
		Gram negative			Gram positive	
		E. coli TolC	*E. coli K12*	*PAO1*	*B. subtilis*	*S. aureus*
CBR703	18 µM[b]	14	>25	>25	>25	>25
7	35	9	>25	>25	23	>25
19	19 µM[b]	21	>50	>50	43	>50
26	29	24	>25	>25	>25	>25
3a	n.i	2	>25	>25	5	11
Rifampicin	24 nM[b]	6	7	13	5	0.02

257 No correlation between RNAP inhibition and antibacterial activities was observed, suggesting
258 that the antibacterial activity was due to a mechanism other than RNAP inhibition. The SD in
259 these experiments was < 25 % (most cases: < 15 %). [a] >: MIC-determination was limited due to
260 insufficient solubility of the compound; [b]: IC_{50} value.

261

262 **TABLE 2** Investigation of cytotoxicity in HEK 293 cells.

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

263 The most potent antibacterial compound **3a** was also the most toxic one. Rifampicin: negative
264 control; Doxorubicin: positive control; LD: lethal dose.

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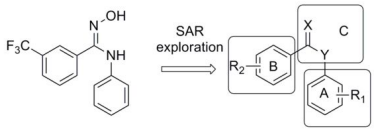

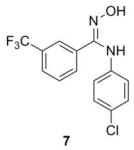
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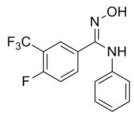
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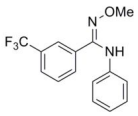
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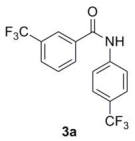
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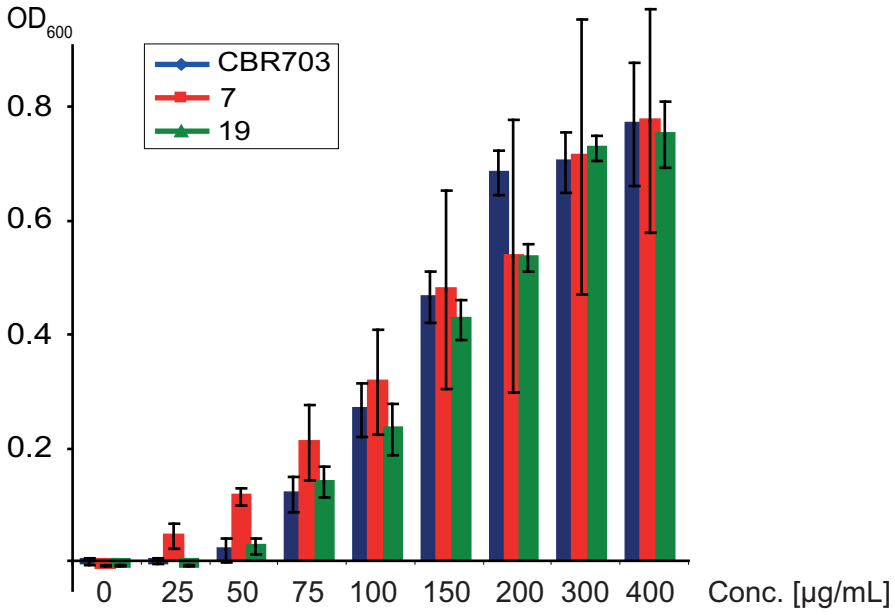

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