First-In-Class Inhibitor of Ribosomal RNA Synthesis with Antimicrobial Activity against *Staphylococcus Aureus*

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Supporting Information

ABSTRACT: We report discovery of the first bacterial ribosomal RNA (rRNA) synthesis inhibitor that has specific antimicrobial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA). A pharmacophore model was constructed based on the protein-protein interaction between essential bacterial rRNA transcription factors NusB and NusE, and employed for an *in silico* screen to identify potential leads. One compound, (*E*)-2-[[(3-ethynylphenyl)imino]methyl]-4-nitrophenol (MC4), demonstrated antimicrobial activity against a panel of *S. aureus* strains, including MRSA, without significant toxicity to mammalian cells. MC4 resulted in reduction of the rRNA level in bacteria, and the target specificity of MC4 was confirmed at both cellular and molecular levels. Results obtained from this work validated the bacterial rRNA transcription machinery as a novel antimicrobial target. This approach may be extended to other factors in rRNA transcription, and MC4 could be applied as a chemical probe to dissect the relationship between MRSA infection, growth rate and rRNA synthesis, in addition to its therapeutic potential.

Infections caused by the Gram-positive pathogen Methicillin-resistant *Staphylococcus aureus* (MRSA) have become one of the most serious public health concerns worldwide.[1] The pharmaceutical arsenal available to control MRSA is limited to vancomycin, daptomycin, and linezolid,[2] for which resistance has already evolved.[3] Therefore, there is an urgent need to validate new antibiotic target, in order to develop novel antimicrobials with potent and specific activities to combat MRSA-associated infections.

In bacteria cells, rRNA comprises up to 80% of total RNA content and transcription of rRNA has been shown to positively correlate with bacterial growth rate to meet the demand for protein synthesis.[4] Although rRNA synthesis is one of the most fundamental requirements for living cells, there is a noticeable discrepancy in this process. In eukaryotic cells the ribosomal genes are transcribed by different types of RNA polymerases, namely RNA Pol I and Pol III.[5] On the other hand, there is only one RNA polymerase in bacteria, which is associated with a number of elongation factors to form so-called "rRNA antitermination complexes", which ensure efficient transcription of the rRNA genes.[6]

NusB and NusE (also known as the ribosomal protein S10 of the 30S ribosomal subunit) are highly conserved essential small transcription factors involved in the formation of rRNA antitermination complexes.[7] The protein-protein interaction between NusB-NusE represents the first regulatory step in rRNA transcription antitermination complex assembly.[8] Once a NusB-NusE heterodimer forms, it interacts with a region of the rRNA leader sequence called boxA.[9] Following binding of the NusB-NusE-boxA complex to RNA polymerase, other factors (such as NusA, NusG and others) will be recruited, among which only NusG has an eukaryotic homolog.[10]

Since NusB/E is essential for bacterial cell viability,[11] we hypothesize that disruption of NusB-NusE heterodimer formation by small molecules will result in reduced rRNA synthesis and bacterial cell proliferation. Previously, by rational design and pharmacophore based virtual screening, we identified small chemical molecule inhibitors with antimicrobial activities, targeting the interaction between bacterial RNA polymerase and the essential housekeeping transcription initiation factor σ.[12] Using a similar approach, we have identified an inhibitor against bacterial rRNA synthesis that has antimicrobial activities against *S. aureus* strains including MRSA.

A bacterial rRNA transcription complex was modelled based on the crystal structure of RNA polymerase elongation complex[13] with a suite of Nus transcription factors NusA, NusB, NusE, and NusG (Figure 1A). NusG binds to the central cleft of RNA polymerase via its N-terminal domain,[14] and its C-terminal domain interacts with NusE,[15] which anchors the NusB-NusE-boxA subcomplex to the downstream face of RNA polymerase (Figure 1A). NusA binds to RNA polymerase near the RNA exit channel (Figure 1A),[16] consistent with its binding to rRNA just downstream of the boxA sequence.[17] The interaction between RNA polymerase-Nus factors and rRNA results in a constrained loop, facilitating rapid and proper folding of the emerging transcript, which is consistent with

previous biochemical observations that the RNA polymerase-Nus factor complex would serve chaperone roles in rRNA synthesis.[18] This assembly also has possible roles in preventing the termination factor Rho from accessing the rRNA transcript,[19] ensuring complete transcription of the relatively large rRNA operons during rapid bacteria cell growth. Recently reported structural information on phage protein λN-dependent transcription antitermination complex also displayed similarities to our rRNA transcription complex model.[20]

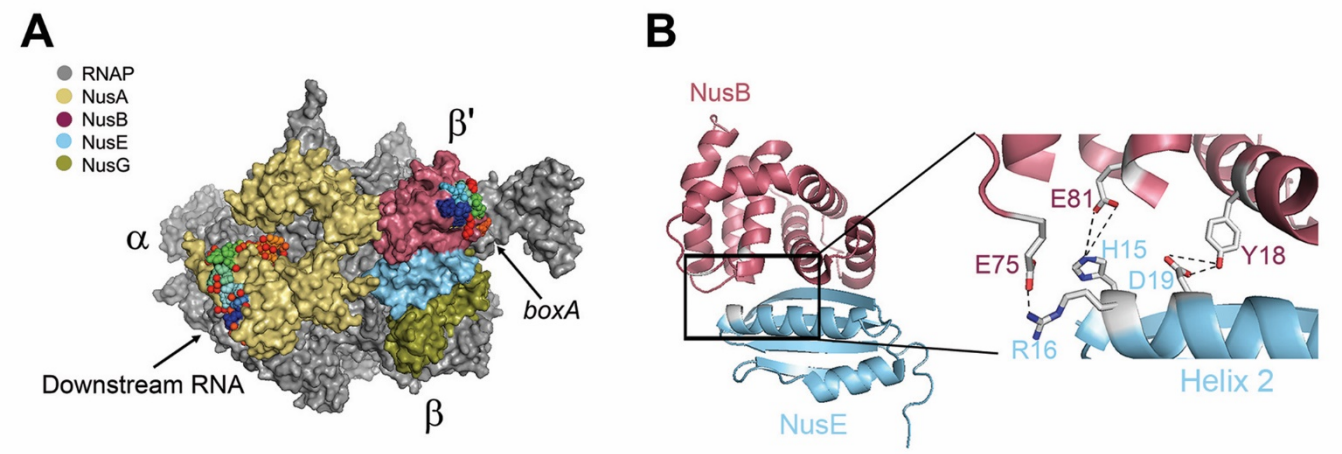

Figure 1. (A) A model of bacterial rRNA transcription complex. (B) NusB-NusE interface.

NusB and NusE present a low interfacial contact area (Figure 1B),[21] and both are highly conserved proteins across prokaryotes (Figure S1). Examination of the published crystal structures of the *Escherichia coli* NusB-NusE heterodimer (PDB: 3D3B) reveals that NusE contains only 18% α-helix, and binds with NusB mainly via interactions with Helix 2 (Figure 1B).[22] The hydrogen bonding interactions occur between NusB E81-NusE H15, NusB Y18-NusE D19 and NusB E75-NusE R16 (Figure 1B expanded view; E. coli amino acid residue numbering), which are highly conserved across prokaryotes (Figure S1 arrows). Additionally, an NMR study of the *Aquifex aeolicus* NusB-NusE interaction also confirmed similar interactions exist in solution.[23]

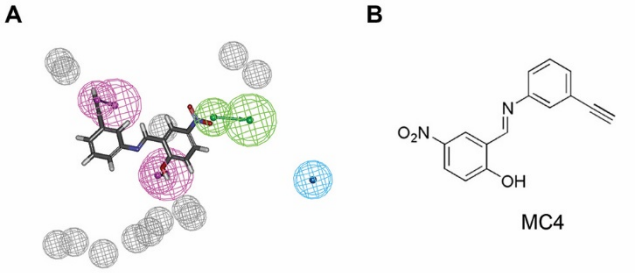

Figure 2. (A) Pharmacophore model with MC4 docked in. (B) Chemical structure of (*E*)-2-[[(3-ethynylphenyl)imino]methyl]-4-nitrophenol (MC4).

The structural information of the NusB-NusE heterodimer co-crystal (PDB: 3D3B)[22] was used to develop a pharmacophore model (Figure 2A). The pharmacophore model comprised two hydrogen donors (pink), one acceptor (green) to mimic the major hydrogen bonds between NusB and NusE as mentioned above, and one conserved hydrophobic interaction (cyan; Figure 2A) between *E. coli* residues NusB L22 and NusE V26. In addition to the interactions, a series of exclusion zones (grey) were added to minimize steric clashes within the shallow pocket that forms the binding site on NusB. The final pharmacophore model was then created using Biovia DS4.5 to map on all the features required.[24] As the pharmacophore model was designed based on the properties of the important amino acid residues on NusE protein responsible for binding to NusB, theoretically, the ideal small molecules capable of docking into this pharmacophore model should be able to bind to NusB, and demonstrate inhibitory activity against the NusB-NusE interaction accordingly.

Based on the pharmacophore model, an *in silico* screen was performed using a virtual compound library constructed by combining the mini-Maybridge library and the Enamine antibacterial library.[25] The top 50-hits from the initial virtual screen were re-mapped against the pharmacophore model and the energy minimized conformations of compounds visually inspected. The compounds displayed poor fitting into the pharmacophore were removed. As a result, we initially short-listed 7 compounds (Figure S2) for wet-laboratory testing.

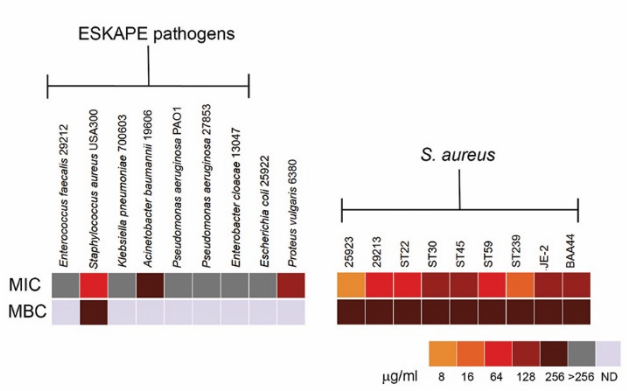

Figure 3. The antimicrobial activity of MC4 against selected pathogenic bacteria. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration. ND, not determined.

We first screened the antimicrobial activity of the 7 compounds against Community-Acquired MRSA strain USA300. Of the analogues evaluated, MC4 (Figure 2B) was found to demonstrate growth inhibition effects with a minimum inhibitory concentration (MIC) of 64 μg/ml (Figure 3). With a molecular weight of 266.3,

MC4 has only been reported of use to form metal complex dye in optical layers for optical data recording.[26] We then tested the antimicrobial activities of MC4 against a panel of representative strains of pathogens, MC4 demonstrated preferred antimicrobial activity against *S. aureus* strains including MRSA over other pathogens tested, with a MIC as low as 8 µg/ml against control strain *S. aureus* 25923 and 16 µg/ml against healthcare-acquired MRSA ST239 (Figure 3). Additionally, MC4 did not show significant cytotoxicity against mammalian cell lines compared to 5-fluorouracil (Table S1).

We analyzed the DNA, rRNA and protein productions due to MC4 treatment in *S. aureus* ATCC 25923 cells during exponential growth. MC4, rifampicin and oxacillin were added at ¼ MIC level, which did not interfere with growth rate of *S. aureus* ATCC 25923 cells. DNA and total protein were extracted and analyzed for the absolute weight. Total RNA was extracted and subject to Agilent 2100 analysis, and the level of major rRNA (the sum of 16S + 23S rRNA) as percentage of total RNA was compared across each treatment group. As shown in figure 4, none of the treatment affected DNA duplication, as previewed by mode of action. In the control cells, the level of major rRNA was around 78% of total RNA (Figure 4).[4] Rifampicin, as a positive control, resulted in reduction of rRNA level consistent with previous observations (Figure 4).[27] Even at ¼ MIC, MC4 showed significant reduction in the rRNA level, lower than rifampicin treated cells (Figure 4). Furthermore, MC4 treatment led to significant reduction of protein level, while rifampicin didn't show this effect, probably because the inhibition of rRNA synthesis by MC4 specifically decreased ribosome production, affecting the ability of protein synthesis. Whereas oxacillin treated cells displayed slightly higher rRNA and protein production levels compared to control cells, this result may be caused by use of sub-inhibitory concentration, which can significantly induce exotoxin gene expression.[28]

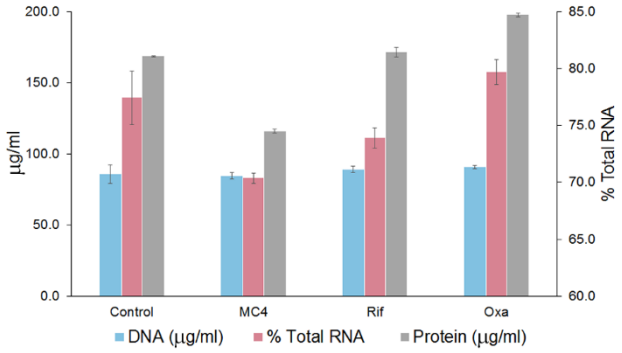

Figure 4. The effects of MC4, Rifampicin (Rif), Oxacillin (Oxa) at ¼ minimum inhibitory concentrations (MICs) on DNA, rRNA (16S + 23S) and protein productions in *S. aureus* 25923 cells.

Finally, we intended to establish MC4's mode-of-action at the molecular level using biochemical and biophysical assays with purified proteins from *B. subtilis* as the NusB and NusE proteins are highly conserved in bacteria. An ELISA-based inhibitory assay was performed as described previously to assess the in vitro inhibition of NusB-NusE heterodimer formation by MC4.[24] NusB was used to coat the 96-well ELISA plate and GST-tagged NusE used as the probe. MC4 showed positive inhibition of the NusB-NusE interaction with IC_{50} ~34.7±0.13 µM. By further testing a series of MC4 analogues, we found that three functional groups on the molecule targeting interactions between NusB E81-NusE H15, NusB Y18-NusE D19 and NusB E75-NusE R16 were compulsory for inhibiting NusB-NusE binding, which confirmed our pharmacophore model, and demonstrated that imine and *p*-nitrophenol of MC4 didn't contribute to the activity. We have also quantified the interaction between MC4 and NusB. A previous report demonstrated that NusB bound to NusE in a 1:1 ratio with a Kd ~1 µM as determined by isothermal titration calorimetry (ITC).[21] In similar experiments using ITC, we found MC4 bound specifically to NusB (Figure S3A) with a one-site binding mode (N = 0.988 ± 0.076), with a K_d = 1.45 ± 0.55 µM, ΔH = -7141 ± 939.8 cal/mol, and ΔS = -1.81 cal/mol/deg. Binding of MC4 to NusE could not be detected in similar experiments (not shown), or between MC4 and NusB variants (Y18A, D76A, D81A) with the three amino acid residues responsible for NusE binding altered to alanine (Figure S3 B-D). These results together suggest the inhibition of NusB-NusE heterodimer formation is achieved via specific interaction between MC4 and NusB as designed. Further experiments will be carried out to resolve the structure of NusB in complex with MC4 for target validation, as well as structure-based lead optimization.

The potential impact of untreatable antibiotic resistant infections on society is profound and there is an urgent need to identify new drug targets.[29] Traditionally, the bacterial ribosome itself (both 30S and 50S subunits) has been one of the most commonly exploited target for antibiotics inhibiting protein synthesis.[30] Recent drug discovery research had validated inhibition of rescuing stalled ribosomes at the end of mRNAs resulted in antibacterial activity.[31] Given ribosome is positively related with fast growth/proliferation and the large difference between eukaryotic and prokaryotic rRNA transcription machinery, it is tempting to hypothesize inhibition of rRNA synthesis would be expected to have a major impact on cell growth/viability. This hypothesis is strengthened by recent findings showing that many anti-cancer drugs inhibit rRNA synthesis or maturation.[32]

In this work we have used a pharmacophore-based *in silico* screen followed by biological confirmation to identify a potential new antibiotic lead. We have targeted an essential interaction between transcription factors NusB and NusE that is required for the formation of highly processive complexes used for the synthesis of rRNA within bacterial cells. One of the shortlisted compounds (MC4) showed specific activity against *S. aureus* strains including MRSA without significant toxicity to mammalian cell lines. It is, to our knowledge, the first compound designed to target bacterial rRNA synthesis that has antimicrobial activities. The detailed effect of MC4 in rRNA transcription/processing, ribosome biogenesis, as well as *S. aureus* virulence is currently under investigation. Although MC4 has been shown to specifically inhibit NusB-NusE interaction at both molecular and cellular levels, any potential off-target effect on bacterial cells remains to be elucidated. Since NusB and NusE are highly conserved in bacteria, the reason why MC4 has preferred antimicrobial activity against *S. aureus* over other pathogens needs to be further investigated.

Additionally, we validated an essential protein-protein interaction between transcription factors in the bacterial rRNA synthesis machinery as a novel antimicrobial target. Other important protein-protein interactions involved in bacterial rRNA transcription, e.g. between NusE-NusG, NusE-RNA polymerase might also have the potential as novel antimicrobial targets.[33] This work paves the way for the structural optimization of MC4, and potentially other compounds from more comprehensive screens, for development as prospective new antimicrobial lead molecules targeting bacterial rRNA synthesis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details and additional data (PDF)

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Author Contributions

X.Y., and C.M. conceived the idea. X.Y., M.J.L, A.C.M.Y., and C.M. performed experiments. X.Y., M.J.L, P.J.L., P.K.S.C, M.I., and C.M. analyzed data. X.Y., and C.M. wrote the paper.

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Notes

The authors declare no competing financial interests.

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