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DRUG DISCOVERY AND RESISTANCE

Rifamycin inhibition of WT and Rif-resistant *Mycobacterium tuberculosis* and *Escherichia coli* RNA polymerases *in vitro*

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SUMMARY

Mycobacterium tuberculosis (MTB) infects over 9 million people globally and claims approximately 2 million lives annually. Rifampin (Rif) is one of the first-line anti-tuberculosis drugs that inhibits transcription by binding to the β subunit (encoded by the *rpoB* gene) of the prokaryotic RNA polymerase (RNAP). A highly conserved 81 base pair core region among the β subunit of prokaryotes harbors most of the point mutations leading to rifamycin-resistant (RifR) mutations, where the majority of the clinically relevant MTB RifR mutations result from amino acid substitutions of one of the following three amino acids: βAsp435, βHis445, and βSer450 (MTB numbering). In this study, to determine the direct effect of rifamycins on the MTB RNAP, co-overexpression vectors were constructed to co-express the core subunits of wild-type and RifR mutants of MTB RNAP. The three aforementioned amino acids were each mutated to the most prevalent substitution found in the MTB clinical isolates (Asp435Val, His445Tyr, Ser450Leu) in the *rpoB* gene via site-directed mutagenesis. After purification via two-step column chromatography, the *in vitro* activity of the wild-type and RifR mutant MTB RNAPs was assessed via rolling circle transcription assay. The apparent IC_{50} values for three key rifamycins (rifampin (Rif), rifabutin (Rbn), and rifaximin (Rfx)) were determined and these results indicate that the mutant RNAPs demonstrate approximately 10^3-fold or greater loss of affinities for rifamycins relative to wild-type MTB RNAP.

Along with the MTB RNAPs, rifamycin inhibition of the *Escherichia coli* RNAP counterparts was also assessed. Previously, it has been reported that Gram-positive bacteria (particularly mycobacteria) are more sensitive to rifamycins than Gram-negative bacteria. Under our experimental conditions, the rifamycin IC_{50}s for wild-type and RifR mutants of MTB and *E. coli* RNAPs (wild-type and corresponding mutants) were very similar; therefore, the difference in sensitivity toward rifamycins does not reside in the RNAP itself. The correlation between the sensitivity of rifamycins and permeability into cells was evaluated using the wild-type *E. coli* strains (TG2 and DH5α) and a mutant *E. coli* strain with efflux pump defects (EC2880, *tolC*$^-$/*imp*$^-$). The MICs were drastically lower in the EC2880 strain, consistent with previous reports that the differential sensitivity of MTB and *E. coli* to rifamycins is not related to the RNAP, but rather has to do with efflux pumps in *E. coli*. Future work will focus on the elucidation of the molecular interaction of these MTB RifR mutants with rifamycins to provide insight to the design of novel rifamycins.

1. Introduction

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (MTB), is currently one of, if not, the biggest global health burden. TB exists in two forms (latent and active) with one-third of the world's population infected by the latent form. In 2009, approximately 9.4 million new cases of TB (1.1 million amongst HIV infected individuals) were reported, along with 1.7 million deaths caused by active TB. Furthermore, the emergence of multi-drug resistant TB (MDR TB) increased to 440,000 cases in 2008.[1] MDR TB strains are resistant to at least two of the primary anti-tuberculosis antibiotics; whereas, extensively-drug resistant TB (XDR TB) strains are resistant to second-line drugs as well.

Rifampin (Rif) is one of the first-line anti-tuberculosis drugs (Figure 1). Rif (a semi-synthetic rifamycin derivative) is a broad-spectrum antibiotic, which is more effective against

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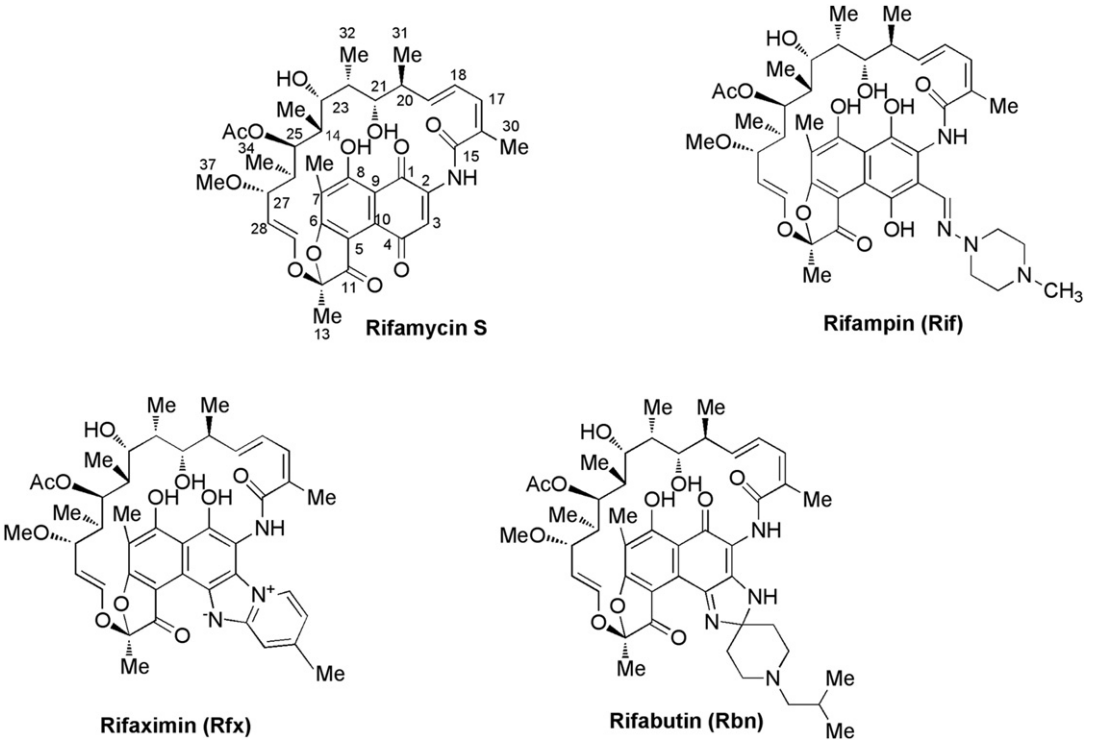

Figure 1. Structures of key rifamycins (typical rifamycin numbering system is shown with rifamycin S). Additional rifamycins mentioned in the paper but not pictured include: rifamcyin SV (the reduced quinone form of rifamycin S) and rifapentine (derivative of rifampin with a cyclopentyl group attached to the piperazine instead of the methyl group).

Gram-positive (particularly mycobacteria) than Gram-negative bacteria.[2] First introduced in the late 1960s, Rif was seen to reduce the duration of TB treatment from 18 to 9 months.[3,4] Rif inhibits the bacterial DNA-dependent RNA polymerase (RNAP), where the catalytic core of the RNAP is composed of the following subunits: α_2, β, β′, and ω. Rif binds to the β subunit (encoded by the *rpoB* gene) and sterically blocks the growing RNA chain during transcription.[3,4] RNAP is a vital enzyme necessary for the production of all of the RNA in eubacteria, hence making RNAP an attractive target for antibiotic development.[5]

However rifamycin-resistant (RifR) mutations arise spontaneously at a frequency of 10^{-8} where approximately 95% of these mutations are within 4 regions (N-terminal cluster and clusters I, II, III) of the β subunit (Figure 2).[3,6] These mutations mainly consist of single amino acid substitutions and a few deletions or insertions of residues.[7,8] All but one of the 15 different residues that have been identified from MTB RifR clinical isolates are mapped to the 81 base pair region of cluster I (highly conserved among prokaryotes).[8,9] From the crystal structure of *Thermus aquaticus* (Taq) core RNAP bound to Rif, the residues that directly interact with Rif (via hydrogen bonding or van der Waals interactions) were determined and the majority of these residues are found in Cluster 1 (Figure 3).[3,6] Among these residues, the individual substitutions of three residues (Asp435 (7.4%), His445 (20%), and Ser450 (42%); MTB numbering) together account for 84% of MTB RifR strains found in clinical isolates. The most abundant amino acids substituted in place of these residues are as follows: 435 Val, 445 Tyr, and 450 Leu, respectively.[8,9]

In general, TB and the problem with resistance have been studied for decades along with rifamycins but *in vitro* data for

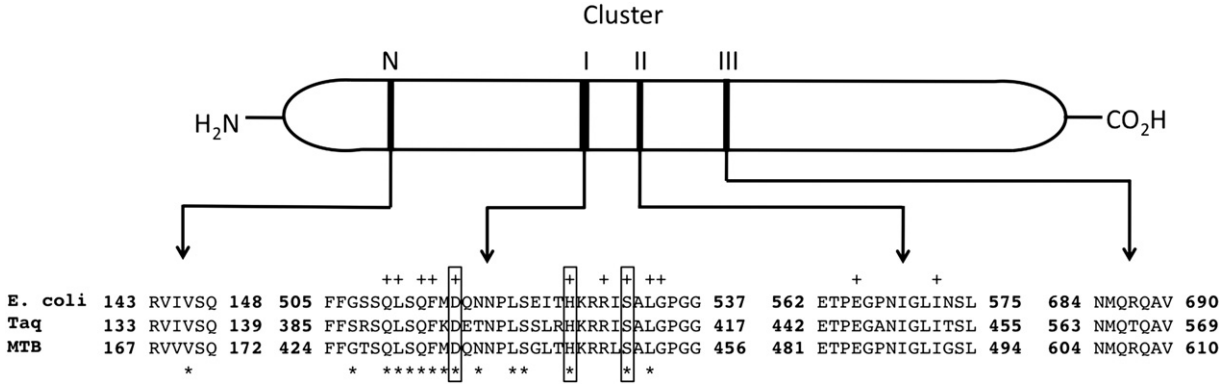

Figure 2. Selected sequences from rifamycin-resistant (RifR) regions of the RNAP β subunits from *E. coli*, *Thermus aquaticus* (Taq), and *Mycobacterium tuberculosis* (MTB) (Cluster N and Clusters I, II, and III). The 12 amino acids of the β subunit that interact directly with rifampin are indicated with + above the *E. coli* sequence. The 15 positions that have been identified in MTB RifR clinical isolates are indicated with * below the MTB amino acid sequence. Substitution of the three amino acids that account for 84% of the RifR clinical isolates are each outlined in a box. (Data from Campbell, 2001).