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DOMAIN 4 SYNTHESIS AND PROCESSING OF MACROMOLECULES

Inhibition of RNA Polymerase by Rifampicin and Rifamycin-Like Molecules

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ABSTRACT RNA polymerases (RNAPs) accomplish the first step of gene expression in all living organisms. However, the sequence divergence between bacterial and human RNAPs makes the bacterial RNAP a promising target for antibiotic development. The most clinically important and extensively studied class of antibiotics known to inhibit bacterial RNAP are the rifamycins. For example, rifamycins are a vital element of the current combination therapy for treatment of tuberculosis. Here, we provide an overview of the history of the discovery of rifamycins, their mechanisms of action, the mechanisms of bacterial resistance against them, and progress in their further development.

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INTRODUCTION

While a handful of natural and synthetic compounds are known to target bacterial RNA polymerases (RNAPs), only two of them have managed to make it into clinical use: rifamycins and lipiarmycin (1). The most clinically important and extensively studied class of antibiotics known to inhibit bacterial RNA polymerase is the rifamycins (RIFs). RIFs are a vital element of the current combination therapy for treatment of tuberculosis (TB), an infection caused by *Mycobacterium tuberculosis* (2). The mechanism of RNAP inhibition by RIFs is believed to be identical for various bacterial RNAPs, and most of the studies have been performed on *Escherichia coli* RNAP as a common model system (3).

HISTORY OF RIFAMYCINS

Ansamycins are a group of naturally derived antibiotics which are named after their characteristic basket-like structures, where an aliphatic ansa-chain (*ansa* = handle in latin) spans an aromatic naphthalenic (e.g., RIF) or benzenic moiety (e.g., geldanamycin) from its nonadjacent positions (4). RIFs, the first antibiotics found to inhibit bacterial RNAPs, were originally isolated from *Amycolatopsis mediterranei* in 1959 (5–7). Rifamycin B (RIF B) was the first of a kind that was stable enough to be purified from a mixture of RIFs (8). Although RIF B has poor antimicrobial activity (likely due to its inability to penetrate the bacterial cell envelope), it is reversibly converted to

RIF O in aqueous oxygenated solutions. RIF O is then hydrolyzed to RIF S, losing a glycolic acid residue. The reduction of RIF S results in RIF SV (Fig. 1) (9, 10). It is believed that the observed activity of RIF B is due at least in part to the transformation into active products (RIF O, S, and SV) (11).

RIF SV was the first ansamycin that found clinical application. RIF SV has potent activity, especially against a spectrum of Gram-positive bacteria and a clinically significant pathogen, *M. tuberculosis*. However, RIF SV has the limitation of oral administration due to low gastrointestinal absorbance (12). Therefore, comprehensive semisynthetic RIF programs aimed at finding a compound with enhanced oral absorption, prolonged existence in blood, and better activity against both Gram-positive and Gram-negative bacteria. Rifampicin (RMP) was the result of preparation and screening of several hundreds of semisynthetic RIFs (13). Since the introduction of RMP, it has remained the first-line treatment for mycobacterial infections, including TB.

RIFAMYCINS IN CLINIC

TB is the ninth leading cause of death and the main cause of death by a single infectious agent worldwide. In 2018, 10 million people fell ill with TB, and about 1.2 million (HIV-negative patients) died because of TB. The current treatment for TB is 6-month combination therapy with RIF, isoniazid, ethambutol, and pyrazinamide. Drug-resistant TB is a serious threat, exacerbated by the fact that as recently as 2018, approximately half a million new

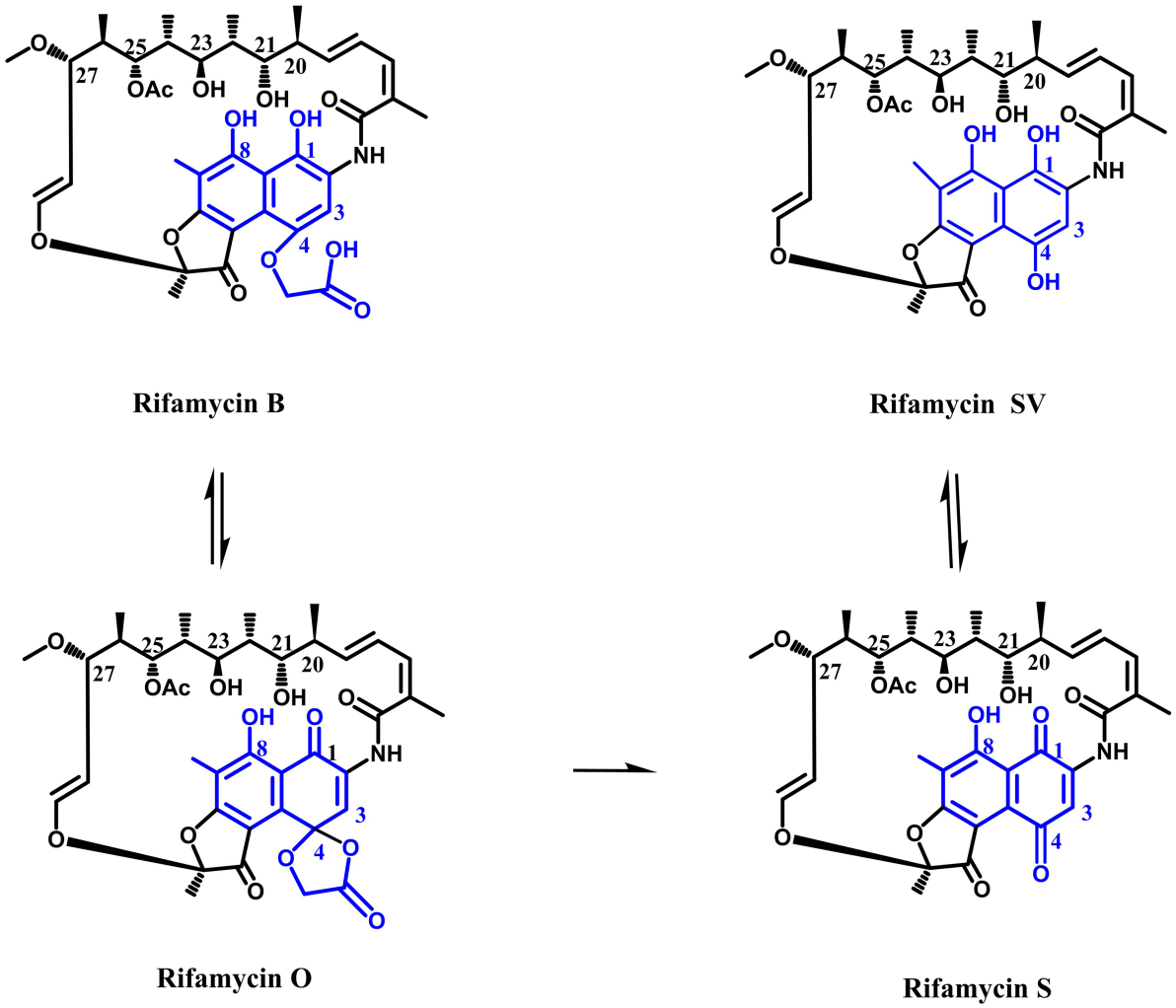


Figure 1 The chemical pathway for conversion of RIF B to RIF SV. The ansa chain and naphthalene moiety of molecules are shown in black and blue, respectively.

cases of RMP-resistant (RMP^r) TB were recorded, 78% of which were multidrug-resistant TB (MDR-TB; resistance to RMP and isoniazid). The RMP^r-TB and MDR-TB require longer treatments, which typically last for 20 months. The role of RIFs as the main first-line anti-TB drug is so critical that the WHO recommended the Xpert MTB/RIF assay for simultaneous detection of TB and RMP resistance (14). RMP is also one of the relatively few anti-TB drugs with sterilizing activity, the capacity to kill the mycobacteria that remain after the initial phase of treatment (e.g., the population of bacteria that undergo sporadic metabolism) (15). The sterilizing activity of RIFs enables further shortening of the treatment if higher doses are tolerated. Higher doses of RMP (up to 35 mg/kg) are in trials to reduce the treatment period for drug-susceptible TB (14). Five different RIFs are currently marketed in various countries: RMP, rifapentine (RPT), and rifabutin (RBT) for the systemic treatment of mycobacterial infections, rifaximin (RXM) only for travelers' diarrhea), and RIF SV (with limited availability) (16).

MECHANISM OF ACTION OF RIFs

The antibacterial activity of RIFs is due to inhibition of DNA-dependent RNA synthesis (17). This inhibition is a result of strong binding of RIFs to prokaryotic RNAP, ranking them as the most potent inhibitors of bacterial RNAP (the 50% effective concentration [$EC50_{RMP}$] for *E. coli* RNAP is ~20 nM). The binding constant of RMP for eukaryotic RNAP is at least 100 times higher than its binding constant for prokaryotic enzymes. Inhibition of RNAP is the common mechanism of action (MOA) among all structurally related RIFs with antibacterial activity (15, 18, 19). The antimicrobial activity differences of RIFs in Gram-positive and Gram-negative bacteria are not related to their binding site on RNAP but are due to other factors like efflux pumps in *E. coli* (20). The binding of RIFs to RNAP was initially verified by mapping almost all RMP^r mutations to the *rpoB* gene encoding the β subunit of RNAP (21).

Studies on the MOA of RIFs revealed that they inhibit RNA synthesis at the very early stages of transcription (15, 22, 23) and that RMP is no longer active when RNA synthesis progresses beyond an early stage (24). It was also determined that RMP binds to core *E. coli* RNAP and does not need the σ-subunit for its binding (25). McClure and Cech discovered that RMP inhibition induces the release of dinucleotide from *E. coli* RNAP if transcription is started with nucleoside triphosphate, whereas trinucleotide is released from transcription complexes started with smaller nucleoside mono- or diphosphate. Based on this observation, they proposed that RIFs sterically block the extension of nascent RNA at transcription initiation (26). The well-studied RMP-*E. coli* RNAP model was used as a prototype for analyzing the RMP binding to other bacterial RNAPs, mostly through investigation of the RMP-resistant mutants (3, 27–30). Resolving the crystal structure of RMP bound to RNAP was a breakthrough in the investigation of RIF's MOA. According to the structure, the RIF-binding pocket is located in the β subunit of *Thermus aquaticus* RNAP within the DNA/RNA binding channel in 12-Å proximity to the Mg^{2+} ion at the active site (3), which is consistent with previous biochemical observations with *E. coli* RNAP (31, 32). Based on the crystal structure, binding of RMP to RNAP blocks the formation of the second or third phosphodiester bond (Fig. 2) (3).

The steric model alone did not explain the differences observed in RIFs. For example, a semisynthetic RIF, rifalazil (RLZ), and RMP are not completely cross-resistant, or RLZ and RBT can develop different resistant mutations compared to RMP (33). Based on the crystal

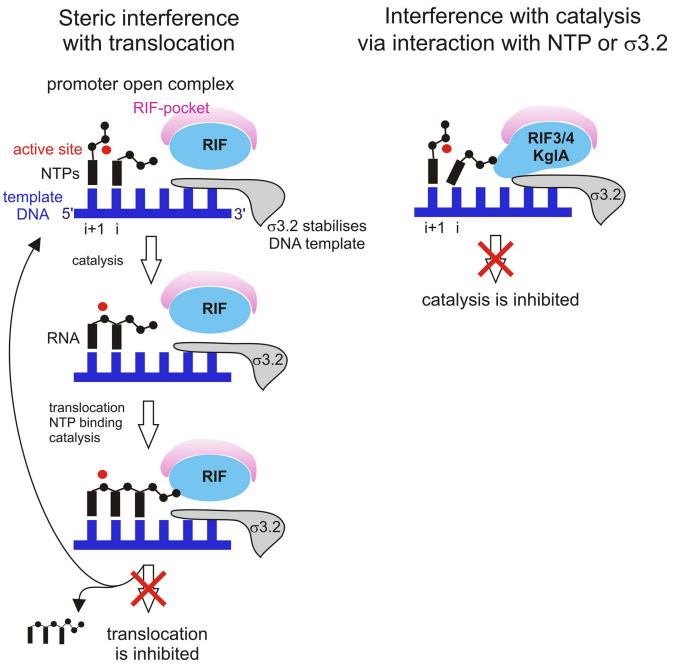


Figure 2 Mechanisms of action of different RIFs. A RIF (with or without groups at C-3/C-4 or KglA) bound at the RIF-binding pocket either sterically blocks progression of the growing RNA chain, resulting in abortive synthesis (left), or inhibits the first phosphodiester bond formation by interfering with initiating NTP or with σ region 3.2 that stabilizes the template DNA.

structures of *Thermus thermophilus* RNAP holoenzymes in complex with RBT and RPT, which lacked catalytic Mg^{2+} from the active center, Artsimovitch et al. proposed an allosteric inhibition model in which instead of or in addition to the steric model, RIFs allosterically reduce the affinity of catalytic Mg^{2+} ion to the RNAP active center (19, 34).

Later biochemical and structural studies contradicted the allosteric model of RIF inhibition. Feklistov et al. showed that RIFs do not affect the affinity of Mg^{2+} to RNAP, and a high concentration of Mg^{2+} does not confer resistance to RIFs (35), as was proposed for the allosteric mechanism. The crystal structures of the *E. coli* RNAP σ^{70} holoenzyme in complex with RMP as well as two RIF derivatives which similarly to RBT inhibit the first phosphodiester bond displayed the binding of RIFs to a RIF-binding pocket without inducing the loss of Mg^{2+} from the active center (36). Accordingly, the authors suggested that a potential interaction of RIF "tails" with a loop-like domain of σ subunit, σ region 3.2, is responsible for inhibiting the formation of the first phosphodiester bond by some RIF derivatives (36). The σ region 3.2 stretches toward the RNAP active center and plays an important role in transcription initiation via direct interaction with template DNA and stabilizes the initiating nucleoside triphosphates (NTPs) in the RNAP active site (37). Therefore, disengagement of the σ region 3.2 from its position by RIFs may reposition the template DNA relative to the active site and impair the binding of, first, NTP, which consequently may inhibit the formation of the first phosphodiester bond (36–39).

Recently, crystal structures of *M. tuberculosis* RNAP containing variable synthetic RNA oligomers (2-, 3-, and 4-RNA nucleotide RNA) in complex with RMP demonstrated that it does not expel the catalytic Mg^{2+} from RNAP (40). The recent studies of kanglemycin A (KglA), a rifamycin molecule with no C-3/C-4 side groups, showed that it does not displace the Mg^{2+} from the RNAP active center while inhibiting the first phosphodiester bond via a steric and/or electrostatic clash of the additional acid moiety of the ansa bridge with γ and/or β phosphates of the initiating NTP (Fig. 2) (41, 42). Overall, the discrepancy in the MOA of different RIFs could be explained by direct interference of large groups of some RIF molecules (e.g., RBT) with the initiating dinucleotide or other proximal residues such as the σ region 3.2 (rather than allosterically with Mg^{2+} of the active center), which also would appear as inhibition of synthesis of the first phosphodiester bond (Fig. 2). It should be mentioned that some discrepancies in interpretation of biochemical results may also be caused by the usage of different initiation substrates in different studies, in particular, NTP versus NpN dinucleotide primers (lacking triphosphate moiety at their 5′ end), which have different sizes and charge distributions, which, in turn, may strongly affect their interactions with various RIFs.

The sensitivity of different bacterial RNAPs to RIFs is influenced not only by the binding region (RIF-pocket) but also by other regions which indirectly change the conformation of the binding site (43). The *M. tuberculosis* and *Mycobacterium avium* RNAPs have been shown to be more sensitive than *E. coli* RNAP to RMP (44, 45). As the RIF-binding pocket is highly conserved among all bacteria, such differences are thought to be due to structural differences in the nonconserved regions surrounding the RIF-binding pocket of RNAPs. For instance, a chimeric *E. coli* RNA polymerase carrying β-subunit regions I and II of *M. tuberculosis* enzyme was as sensitive as wild-type *E. coli* enzyme in response to RMP, suggesting that regions outside the RIF pocket can determine sensitivity to RIFs (43). Similarly, *T. aquaticus* RNAP is about 100-fold less sensitive to RMP compared to *E. coli* RNAP (42, 46), while amino acids of *T. aquaticus* RNAP that directly interact with RIF are identical to those in *E. coli* RNAP. Therefore, the difference is also thought to be due to changes in the regions outside the RIF-binding pocket (3, 47).

STRUCTURE-ACTIVITY RELATIONSHIP OF RIFs

Almost all RIFs form four critical hydrogen bonds with RNAP residues through oxygens at C-1 and C-8 on the naphthalenic ring and C-21 and C-23 hydroxyl groups. A particular spatial arrangement of the above four oxygens is important for the binding. Oxygens at C-1 and C-8 make hydrogen bonds with RNAP residues S531/Q513 and R529, respectively, while hydroxyl oxygen at C-21 makes hydrogen or van der Waals interactions with three residues of RNAP: H526, D516, and F514 (*E. coli* numbering is used here and throughout the paper). The hydroxyl oxygen at C-23 together with carbonyl oxygen of C-25 make hydrogen bonds to F514 (3, 48). In KglA, because of different orientation of the ansa chain compared with other RIFs, the hydroxyl group at C-23 does not form a hydrogen bond with βF514. Instead, the unique sugar side chain of KglA at C-27 makes contact with βR143 (Fig. 3) (41). Moreover, hydrophobic interactions between RIFs

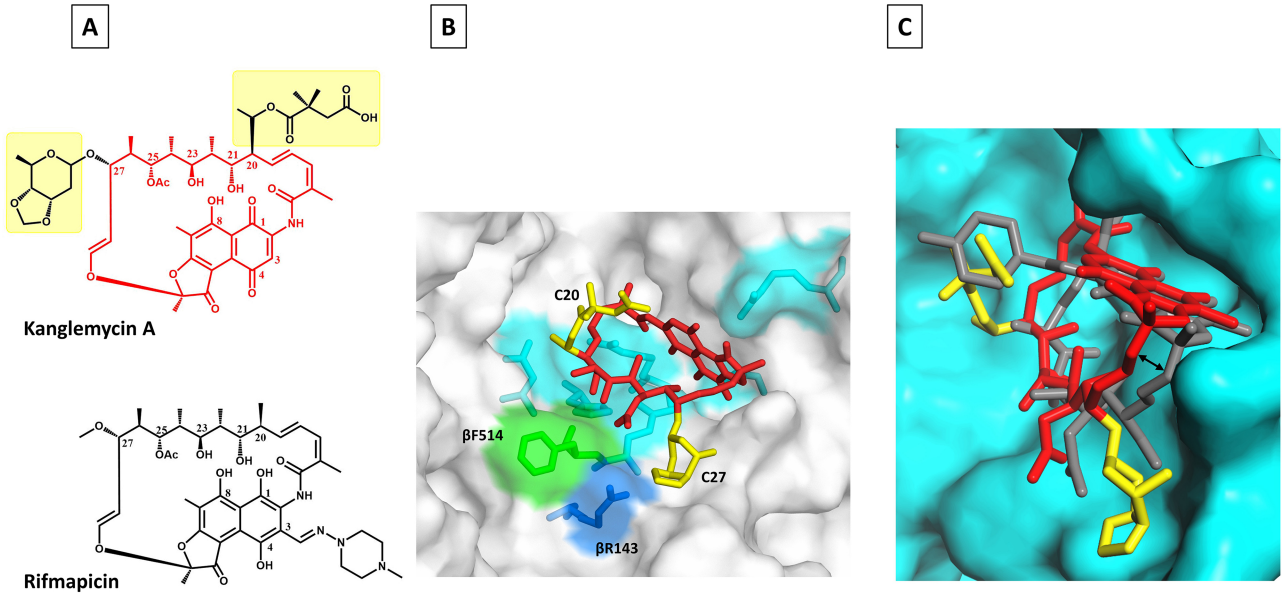


Figure 3 Mode of KglA binding to RNAP compared with RMP. (A) Chemical structures of KglA (in red with C-20 and C-27 side chains highlighted in yellow) and RMP (black). (B) A close-up view of KglA in the RIF-binding pocket of *T. thermophilus* RNAP (PDB: 6CUU). KglA is shown as a stick model (red) with its deoxysugar and succinate groups shown in yellow. RNAP is shown as a transparent surface model (gray), and RNAP β residues, which form the RIF-binding pocket, are shown as stick models. KglA binds to the same residues that RMP binds (cyan) to, with the exception of βF514 (green). KglA makes additional binding with βR143 (blue). (C) A side view of KglA in the RIF-binding pocket shown in panel B (PDB: 1YNN and 6CUU). The RNAP β subunit is shown in cyan. KglA (red and yellow) is overlaid on RMP (gray). Compared with RMP, KglA maintains a larger distance from the RIF-binding pocket (depicted by the two-headed arrow) (41).

and RNAP residues E565, I572, G534, L533, L511, and Q510 contribute to the binding of RMP (3).

MODIFICATIONS OF THE RIF ANSA BRIDGE

Early studies showed that even minor modifications of the ansa bridge, such as acetylation of C-21 or C-23 alcohols, led to activity loss of RIFs (15). Other examples of unsuccessful modifications in the ansa bridge include the epoxy side chain derivatives (e.g., 18,19-epoxy RIF S), RIF SV analogues with a keto group at C-21 instead of hydroxyl and an extra hydroxyl at C-20 (49), derivatives with a cyclic 3-14 linkage to the nitrogen of the macrocyclic ring, and RIFs with open rings, such as RIF W (50). Very few derivatives of RIFs with ansa bridge modifications have been shown to retain their activity due to only minor conformational changes of the ansa bridge. The hydroxylated C-25 derivative of RIF is one of the modifications of the side chain which is tolerated (49, 51). The 24-desmethyl rifampicin has also been claimed to have the same or enhanced activity against some bacteria compared to RMP (52). The considerable potential of RIF ansa bridge modifications to improve their activity was revealed after the discovery of the KglA MOA (41, 42). KglA, a rifamycin molecule with unusual ansa bridge modifications (C-27 β-O-3,4-O,O′-methylene digitoxose and C-20 2,2-dimethyl succinic acid) is more effective against RMP^r RNAPs and bacteria compared with the commonly used RMP. KglA binds to the RIF-binding pocket, but the unique ansa bridge groups of KglA establish additional contacts with the RIF-binding pocket and influence the overall binding mode of KglA in the pocket (Fig. 3) (41). The KglA scaffold provides a rationale for the further development of new RIFs with activity against the RMP^r bacteria.

MODIFICATIONS OF THE NAPHTHAQUINONE RING

Modifications at Position C-4 of RIFs

Generally, modifications in the naphthalene ring of RIFs are well tolerated. The first observation indicating the possibility of modifications in the naphthalene ring was the equal RNAP activity of RIF S, RIF B, and RIF SV (15, 53). RIF B, a potent inhibitor of bacterial RNAP, shows poor penetration through the bacterial cell wall due to free carboxylic acid at C-4. Structure-activity relationship studies noted that RIFs with free carboxyl groups at C-3 or C-4 have reduced ability to penetrate the cell wall and

therefore possess reduced activity against bacteria (54). The first successful modifications at C-4 were the amide and hydrazide analogues of Rif B with enhanced *in vitro* and *in vivo* activity (55). The first RIF B derivative in clinical use was a diethylamide analogue (rifamide), though it was replaced by RMP because of its poor pharmacokinetics (13, 56).

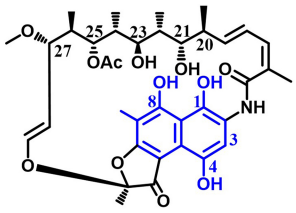
Modifications at Position C-3 of RIFs

Most of the initial successful structure-activity relationship studies concentrated on C-3 modifications of RIFs. A range of substitutions at the C-3 position of RIF S or SV have been shown to maintain their activity against bacteria (57–59). Dampier and Whitlock showed that electronegative groups at C-3 enhance the activity of RIFs, while the electron-donating groups at C-3 reduce the activity against RNAP (60). Early works showed that almost all changes in RIFs, including the naphthaquinone ring modifications, which change the conformation of the ansa bridge, lead to loss of activity (58, 61–63). Although RIF O retains its inhibitory activity against RNAP despite conformational changes in the ansa chain it is suggested that the *in vitro* activity of RIF O could be at least partly due to the hydrolyzed product, RIF S (64, 65). RMP was the result of many hundreds of imine, hydrazide, and oxime derivatives of 3-formyl RIF SV (Fig. 4). Due to its potency against *M. tuberculosis* and improved pharmacokinetic properties, RMP has remained the mainstay in TB therapy since its introduction in 1968 (13). After successful synthesis of RMP, many structure-activity relationship studies moved toward the synthesis of RMP analogues with enhanced half-life to permit intermittent dosing. These studies resulted in the synthesis of many C-3 substituted RMPs (66). RPT, which has a cyclopentyl group instead of a terminal methyl group at C-3, was the best derivative (Fig. 4). Not only is RPT as active as RMP against *M. tuberculosis*, but its longer half-life enables twice-weekly dosing for TB patients (67). However, RPT is completely cross-resistant with RMP (68, 69). RPT is currently used in combination with isoniazid to treat latent *M. tuberculosis* infections (70).

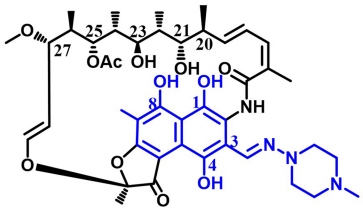
Modifications of C-3/C-4 in RIFs

A variety of RIF analogues with an additional ring linking C-3 and C-4 have been synthesized. The antibacterial activity of different C-3/C-4 analogues of RIFs can differ by several orders of magnitude, and therefore, most of the recent attempts have been focused on making such derivatives of RIFs (71). Rifazine was one of the first rigidified benzannulated analogues of RIF which retained its activity against RNAP (Fig. 4). Rifazine has considerably higher potency against *Staphylococcus aureus* compared with RIF SV (49, 72). Rifaximin (RXM) was another RIF analogue with an additional ring (Fig. 4) which had similar activity as RMP in cell culture, though its activity against *Staphylococcus epidermidis* RNAP was about half of RMP activity (73). RXM has very low oral absorption due to the two oppositely charged nitrogens in addition to phenolic hydroxyls, which is beneficial for treatment of gastrointestinal infections where a high fecal concentration of drug is required (74, 75). RXM is approved in many countries to treat uncomplicated traveler's diarrhea (*E. coli*-born irritable bowel syndrome with diarrhea) (75). Although resistant mutations for RXM are selected at a lower rate compared with RMP, cross-resistance between the two drugs is inevitable (76, 77).

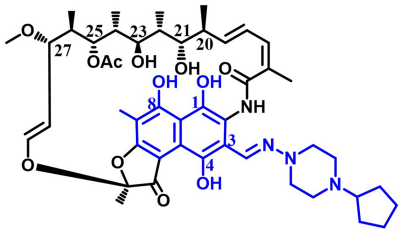
A milestone in the optimization of RIF derivatives was the synthesis of spiro-piperidyl RIF analogues, out of which RBT was the most promising (Fig. 4) (78). The crystal structure of *T. thermophilus* RNAP holoenzyme in complex with RBT showed that, similar to RMP, the ansa moiety of RBT makes contact with β residues of RNAP, suggesting the same MOA as RMP. It is proposed that the C-3/C-4 tail of RBT makes contact with the σ-subunit of RNAP, which enables RBT to inhibit the elongation of RNA at earlier stages of transcription compared to RMP. However, there is no strong biochemical evidence to support that binding to the σ-subunit is responsible for this variation (19, 35). RBT is more potent against RMP-susceptible *M. tuberculosis* than RMP (20), but most of the RMP^r *M. tuberculosis* strains are also resistant to RBT, albeit with lower MICs than those for RMP (79). The *in vitro* activity of RBT against RMP^r RNAPs also confirmed its partial cross-resistance with RMP (20). Further studies of large numbers of clinically isolated RMP^r strains indicated the potential of RBT for treatment of patients diagnosed with specific RMP^r-TB (80, 81). One of the main drawbacks of RIFs is the interaction with human pregnane X receptor (hPXR), which induces the hepatic cytochrome P450 (Cyp450) and other proteins involved in xenobiotic metabolism. This is of great importance in patients coinfected with HIV and *M. tuberculosis*, as certain antiviral drugs are metabolized by Cyp450. Because of the low drug-drug interactions of RBT, especially with antiviral drugs, and the high level of *in vivo* toxicity, it is used as an alternative therapy against several mycobacterial infections, including *M. avium*-intracellulare complex



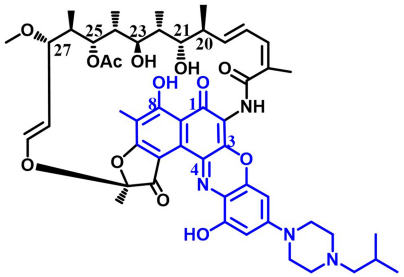
Rifamycin SV



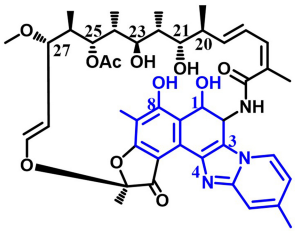
Rifampicin (RMP)



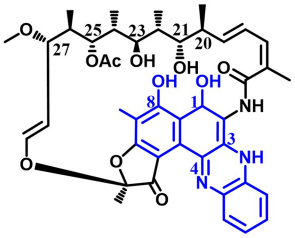
Rifapentine (RPT)



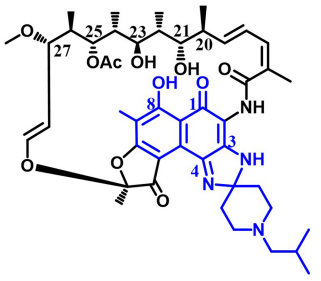
Rifalazil (RZL)



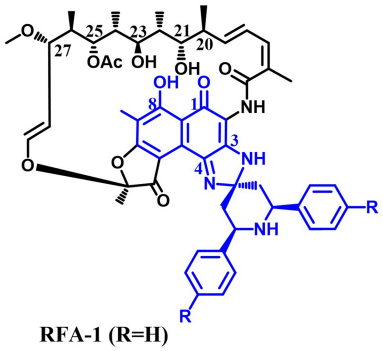
Rifaximin (RXM)



Rifazine



Rifabutine (RBT)



RFA-1 (R=H)

RFA-2 (R=F)

Figure 4 Chemical structures of different RIFs. The ansa chain and naphthalene moiety of the molecules are shown in black and blue, respectively.

(MAC) in patients with AIDS, and for treatment of MDR-TB (82, 83). More recently, synthesis of RBT analogues resulted in two promising candidates, RFA-1 and RFA-2 (Fig. 4), which display MIC values up to 100 times lower than that of RMP and 20 times lower than that of RBT against MDR-TB (84, 85). Preliminary molecular modelling calculations on an RMP^r *M. tuberculosis* RNAP showed increased interaction energy between the RFA-1 compared to RBT and RMP. Therefore, the enhanced antimicrobial activity of the two derivatives against RMP^r strains derives from their tighter binding to RNAP as a result of additional enzyme-ligand contacts (85).

Another breakthrough in RIF analogues was the synthesis of 3′-hydroxy-5′-aminobenzoxazinorifamycin derivatives. The benzoxazinorifamycins (bxRIFs) showed enhanced *in vivo* activity against slowly growing mycobacteria, including *M. tuberculosis* and MAC compared to RMP, and provided better absorption from the gastrointestinal tract. Among such derivatives, KRM-1648 (rifalazil [RLZ]) was the most promising analogue due to its excellent potency against *M. tuberculosis* in addition to the improved pharmacokinetic characteristics (Fig. 4) (86). RLZ strongly inhibits a spectrum of Gram-positive bacteria with MICs lower than or similar to those for RMP. Although the frequency of spontaneous mutation for RLZ is almost same as for RMP (87), it has somewhat enhanced activity against some of the isolates which are resistant to RMP and RBT (36, 88–90). Fujii et al. showed that RLZ similarly inhibits RNAPs of different bacterial origins *in vitro* and concluded that its improved antibacterial activity depends on cell wall permeability of the target bacteria (45). Despite many exceptional characteristics of RLZ, including high potency (up to 250-fold more potent than RMP) (91), its activity against some RMP^r strains (88), high volume of distribution and tissue level (92), and lack of hepatic Cyp450 induction (93), RLZ development was suspended due to high toxicity observed in phases I and II of clinical trials (94).

In a study aimed at the synthesis of novel bxRIFs, Gill et al. showed three novel RLZ derivatives which have improved binding affinity to wild-type and rifamycin-resistant (RIF^r) RNAPs of *M. tuberculosis* (94). It is suggested that bxRIFs are capable of making additional contacts with RNAP (possibly with σ region 3.2 and other regions of the RNAP complex) (36). However, all analogues exhibited high human pregnane X receptor (hPXR) activation and cytotoxicity and had low antitubercular activity in cell culture (94).

MECHANISM OF RESISTANCE TO RIFs

Shortly after the discovery of RIFs, the occurrence of resistant mutations creating a high level of resistance was observed in the laboratory and in infected patients (95, 96). Bacteria develop resistance to RIFs at frequencies of 10^{-10} to 10^{-7} depending on the organism, methodology, and type of RIF molecule (16, 97). Because of the rapid emergence of resistant isolates, RMP is used in drug combinations almost exclusively for treatment of TB (4).

Mutations Affecting the β Subunit

Almost all mutations conferring resistance to RIFs map to the *rpoB* gene (which encodes the β subunit of RNAP) in *M. tuberculosis* (29, 98), *E. coli* (22, 99), and other microorganisms examined (100–102) (Fig. 5). Although *E. coli* is not the main therapeutic target for most RIFs, well-studied RNAP of this organism in addition to the homology of its RNAP to the *M. tuberculosis* RNAP makes it a good model for genetic and physiological studies. RIF^r spontaneous point mutations, mostly as single amino acid changes, occur within four regions of *rpoB* (known as the RIF resistance-determining region, RRDR): N-terminal cluster (cluster N, amino acids 146 to 148), cluster I (amino acids 504 to 538), cluster II (amino acids 562 to 575), and cluster III (amino acids 684 to 691) (Fig. 5) (103, 104). The vast majority of such mutations (~95%) are within the 81-base pair region of cluster I, which is highly conserved among bacterial RNAPs but different from archaeal or eukaryotic RNAPs (98, 105). According to crystal structure of the *T. aquaticus* RNAP bound to RMP, almost all residues that directly interact with RMP are encoded by cluster I. Almost all residues involved in direct interaction with RMP are susceptible to mutations resulting in RMP resistance (Fig. 5) (3).

While point mutations have been found in 33 codons of RRDR, only three mutations, S531L, H526Y, and D516V, account for around 41%, 36%, and 9%, respectively, of all clinically isolated RIF^r-TB strains (98). The three most frequent RMP^r mutations have direct effect on interactions with oxygens at C-8 and C-21 of RMP (Fig. 5) (3). Depending on the point mutations, the mechanism of resistance to RIFs varies. For example, the βS531L does not impose a significant structural or functional impact on RNAP in the absence of RMP. The βS531L disorders the RIF-binding pocket upon RMP binding and therefore reduces the binding affinity for RMP. The collision between the leucine at β531 and the β subunit fork loop 2 (a conserved loop of the active center which plays a role in

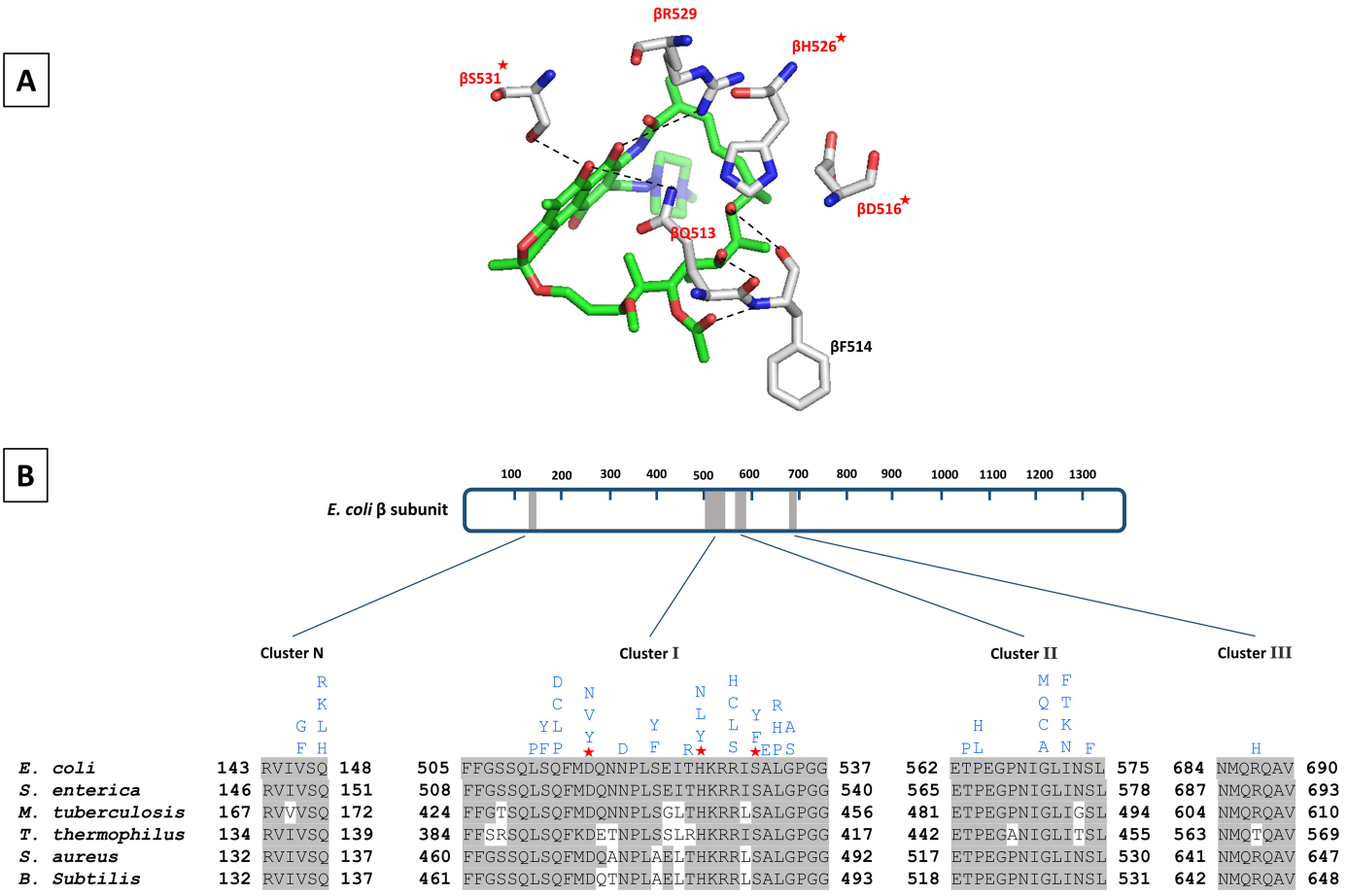


Figure 5 Rifamycin resistance-determining regions of different bacterial RNAPs. (A) Schematic representation of RMP in the stick model (green) bound to residues of *E. coli* RIF-binding pocket (gray stick model; PDBID: 5UAC) (48). The hydrogen bonds between RMP and residues are shown as dashed lines. Amino acid residues that are mutated in clinical RIF^r isolates are highlighted in red. The three residues which are most frequently mutated to confer RIF^r clinical isolates of *M. tuberculosis* are marked by an asterisk. (B) The schematic on top represents the primary sequence of the *E. coli* β subunit. The amino acid numbering is depicted. Gray boxes represent the four clusters (RMP resistance-determining regions; RRDRs) where RIF^r mutations occur. A sequence alignment showing these clusters in *E. coli*, *S. enterica*, *M. tuberculosis*, *T. thermophilus*, *S. aureus*, and *B. subtilis* is depicted below the schematic bar. Amino acids that are identical to *E. coli* are highlighted in gray. Mutations that confer RIF^r in *E. coli* are indicated above the sequence.

DNA strand separation) is suggested to be responsible for disordering the RIF-binding pocket. The H526Y mutation significantly changes the RIF-binding pocket and therefore causes a significant steric conflict for binding of RMP to RNAP. As a result, mutations at βH526 lead to very high levels of resistance to RMP. The less frequent mutation, βD516V, reduces the affinity of RIF binding only by changing the electrostatic surface of the RIF-binding pocket (48, 106). Other RIF-resistant *rpoB* mutations which do not make direct interactions with RIF are thought to act by changing the conformation of the RNAP.

Many studies focused on binding surfaces away from the RIF-binding pocket to target the RIF^r bacterial RNAPs (40, 107). However, the RIF-binding pocket is still a promising target for the development of new RIFs with activity against RIF^r pathogens. For example, the additional contacts of KglA with the RIF-binding pocket induces conformational changes of KglA which, compared with RMP, positions the drug slightly away from the RIF-binding pocket (Fig. 3). The increased distance between KglA and RNAP enables it to maintain its inhibitory activity against the most frequent RIF-resistant bacterial RNAP (βS531L). Due to additional contacts and slightly different modes of binding to RNAP, it was suggested that only an unlikely event of two concurrent mutations within the RIF-binding pocket can produce resistance to KglA (41). However, such mutations impose a great fitness cost and may compromise the transcriptional integrity and therefore make the pathogen nonviable (108).

Other Mechanisms of Resistance to RIFs

Some bacteria, such as certain soil-dwelling actinomycetes, are intrinsically resistant to RIFs. An example of such intrinsic resistance is the presence of asparagine at consensus codon 531 in comparison to serine in susceptible bacteria (109). Various RNAP-independent mechanisms have been described for RMP resistance, though mostly in clinically insignificant organisms. For example, the removal of RIFs by efflux pumps has been shown in different strains, including *M. tuberculosis* (110, 111). In *Nocardia farcinica*, an RMP monooxygenase (ROX) is recognized as a secondary mechanism of RMP^r which, by adding an oxygen to the C3 side chain of RMP, converts it to a compound with lower antimicrobial activity (112). Furthermore, in certain species of mycobacteria, including the emerging pathogen *Mycobacterium abscessus*, the high level of resistance to RMP is partly associated with the ADP-ribosyltransferase (Arr) enzyme that can modify RMP with ADP-ribosyl, a group transferred from NAD^+, which results in RIF inactivation (113). Since the ribosylation of RMP by Arr occurs at position C23, the presence of bulky side chains in the vicinity of the ribosylation site is suggested to create a steric hindrance for RMP inactivation. For instance, different 3-morpholino RIFs which possess a bulky carbamate group at C25 are resistant to RIF inactivation by Arr (114). KglA and its congeners which carry a relatively large sugar side group at C-27 (41, 42) are potential candidates for developing RIFs with the ability to overcome Arr-mediated RIF inactivation.

Fitness Cost of RIF^r Mutations and Fitness-Compensatory Mechanisms

Most mutations conferring antibiotic resistance come with a cost, meaning the bacterial strains carrying resistant mutations have compromised fitness compared with the ancestral strains (115–118). In the absence of antibiotics, resistant bacteria bearing reduced fitness should be outcompeted by the drug-sensitive bacteria and be eliminated from the population. However, the fitness cost of antibiotic resistance is counterbalanced by compensatory evolution. Rapidly evolving secondary mutations provide the resistant bacteria the ability to become as fit as susceptible strains and hence stabilize the resistant bacterial population in the absence of antibiotic (116).

Almost all Rif-resistant mutations impose reduced fitness. The fitness cost of RIF^r mutations is generally inevitable, as the process targeted by RIFs, the transcription, is vital to all aspects of bacterial life, including the growth and virulence (117–122). The fitness cost of RIF^r mutations and their corresponding prevalence in nature have a negative correlation. Therefore, only the RIF^r mutations with low fitness costs are found frequently in resistant clinical isolates (48, 123). Fitness in bacteria critically depends on their surrounding envi-

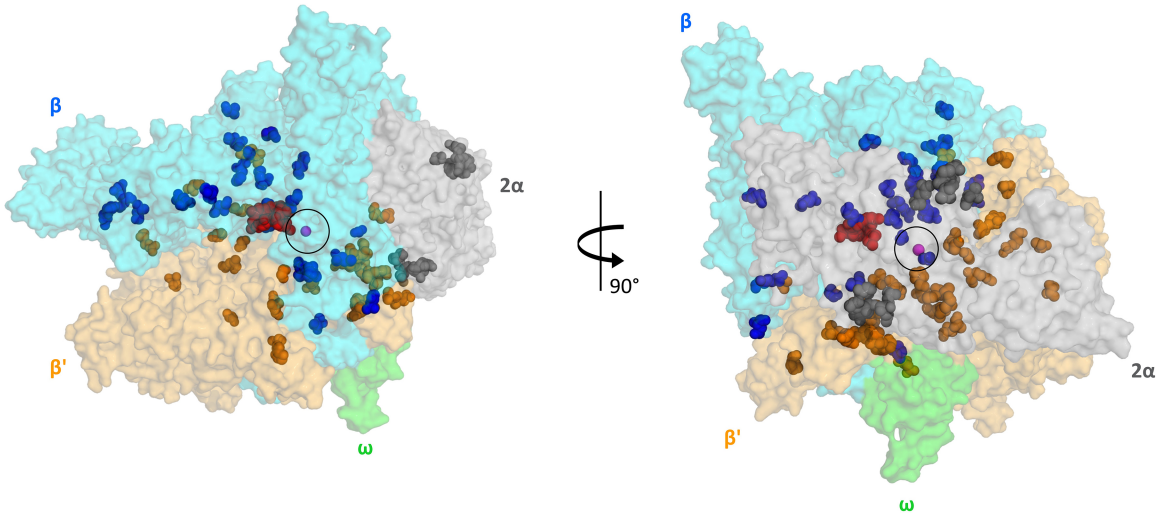


Figure 6 Two views of the RIF compensatory mutations from different RIF^r bacteria (108, 121, 122, 128, 131) mapped onto the crystal structure of the *E. coli* RNAP-RMP complex (PDB:5UAC). The RNAP core enzyme is illustrated as a transparent surface (α subunits, gray; β subunit, cyan; β′ subunit, bright orange; ω subunit, gray). The active center of RNAP, marked by the presence of catalytic Mg^{2+} (magenta sphere) is circled. The RMP molecule is depicted as red spheres. Compensatory mutations found on the α, β, and β′ subunits of RNAP are shown as gray, blue, and orange spheres, respectively.

Table 1 List of secondary mutations in different RIF^r bacteria

Strain	RMP^r mutation	Secondary mutations in α	Secondary mutations in β	Secondary mutations in β′	Reference
Salmonella enterica	βS531	αA189E αT196S	βN339H βD340Y βE546k βG809A βQ1264R	β′H419P β′A446V β′D622E β′R634P β′T674I β′I937T β′S942L β′R943H β′R1075C β′R1075L β′G1136A	128
Salmonella enterica	βR529	αR191C	βD516G βP560L βP564S βE565A βR637C βH673Y	β′P64L β′L770P β′R1075P β′R1075H β′G1136 β′R1140H β′V1198E	122
E. coli[a]	βD516		βS574Y βH554Y βS574Y		121
E. coli[a]	βP564		βR211P βS574F βL194R		121
E. coli[a]	βL511		βD516G		121
E. coli[a]	βI572		βG556G		121
M. tuberculosis[c]	βS531	αA189V αR191W αV192G αT196A	βT1286I βQ490R βP25S	β′G257R β′G257S β′N341S β′P359R β′P359V β′F377L β′K370R β′V408G β′P420_V421insA β′L432V β′L441P β′L452V β′D622E β′E658G β′E658D β′E658A β′P998R β′P998S β′A1312V β′N1350S β′A1312V	108
M. tuberculosis[b]	βS531			β′D410Y β′F377C β′G257R β′H450Q β′V408G β′V408A β′I416T β′Q448E	131

(continued)

Table 1 List of secondary mutations in different RIF^r bacteria *(continued)*

Strain	RMP^r mutation	Secondary mutations in α	Secondary mutations in β	Secondary mutations in β′	Reference
M. tuberculosis[b]	βD516			β′V408G	131
M. tuberculosis[c]	βH526	αE29K	βE472G βv650A	β′A919G	108
M. tuberculosis[c]	βQ513		βN856S βR841H		108

[a]The *rpoAC* genes were not sequenced for compensatory mutations.
[b]The *rpoAB* genes were not sequenced for compensatory mutations.
[c]Only the mutated residues which are identical between *E. coli* and *M. tuberculosis* were included.

ronment. For example, RIF^r mutants, which reduce the bacterial fitness in exponential growth, can be advantageous in the later stages of growth (e.g., aging colonies), at high temperatures, or in low-glucose environments (124, 125). The fitness costs of RIF^r mutations could be ameliorated by secondary mutations within RNAP β, β′, or α subunits and occur at higher frequencies than the mutations resulting in reversion of primary RIF^r mutation (Fig. 6 and Table 1) (108, 122, 126, 127). The secondary mutations are concentrated in particular structural regions of RNAP and may act by different mechanisms, including restoring the properties of the RIF-binding pocket, changing the interaction of enzyme with substrate or RNA, or affecting the interactions between different RNAP subunits (e.g., mutations in the α-β′ interface) (108, 122, 127, 128). The secondary mutations do not considerably change the susceptibility of bacteria to RIFs, with the exception of those that are known RIF^r mutations (e.g., βD516G) (122).

A combination of different factors determine the recurrence of RIF^r mutations. In addition to low fitness cost, a high level of resistance to RIF, and acquiring secondary mutations to further reduce the fitness cost, all contribute to the success of specific RIF^r mutations in clinical isolates of *M. tuberculosis* (129). Although the compensatory mutations play key roles in developing clinical RIF^r bacteria with high fitness (118, 120, 127, 130), our knowledge of the MOA of such mutations is very limited. Investigating the MOA of compensatory mutations in different organisms could possibly shed new light on the activities of RNAP and allosteric switches within the enzyme and open new doors to potential novel approaches for tackling antibiotic resistance.

Although the importance of RIF^r fitness compensatory mutations has been studied in different organisms, *E. coli* (121) and *Salmonella* are broadly used as genetically amenable model organisms to study the fitness cost in resistance to RIFs and the genetics of the corresponding compensatory evolution (122, 128, 129). The compensatory mutations found in the model organisms overlap with the mutations found in clinical isolates of *M. tuberculosis*, suggesting the conserved role of fitness compensatory mutations in bacteria (Table 1) (122, 127–129).

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