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Original article

Pharmacophore insights into rpoB gene mutations in *Mycobacterium tuberculosis* rifampicin resistant isolates

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ABSTRACT

This paper reports the susceptibility profile to rifabutin (RFB) **1** and six recently synthesized RFB analogs **3**–**8**, of either rifampicin (RFP) susceptible *Mycobacterium tuberculosis* and resistant clinical isolates from two sources: Mexico and Brazil. Taking into account that about 95% of *M. tuberculosis* strains resistant to RFP present mutations in the *rpoB* gene, with some of these mutations being determinant also to RFB resistance, the RFB analogs were screened for activity against a set of known RFP susceptible and resistant strains. *N*′-Acetyl-RFB **5** and *N*′-(undec-10″-enoyl)-RFB **8** showed the best results, in particular with mutations in the codon 516, 522 and 531 of the *rpo*B gene, and were therefore selected for *in vivo* assessment of their efficacy. Studies conducted with tuberculous Balb/C mice previously infected with Ser531Leu mutated clinical isolate, evidenced both **5** and **8** as promoters of a significant decrease on tubercle bacilli burden in lungs associated with lower tissue damage, thus confirming them as good leads for drug discovery. The SAR of the acylated compounds **5** and **8** envisaging the identification of pharmacophore features, highlights the importance of profiling more clearly the chemistry within the molecular aspects for elucidation of the mode of action of RFB and analogs, in relation to mutations in Multidrug-Resistant (MDR) strains.

1. Introduction

Effective weapons against a wave of new drug-resistant strains are urgent. If we wonder where we do stand on tuberculosis (TB), most researchers would state that we are basically back to where we were before drugs. Novel approaches linking drug candidates to gene studies are needed to overcome the threat posed by the emergence of drug-resistant *Mycobacterium tuberculosis*. More effective agents against multidrug-resistant tuberculosis (MDR-TB) able to shorten the duration of treatment are needed, particularly those targeting the eradication of the latent form of TB.

For the ancient disease TB as with other "neglected diseases" for which the Pharmaceutical Industry has low expectance in terms of revenues, since these are mostly widespread in underdeveloped countries, an exciting drug development pathway is open by targeting genetic make-up in susceptibility. Forty years have passed with no introduction into the market of new anti-TB drug classes, in spite of a huge diversity of attempts, providing identification of new targets, and the discovery of novel agents with novel mode of action (MoA) [1].

In current Directly Observed Therapy (DOT) programs, Rifabutin (RFB) plays a fundamental role in the treatment of patients with active TB. Although rifampicin (RFP) and isoniazid (INH) are by far the most effective anti-TB agents, RFP and RFB are crucial for ensuring success of short-course (6-month) chemotherapy [2]. Namely, patients with INH-resistant TB can respond well to 6-month treatment without INH (on a regimen of RFP, pyrazinamide, and ethambutol), but patients with RFP-resistant TB do

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not respond well to short-course chemotherapy without RFP or RFB [3]. Without RFP or RFB, most anti-TB regimens require at least 18 months of treatment [4], and every attempt should be made to use RFP or RFB in initial anti-TB treatment. In addition, anti-TB drug regimens for patients infected with the human immunodeficiency virus (HIV) pose a special challenge when certain antiretroviral medications are also indicated. RFP should not be taken together with most protease inhibitors or non-nucleoside reverse transcriptase inhibitors. RFB, however, often has to be used in these patients [5]. Nevertheless, a 2010 Cochrane Review [6] concluded that the replacement of RFP by RFB for first-line treatment of TB is not supported by the current evidence, and also that HIV positive people with TB, the group most likely to benefit from the RFB use, are still under-represented in trials to date, and further trials in this group would be useful.

In general, the major problem in the clinical use of RFB and other rifamycins (Rifs), is that their versatility and efficiency are limited by the rapid emergence of resistant strains, mostly as a consequence of mutations that occur on the active side of its molecular target: the ribonucleic acid polymerase enzyme (RNAP). To overcome this problem, it is necessary to understand the detailed MoA and the bacterial resistance to these drugs at the molecular level. A key breakthrough was the recent determination of the crystal structures of several inhibitors with the RNAP [7].

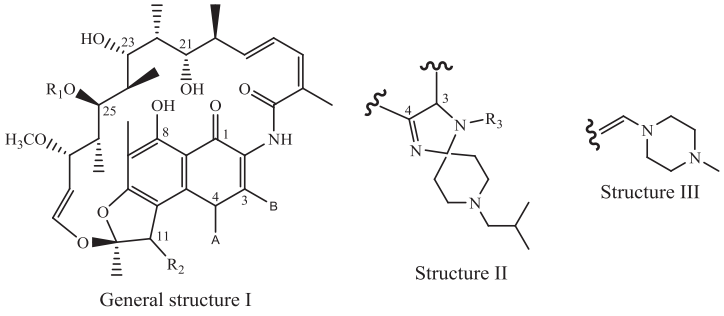
Rifs specifically inhibit bacterial transcription, by binding to RNAP. Also known as DNA-dependent RNA polymerase (DDRP), it is an intricate enzyme with a $\alpha_2\beta\beta'\omega$ subunit structure [8]. The exact mechanism by which Rifs interfere with the process had long remained elusive until 2001, when Campbell et al. [9], showed that Rifs bind to the β-subunit encoded by the *rpo*B, 12 Å away from the Mg^{2+} ion at the RNAP active site, in the DNA channel, physically blocking the RNA elongation when the transcript is 2–3 nucleotide long [9]. These findings supported the steric-occlusion model for Rifs action, which basically indicates that the stronger the interactions between the Rifs and a restricted number of specific amino acid (aa) residues, encoded by the *rpo*B of the RNAP, the better it would halt transcription.

Specific parameters and constrains on the chemical transformation of anti-TB leads/current drugs have evolved from a few SAR studies [10,11], and are evolving still scarcely through *in silico* studies [12]. Drug development with an existing drug such as RFB as the lead compound, with efficacy improvement through structure-based successful manipulation, can still be considered an attractive strategy from the economic, pharmaceutical and clinical points of view [13,14]. Successful results have been reported with ongoing RFB analogs [14,15].

RFB **1** is a semi-synthetic spiropiperidyl derivative of the ansamycin family of antibiotics [16–18]. For Rifs it is well known that modifications of the ansa bridge conformation, or at O1 and O8 of the central chromophore, and O21 and O23 hydroxyl groups, reduce substantially the *in vitro* inhibitory activity. But where do we stand exactly with what we have learned about the MoA, and where do we go using improved tools from what we achieved with RFP and RFB? For decades the pharmacophore concept has always been the driver for SAR studies on ansamycins [17–19] and is defined by IUPAC as "the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response". As pointed out by Leach et al. [20], central to the pharmacophore concept is the notion that the molecular recognition can be ascribed to a set of common features such as hydrogen-bond donors, hydrogen-bond acceptors, positively and negatively charged groups, and hydrophobic regions. As such, there is a link with contemporary pharmacophore models through the principles of bioisosterism and 3D spatial molecular and conformational relationships, leading to sophisticated computer algorithms. We focus here the challenges and constraints within the key problem of proposing the ansamycin pharmacophore mapping for a set of active molecules and their biological activities on both RFP susceptible *M. tuberculosis* and resistant clinical isolates with specific mutations.

Positions 3 and 4 of the naphthoquinone cromophore (cf. Fig. 1) are considered the centre of the pharmacophore and have been extensively modified by semi-synthesis to improve the pharmacological properties of Rifs. Differences between 3-tailed Rifs as RFP and rifapentine (RPN), and both 3,4-tailed Rifs (e.g. RFB), such as the inhibition by the former of only both the second and third phosphodiester bond formation, while the later inhibit also the first phosphodiester bond formation, fail to be explained by the proposed simple steric hindrance mechanism [21]. Also difficult to explain has been the existence of *rpo*B mutants which are resistant to RFP but yet susceptible to RFB **1** [22], regardless of the presence of point mutations in residues of the RNAP, that are known not to interact with Rifs tail (either at positions 3 or 4 or even both substitutions), where most of the differences between Rifs are located [9].

Based on these observations which were difficult to explain in the light of the steric-occlusion model, Artsimovitch et al. [21], proposed a different mechanism based on a 2.5 Å structural biology



1: Rifabutin (R_1: $COCH_3$; R_2: =O; A and B together are part of spiro structure II where R_3: H)
2: Rifampicin (R_1: $COCH_3$; R_2: =O; A: OH; B: structure III)
3: 25-Deacetyl-rifabutin (R_1: H; R_2: =O; A and B together are part of spiro structure II where R_3: H)
4: 25-Deacetyl-rifabutinol (R_1: H; R_2: OH; A and B together are part of spiro structure II where R_3: H)
5: N'-Acetyl-rifabutin (R_1: $COCH_3$; R_2: =O; A and B together are part of spiro structure II where R_3: $COCH_3$)
6: N'-Acetyl-rifabutinol (R_1: $COCH_3$; R_2: OH; A and B together are part of spiro structure II where R_3: $COCH_3$)
7: N'-Palmitoyl-rifabutin (R_1: $COCH_3$; R_2: =O; A and B together are part of spiro structure II where R_3: $COC_{15}H_{31}$)
8: N'-(Undec-10"-enoyl)-rifabutin (R_1: $COCH_3$; R_2: =O; A and B together are part of spiro structure II where R_3: $COC_{10}H_{19}$)

Fig. 1. RFB **1** and some of its previously synthesized derivatives **3**–**8** (to access the full list please see Figueiredo et al.[15], doi:10.1016/j.bmc.2008.12.006).

model with RPN and RFB bound to the RNAP from *Thermus thermophilus*. Since no substantial conformational changes in the active site upon Rifs binding occur, Artsimovitch et al. proposed that the inhibition of RNA transcription is mostly the result of at least two allosteric pathways, resulting from Rifs binding to the RNAP, disfavoring catalytic Mg^{2+} coordination and thus compromising the phosphodiester bond formation [8,21]. Although not ruling out the steric-occlusion model as a component of the inhibition mechanism, it was postulated that the allosteric modulation of the RNAP catalytic reaction should be an essential component of transcription control by Rifs.

More recently Feklistov et al. [23], supported the steric-occlusion model. The validity of the proposed allosteric model was tested and it was found out that rifamycins have no effect on metal ion binding to the active center and that mutants resistant to Rifs are so as a consequence of Rifs not binding tightly to the enzyme. This MoA implicates that inhibitors with additional tighter binding interactions to a resistant mutant will increase their free energy of binding, thus enabling to overcome resistant mutations. In practice, these mutations will only reduce the binding affinity of the inhibitors for RNAP.

It is of common knowledge that in *M. tuberculosis* approximately 95% of RFP-resistant clinical isolates carry mutations in the rpoB gene [18]. The level of RFP resistance depends on the site and kind of substitution identified in the rpoB gene. Knowing that the cross-resistance of *M. tuberculosis* strains to RFP and RFB **1** is incomplete, there is always the perspective of devising new RFB **1** analogs through a SAR-based approach analysis of the interactions of these Rifs with the DDRP. The data published on Distance-Restrained Docking of Rifampicin and Rifamycin SV to RNA should also be taken as Ref. [24]. RFB **1** remains particularly noteworthy, because it is about four to eight-fold more potent than RFP for TB and *Mycobacterium avium* treatments, while exhibiting a quite acceptable balance of pharmacokinetic/pharmacodynamic (PK/PD) key clinical properties and toxicity [25,26].

Desired analogs should bear polar groups able to allow hydrogen bonding with the RNAP main chain atoms, or flexible hydrophobic groups that would rely on non-specific van der Waals interactions [21]. Although several residues of the binding pocket and surrounding distorting positions at the *rpoB* gene region have already been identified, where point mutations responsible for RFP resistance occur [9], the molecular details of ensuing events remain rather unclear, even after quite promising improved activity against RFP-resistant strains of a few RFB analogs has been reported [14].

Hoping to contribute to the precedent discussion, we report here the experimental *in vivo* and *in vitro* results from recently developed RFB analogs [15] against *M. tuberculosis* strains with and without *rpoB* mutations [15], and discuss potential pharmacophore features in the light of the current knowledge according to a structure–activity relationship (SAR) perspective.

2. Material and methods

2.1. Chemistry

RFB analogs were all synthesized: deacetyl analogs **3** and **4** ($R_1 = H$) that were previously referred as RFB metabolites in urine [27], furanol analogs **4** and **6** ($R_2 = OH$) already reported [18] as well as acyl derivatives in RFB dihydroimidazole ring ($R_3 \neq H$), by introduction of a variable carbon chain length leading to compound **5**–**7** [15]. Molecular structures of those are depicted in Fig. 1. *C* log *P* values were predicted using the ALOGPS 2.1 program (http://146.107.217.178/lab/alogps/start.html) [15].

2.2. Sample preparation

RFP (Sigma®), RFB **1** (Mycobutin®; Pfizer) and the six RFB analogs **3**–**8** were dissolved in dimethyl sulphoxide at 10 g/l and stored at −70 °C. Working solutions were prepared from the stock solutions by dilution with Middlebrook 7H9 (4.7 g of Middlebrook 7H9 broth base [Difco-Becton Diskinson®], 2 ml of glycerol in 900 ml of water) enriched with 10% oleic acid, albumin, dextrose and catalase (OADC-BBL®).

2.3. Antimicrobial activity evaluation

The antimicrobial activity of the RFB analogs **3**–**8** was evaluated against *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) and on clinical isolates resistant or susceptible to RFP maintained in Ogawa–Kudoh medium for 14 days. The bacterial suspensions were prepared in sterile water containing 3 mm glass beads. The suspension was homogenized with a vortex mixer and the turbidity was adjusted in agreement with tube no. 1 of the McFarland scale (3.2×10^8 CFU/ml, colony-forming units). The inoculum was prepared by diluting the bacterial suspension 1:25 in Middlebrook 7H9 OADC medium. The minimum inhibitory concentration (MIC) of each drug was determined using the REMA method [28]. In brief, the assay was accomplished in micro plates (96 wells) using resazurin as indicator of cellular viability, 7H9 OADC medium, RFP, RFB **1**, and RFB analogs. Drugs were dissolved in DMSO and serial two-fold dilutions of the drugs (ranging from 0.06 to 2.0 mg/l) were prepared and dispensed into each well with RFP and RFB **1** as controls.

2.4. Sequencing of the rpoB

Aiming at identifying the molecular basis of resistance in all strains, the 159 bp fragment which corresponds to the hotspot region of 81 bp of *rpoB* gene, was sequenced. This region involves codons 507–533 and is also referred to as the RFP Resistance Determining Region (RRDR). DNA was extracted using the cetyl trimethylammonium bromide method [29]. The *rpoB* gene was amplified as previously described [30], with primers RIF1 5′GGTCGCCGCGATCAAGGAGT3′ and RIF2 5′TGCACGTCGCGGACCTCCA3′ (Invitrogen®). The amplification was carried out in the Gene Amp PCR System 9700 (Applied Biosystems®). PCR products were purified with MicroSpin® S-300 (Amersham Biosciences®), following the manufacturers protocol and the purified products were quantified in GeneQuant® (Pharmacia®). DNA-sequencing was accomplished using ABI PRISM BigDye® Terminator v 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems®) with AmpliTaq DNA polymerase (Applied Biosystems®), in the ABI Prism 3100 DNA sequencer (Applied Biosystems®). Nucleotide sequences were analyzed using Chomas version 1.45 (Technelsyium, 2004) and BLAST (Basic Local Alignment Search Tool) (NCBI/BLAST).

2.5. In vivo activity

2.5.1. Experimental model of tuberculosis infection in mice

All animal work was performed according to the Local Ethical Committee for Experimentation in Animals in Mexico. The tuberculosis model has been described in detail elsewhere [31]. Male Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA), and used at 6–8 weeks of age. A MDR (INH and RFP resistant) clinical isolate from a tuberculous patient which is resistant to all primary antibiotics was used to infect the mice. This clinical isolate was characterized by the mutation Ser531Leu in *rpo*B. This strain was cultured in Youman's modified medium of Proskauer and Beck. Colonies were harvested after 4 weeks and suspended in phosphate-buffered saline (PBS) containing 0.05% Tween 80 by shaking for 10 min with glass beads. The suspension

was centrifuged for 1 min at 350 *g* to remove large clumps of bacilli. Then, a preliminary bacterial count was achieved by smearing the supernatant at a known volume to area ratio, and counting 10 random fields after staining by the Ziehl–Neelsen technique. The suspension was finally diluted to 2.5×10^5 bacteria in 100 μl of PBS and stored at −70 °C. Before use, bacteria were recounted, and viability checked as described [32].

In order to achieve intra-tracheal infection, mice were anaesthetized with 56 mg/kg intraperitoneal pentothal. The trachea was exposed via a small midline incision, and 2.5×10^5 viable bacteria in 100 μl of PBS were injected. The incision was then sutured with sterile silk, and the mice were maintained vertical until the effects of the anesthetic had worn off. Infected animals were housed in groups of 5 in cages fitted with micro-isolators.

2.6. Drug administration

Animals surviving 60 days after infection were randomly allocated to the required treatment groups. Subsequent treatment was started 60 days after infection, and groups of these animals were sacrificed at 1 and 2 months intervals. All data points are means (±SD) from 4 to 6 animals for a representative experiment. Individual drugs were considered effective in their *in vivo* antimycobacterial activity against *M. tuberculosis* if the drug-treated groups showed statistically significant (at least $P \leq 0.05$ level) differences from the untreated control groups. The dosages were determined according to the *in vitro* MICs previously obtained [15], the drug concentration being thereafter adjusted according to the estimated number of bacilli in mice lungs after 2 months of infection. This amount of drug was triplicated considering its dilution after absorption and systemic distribution after oral administration. Two different dosages (5 and 15 mg/kg of body weight) were tested. Elected best profiled RFB analogs **5** and **8** were administered as a suspension, first dissolving the drug in a small volume of ethanol, and then further diluting in 0.05% sodium carboxymethyl cellulose and 0.04% Tween 80 in a total of 0.1 ml administered intra-gastric with a canula daily until the end of the experiments [33].

2.7. Assessment of colony-forming units in infected lungs and preparation of tissue for histology and morphometry

One lung was immediately frozen by immersion in liquid nitrogen and used for colony counting, while the other was perfused with 10% formaldehyde and used for histopathological analysis. Frozen lungs were disrupted in a Polytron homogenizer (Kinematica, Luzern, Switzerland) in sterile 50 ml tubes containing 3 ml of isotonic saline. Four dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Lab code 0627-17-4) enriched with OADC also from Difco code 07-22-64-0. Incubation time was 21 days. Four animals were sacrificed at each time point, in 2 different experiments, so that data points are the means from eight animals.

For histological study, after 2 days of fixation, parasaggital sections were taken through the hilus, and these were dehydrated and embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The area of granuloma and the % of lung affected by pneumonia were measured using a Zidas Zeiss image analysis system. Measurements were done blind, and data are expressed as the mean of 3–4 animals ± SD.

3. Results

3.1. In vitro activity

Strain H_{37}Rv and three clinical *rpo*B wild type isolates (MA03, 359 and 361) were susceptible (MIC ≤ 1.0 mg/l) to RFP and to RFB **1** and all its analogs. Among RFP-resistant strains, only one (353) had no mutation in the sequenced region. This strain showed MIC ≥ 2.0 mg/l to RFP and RFB analogs **3**, **4**, **5**, **7** and **8**. Interestingly it was susceptible to RFB **1** and its *N*-acetyl-derivative **6** (Table 1).

Table 1 summarizes the susceptibility/resistance profiles of H_{37}RV, together with mutated and non-mutated clinical *M. tuberculosis* isolates toward RFP, RFB 1 and developed RFB **1** analogs **3**–**8**.

Only for mutants His526Asp neither RFP, RFB nor any of the RFB analogs were effective.

Strains 307, 311 and 349 with mutation Ser531Leu and strain 308 with mutation His526Asp showed higher resistance levels to RFP, RFB **1** and its analogs **3**–**8**, with MICs ranging from 1.0 to more than 2.0 mg/l. Strain 309 (His526Arg) was susceptible to both RFP and RFB **1** and also to 25-deacetyl-rifabutin **3**, but not to 25-deacetyl-rifabutinol **4** and *N*′-acetyl-RFB **5**.

Strain 344 (Ser522Leu) with the same aa change than strains 307, 311 and 349 but in a different codon, have dissimilar resistance profiles, showing susceptibility to RFB **1** and analogs **5**, **6** and also **8**, the *N*′-acylated derivatives with the exception of the most

Table 1
In vitro assays with RFB derivatives at 2.00 mg/l to 0.06 mg/l.

Mutation	Amino acid change	Strains and isolates	MIC (mg/l)							
			RFP	RFB 1	3	4	5	6	7	8
No mutation	–	H_{37}Rv	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
	–	MA03	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
	–	353	>2.00	0.12	>2.000	2.00	0.12	>2.00	>2.00	>2.00
	–	359	0.12	<0.06	<0.06	0.25	<0.06	<0.06	<0.06	<0.06
	–	361	<0.06	<0.06	<0.06	0.12	<0.06	<0.06	<0.06	<0.06
TCG531TTG	Ser531Leu	307	>2.00	2.00	>2.00	>2.00	2.00	>2.00	>2.00	>2.00
		311	>2.00	2.00	>2.00	2.00	>2.00	>2.00	>2.00	>2.00
		349	>2.00	1.00	>2.00	>2.00	2.00	>2.00	>2.00	>2.00
CAC526GAC	His526Asp	308	>2.00	>2.00	>2.00	>2.00	>2.00	>2.00	>2.00	>2.00
CAC526CGC	His526Arg	309	0.06	0.12	0.25	2.00	0.25	2.00	>2.00	1.00
TCG522TTG	Ser522Leu	344	>2.00	0.06	2.00	>2.00	0.06	0.50	>2.00	0.50
GAC516GTC	Asp516Val	363	>2.00	0.06	1.00	>2.00	0.06	0.50	>2.00	0.25
GAC516GTC	Asp516Leu	352	>2.00	<0.06	0.12	0.50	1.00	>2.00	>2.00	0.25
GAA514AAA	Phe514Phe									
GAC516GTG	Asp516Val	343	>2.00	1.00	>2.00	>2.00	>2.00	>2.00	>2.00	2.00
TCG522TTG	Ser522Leu	364	>2.00	0.25	>2.00	>2.00	0.25	>2.00	>2.00	2.00
CGC529AAG	Arg529Lys Leu530Met	80,014	2.00	0.12	1.00	2.00	0.25	0.50	2.00	0.50
TTG530ATG										

lipophilic one. With respect to double mutated strains, isolate 352 can be considered as a single mutated strain (Asp516Leu), since mutation Phe514Phe corresponds to a silent mutation. Therefore when comparing the results of this strain with those of isolate 363 (Asp516Val), a similar susceptibility profile is observed.

Strains 343 and 364 with the double mutations Asp516Val and Ser522Leu showed different susceptibility. Isolate 343 was only susceptible to RFB and 364 toward RFB **1** and derivative **5**. Another double mutation strain 80014 (Arg529Lys and Leu530Met) was resistant to RFP and susceptible to RFB **1** and analogs **5**, **6** and **8**.

In general RFP-resistant strains were susceptible to RFB **1** and to RFB **5**, this trend followed by RFB **8**.

3.2. In vivo assays

Analogs RFB **5** and **8** were selected to treat mice with tuberculosis and evaluate their efficiency determining pulmonary bacilli loads by counting CFU, and quantifying the extension of tissue damage by the determination of the percentage of lung surface affected by pneumonia.

The treatment began after 2 months post-infection, because at this time post-infected animals were suffering of late progressive disease, with high amount of live bacilli and lung consolidation. Fig. 2 summarizes the results obtained when animals were infected by the intra-tracheal route with a high dose of *M. tuberculosis* RFP-resistant clinical isolate in order to induce progressive disease. The assay involved 3 animal groups for 2 months. The other group received a daily dosage of 5 mg/kg RFB **5**, a second one received the same daily dosage of but of RFB **8** while the third one (the control group) was given only diluent by gavage. Groups of 6 mice were sacrificed after 1 month (white bars) and 2 months (black bars) of treatment, the lungs were removed and processed for bacilli colony-forming units (top panel) and histological analysis by the determination of the percentage of lung surface affected by pneumonia using automated image analysis (bottom panel). The RFB **5** and RFB **8** one month treatment has induced more than three times lower bacilli burden comparing with the control at the same time. After two months treatment the bacilli burden between the groups receiving treatment with RFB **5** and RFB **8** and the control, had been reduced to about half. RFB **8** after one month treatment, is slightly more potent than RFB **5**. Although it was not significant, after 2 months of treatment with RFB 5 or RFB 8, the lungs of mice showed 30% and 15% lesser surface area affected by pneumonia respectively than control non-treated animals.

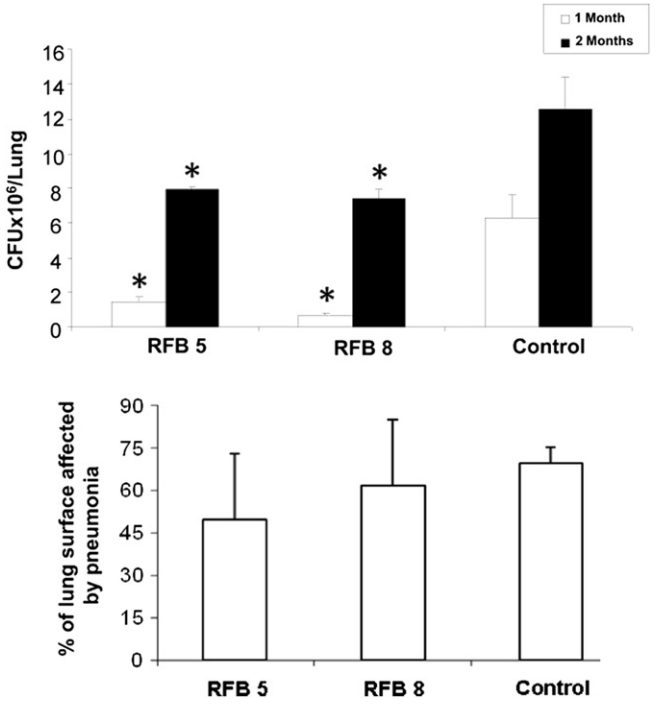


Fig. 2. Control corresponds to groups of Balb/c mice infected with a MDR-MTB strain but not included in a treatment regimen. The one month treatment results reproduce others previously reported [15], in a study conducted in parallel, with RFB **1** as a standard, involving only one month of monotherapeutic treatment. Here we report specifically the effect of continuing administration for 1 and 2 months of RFB **5** and RFB **8** at the dosage of 5 mg/kg on bacterial loads and pathology during advanced disease in the lungs. Each point corresponds to the mean and SD of four mice per group. Asterisks represent statistical significance between treated and control groups ($p < 0.05$).

4. Discussion

A molecular weight below 500 (mean 360), 48 atoms and a preferred value of log *P* 1.3–4.1 (mean 2.3) are generally desirable antimycobacterial candidate attributes (7). Calculated [15] computational value *C* log *P*, as a primary predictive index of LHB (Lipophile Hydrophile Balance) which represents the octanol solubility of each compound in the presence of aqueous solute, was considered for the set of analogs under analysis. The less lipophilic in the group of tested compounds were 25-deacetyl-rifabutin **3** (*C* log *P* 3.68) and 25-deacetyl-rifabutinol **4** (*C* log *P* 3.47). On the other hand, RFB **1** has similar LHB to *N*′-acetyl-rifabutinol **6** (*C* log *P* 4.34) and quite similar to *N*′-acetyl-rifabutin **5** (*C* log *P* 4.35). Higher *C* log *P* values were found for *N*′-palmitoyl-rifabutin **7** (*C* log *P* 7.50) and *N*′-(undec-10″-enoyl)-rifabutin **8** (*C* log *P* 7.33) which hold the largest hydrophobic C15 and C11 respectively, *N*′-alkyl substituent in the molecular structure of the rifabutin backbone.

Thus, taking into account the effect of lipophilicity of the RFB analogs **3**–**7** under study to targeting the MDR-TB, as an indicator of hydrophobic pharmacophore features, it is noticeable that the less lipophilic RFB analog **3** showed lower activity to resistant strains than the parent compound RFB **1**, and the most lipophilic, the *N*′-acylated compound **7**, presented no activity against mutant *M. tuberculosis* clinical isolates at the tested concentrations, according to Table 1.

Analogs having a LHB comparable to RFB **1**, that is, compounds *N*′-acetyl-RFB **5** and *N*′-acetyl-rifabutinol **6**, were active against susceptible and some resistant strains. Being a good indicator of drug permeability, hydrophobic features do not explain the differences encountered but at least compounds with LHB similar to RFB **1** seem better suited for further development.

Trying to understand the pathogen interaction with RFB **1** and its analogs, a quite dissimilar activity profile to both RFP susceptible or resistant strains was noticed. *M. tuberculosis* isolate 353, without mutations in *rpoB* was resistant to RFP and to the remaining RFB analogs tested with the exceptions of RFB **1** and *N*′-acetyl-RFB **5**. Despite the absence of apparent genetic alterations, RFP-resistant strains had already been described by other authors [34,35] and might be considered as a consequence of additional mutations at another target site located outside the core region of *rpo*B. However, other molecular mechanisms such as changes in permeability, increase of efflux or mutations in other RNAP subunits should not be excluded.

A genetic alteration in another locus outside the Rifamycin Resistance Directed Region (RRDR), the RNAP region that was evaluated in this study, may also be a reason for the different susceptibility against RFB **1** and RFB **5**, shown by strains 343 and 364 sharing the same double mutation (Asp516Glu and Ser522Leu). In fact, it has been observed that mutation Ser522Leu increases the

mutation frequency and changed the spectrum of mutations outside the 81 bp hotspot [36].

Regardless of mutation His526Arg, strain 309 was susceptible to RFP, RFB **1** and analogs **3** and **5**, while isolate 308 with mutation His526Asp presented high-level resistance to all the compounds tested. According to our belief, this suggests that either aspartic acid could have a critical role at the 526 codon site, or a proximity of the aa codified by codon 526 of the antibiotic molecule as well as its nature and size for a role in the interaction. Other studies have also shown that resistant phenotype of strains with change at codon 526 depends on the type of aa substitution [35,37]. The reason for this difference of susceptibility may result from the highest resemblance from the physicochemical view point of histidine and arginine, both possessing positively charged terminal chains whereas the carboxylate group of aspartic acid is negatively charged, as depicted in Fig. 3. These are undoubtedly significant positive and negative features to be considered in the pharmacophore preview.

Both His526 and Arg529 are very important β-subunit residues, involved in RNAP/Rifs complex, through hydrogen bonding with the Rifs ring system oxygen atoms as determinants. His526 mutants are considered "affinity mutants" since they interfere with crucial protein polar/hydrophobic RNAP/Rifs interactions. According to Artsimovitch et al. [21] substitution of this side chain residue by a smaller one, as is here the case with strain 308 (replacement of His by Asp), confers, as we have testified, strong Rifs resistance.

The residue Ser531 seems also a crucial point for the donor/acceptor feature-point positioning interaction of the drug ligand with DNA-dependent RNAP, judging by the high levels of resistance shown to all the compounds tested. Indeed, codon 531 mutation has been described as being most frequently responsible for RFP resistance in different studies, resulting in strains invariably resistant to RFP, RFB **1** and other Rifs [37–39]. These β subunit residue mutants are referred to [21] as the most prominent example of "steric" mutants, responsible for reducing the conformational space

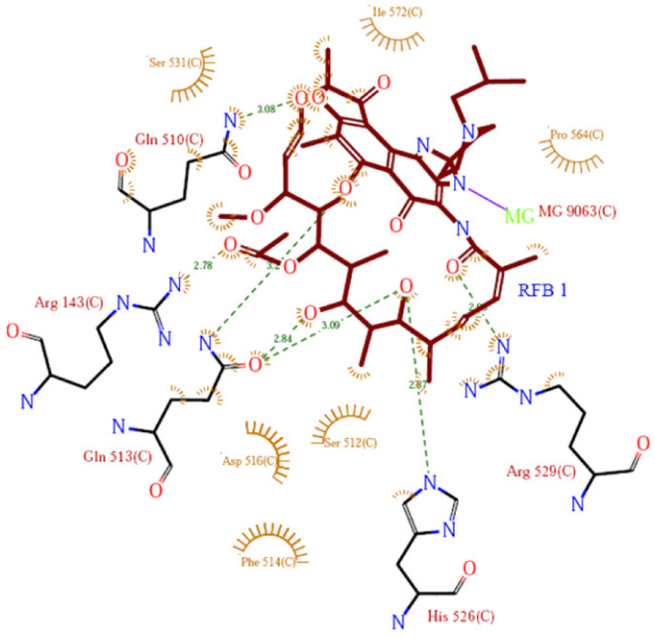


Fig. 3. Adapted Ligplot diagram (*Escherichia coli* numbering, Campbell et al. [9]) representing the interactions of RFB **1** with distance interacting residues of a *Thermus thermophilus* RNAP chain (Chain C) in a crystal (2A68 PDB file: 10.2210/pdb2a68/pdb). Hydrogen bonds are represented by dashed green lines while non-specific van der Waals interactions between the RNAP rpoB aa and the RFB **1** backbone are in orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

variability in Rifs binding site due to a common exceedingly inflexible ring system of Rifs [18,40]. In isolate 311 with Ser531Leu mutation, aa differ in the terminal group: a hydroxyl group in the case of Ser and an isopropyl group in Leu. Expression of this mutation might have lead to the inexistence of hydrogen bonding because of a non-polar/hydrophobic isopropyl group was present instead of a polar/hydrophilic group. This is, a significant hydrogen-bond acceptor feature to be included in the chromophore through the lone pairs acceptors on probably one of the O1, O8, O21, O23 oxygen atoms (most likely those in the naphthol ring) frequently referred as responsible for the interaction of Rifs with RNAP.

Isolate 344 which has got the same aa substitution as strain 311 although in a different β-subunit residue (Ser522Leu), was resistant to RFP but susceptible to RFB **1** and *N*′-acyl analogs **5**, **6** and **8**, a quite expected result [41]. This apparent absence of cross-resistance between RFP and RFB **1** and analogs **5**, **6** and **8**, in mutations leading to aa change of residue Ser522, seems similar to what is known to happen in mutations affecting the residues Asn519 and Asp516 [37,42]. Since these altered residues cluster is located on the side of the invariant ansa bridge that lies opposite to Rifs tail, the observed differences between the sensitivity to RFP and RFB **1** were until recently considered as the result of the development or not of an allosteric action. Feklistov et al. [23] disruption of such a "romantic" theory leads to the existence or absence of van der Waals interaction between the above mentioned residues and the ansa bridge of Rifs as the best explanation to the previous theoretical approaches.

The author's opinion regarding the above differences is that these could be dictated by the closeness of interacting/non-interacting Rifs' groups belonging to the chromophore ansa bridge and residues Asp 516, Asn519 and Ser522. Consequently, these dissimilarities should be most likely understood as the result of the differences between the conformation assumed by RFB **1** and analogs and RFP, arising not only from the tail differences between RFB **1**/RFB analogs (isobutylspiropiperidyl tail) and RFP (4-methyl-piperazinaminyl tail), but also from particular transformations (deacylations, acylations, reductions) made in RFB **1** analogs. The overall "fitting" of the molecules at the active site should contribute also to global conformational changes. Within this rational mutation Ser522Leu has a remarkable effect to be considered in pharmacophore design.

In isolates 352 and 363 that are characterized by Asp516Val and Asp516Leu point mutations respectively, the replacing residue is uncharged and has thus more apolar/hydrophobic character than the original one. These results are in accordance to Artsimovitch et al. [21] who had already proposed that, despite the location of Asp516 being at an interacting distance with Rifs chromophore, it only established weak van der Waals connections with the Rifs backbone, and therefore its substitution by a smaller Val or isosteric Leu should not dramatically affect the Rifs affinity.

Worthy of note are mutations Asp516Val and Asp516Leu different effects on the susceptibility of the tested compounds: deacyl analogs **3** and **4** (ansa bridge alteration) observed susceptibilities vary in the opposite measure as *N*′-acyl analogs **5** and **6** from strain 363 to strain 352. This may be appropriately explained as the result of proximity to the 516 β-residue of the ansa bridge, favoring the non-interaction of the shorter Val over the interaction of isosteric Leu both of which oppose physicochemical characteristics with respect to the polar hydroxide group of deacyl analogs. The same logical rule may be applicable to *N*′-acyl analogs, where the *N*′-acyl moiety is located on the same side of RFB **1** tail, that accordingly to others [21] interact with a Asp516/σ hairpin loop of another RNAP. In this case, this may favor a better interaction through van der Waals contacts in the pharmacophore space provided by a longer aa (Leu), with an apolar/hydrophobic terminal *N*′-acyl group.

Isolate 80014 with double mutation in codons 529 and 530 is not particularly resistant to some of the screened compounds. We associate this fact with the relatively close chemical equivalence between the aa residues replaced in both cases. The sensitivity to RFB **1** and resistance to RFP have already been documented in other studies with Arg529 mutants [35].

Additionally, in the case of this double mutation it may be considered foreseeable that more than the Leu530 mutation, it could be the motif Arg529 the main responsible for the decreased susceptibility of RFB **1** and analogs **5**, **6** and **8**, and for the resistance development to RFP and analogs **3**, **4** and **7**. The last residue Arg529 establishes a stable hydrogen bond with Asp516 in the Rifs/RNAP complex. The aa change (Arg → Lys), despite the close resemblance from the physicochemical view point, might be considered sufficient to disfavor the hydrogen bond between residues 516 and 529, closer upon Rifs binding points, and consequently the cascade of events that culminates with the inhibition of transcription.

Interestingly, when analyzing the set of RFB analogs **3**–**8**, we notice that in all the cases where the isolate shows at least some susceptibility to RFB **1** (MIC < 1 mg/l), the C11-furanone seems to give a contribution to a lowering outcome of the overall resistance when compared to C11-furanol analogs. These results are in line with the results of 2D-NMR studies reported by Santos et al. [19], showing that NMR coupling constants for C27–C28 of C11 furanol analogs differ from C11 furanone equivalents, indicating a different spatial arrangement of the ansa bridge and in particular of the O21 and O23 oxygen atoms that interact with RNAP active sites. Furthermore, one would expect that the hydroxyl group from furanol to be considered a better hydrogen-bond donor, while for furanone there is a strong argument for excluding such type of oxygen atoms, for which theoretical and crystallographic evidence indicates as very weak acceptors [43]. Recent studies [44] corroborate the main established SAR conclusions, namely the importance of Rif C-8 hydroxyl role in hydrogen bonding in accordance with results we have also reported previously [40].

The concept that Ser531 may be a crucial point for the donor/acceptor feature-point positioning interaction of the drug ligand with DNA-directed RNA Polymerase (DDRP), judging by the high levels of resistance shown to all the compounds tested, prompted further experiments aiming to understand if it would be likely to play an important role in a Ser531Leu mutant pulmonary infected mice model. *In vivo* activity assays discussed here are complementary to other, run in parallel using RFB **1** as comparison, which were published elsewhere [15]. Here the goal was to observe the effect of extending the treatment extension with RFB **5** and RFB **8** for another month (2 months treatment), in order to select the compounds that showed similar to better activity (higher CFU reduction) than RFB 1 after 1 month treatment.

Treatment of infected mice with RFB analogs **5** or **8**, produced a significant reduction of bacilli loads after one month of treatment when compared with control non-treated mice. We observed three-fold more CFU after 2 months of treatment when compared to the first month of treatment, suggesting selection of drug-resistant bacilli after 60 days of treatment. However, this bacilli burden is still significantly lower when compared to control non-treated mice. In fact, animals infected with the RFP-resistant strain showed significant CFU reductions when compared with control animals, although it was more modest, only 40%, along with non-significant lesser tissue damage after 2 months of treatment. Taking into account that we only used these new analogs in monotherapy, the results were considered promising enough and prompted our interest in mutant selectivity studies as selection criteria to run the current experiments for the design of the pharmacophore of new anti-TB drugs, complemented by dose–response studies and pre-clinical studies.

In brief, the present results add some new highlights on the MoA of the RFB *N*-acyl derivatives **5** and **8** which are active *in vivo* against different susceptible strains and some RFP-resistant strains when compared to RFB **1**. Potential applications of a rational strategy based on the construction of appropriate electrostatic and/or C–H hydrogen bonding to complement non-specific van der Waals interactions at the RNAP, would still contribute for valuable pharmacophore elucidation. A mix of steric, chemical, hydrophobic, and dynamic influences that result in a mechanism by which mutations induce drug resistance, needs conformational studies to be validated. Further molecular modeling taking advantage of recent Rifs-*T. thermophilus* RNAP complex data or eventually through co-crystallization of structures with RNA polymerase, should lead to more precise candidate alignment algorithms.

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