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Synthesis and evaluation of rifabutin analogs against *Mycobacterium avium* and $H_{37}Rv$, MDR and NRP *Mycobacterium tuberculosis*

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ABSTRACT

Clinical utility of rifabutin **1** (RBT), a potent antibiotic used in multidrug regimens for tuberculosis (TB) as well as for infections caused by *Mycobacterium avium* complex (MAC), has been hampered due to dose-limiting toxicity. RBT analogs **2–11** were synthesized and evaluated against *M. avium* 1581 and *Mycobacterium tuberculosis* susceptible and resistant strains in vitro. A selection of candidates were also assayed against non-replicating persistent (NRP) *M. tuberculosis*. Subsequent in vivo studies with the best preclinical candidate drugs **5** and **8**, in a model of progressive pulmonary tuberculosis of Balb/C mice infected either with $H_{37}Rv$ drug–sensible strain or with multidrug resistant (MDR) clinical isolates, resistant to all primary antibiotics including rifampicin, were performed. The results disclosed here suggest that **5** and **8** have potential for clinical application.

1. Introduction

Tuberculosis (TB) is still the leading killer infectious disease in the world. Globally, 9.2 million new cases and 1.7 million deaths from TB occurred in 2006, of which 0.7 million cases and 0.2 million deaths were in HIV-positive people. There were an estimated 14.4 million prevalent cases of TB with 0.5 million cases of multidrug-resistant TB (MDR-TB) in 2006.[1]

Globally, the HIV and TB epidemics create a public health crisis of enormous proportions. The therapeutic options for latent TB infection are similar to those for HIV-uninfected persons. However, because rifampicin (RIF) induces the cytochrome *P*450-3A (CYP3A) system in the liver and intestinal wall, it increases the metabolism of both protease inhibitors and non-nucleoside reverse transcriptase inhibitors. This effect is weaker with rifabutin (RBT) than with RIF.[2,3]

Consequently, for the development of new anti-tuberculosis drugs, the following points have currently been recognized to be of particular importance: (1) drugs which display lasting anti-mycobacterial activity in vivo, since they can be administered intermittently and consequently facilitate directly observed therapy and enhance patient compliance; (2) novel anti-tuberculosis compounds to fight MDR-MTB; and (3) the use of new anti-TB drug classes that are able to eradicate the slowly metabolizing and, if possible, dormant populations.[4]

Unfortunately, no new drugs except RBT **1** and rifapentine (RPT) have been successfully marketed for TB during the 40 years after introduction of RIF. The limitations of current therapies are the long treatment duration that is required to sterilize tissues, typically six months. This extended period of treatment is a direct result of the lifestyle of the bacilli, some of which may enter a 'dormant' or 'persistent' phase after the initial infection[5] and the concomitant immune response.

In this context, an important feature of the rifamycins is that they are active against both actively growing and slow-metabolizing non-growing bacilli. Their activity against the latter is thought to be involved in shortening the TB therapy from 12–18 months to 9 months.[6] RBT **1** like RIF inhibits bacterial transcription by binding to the β-subunit of bacterial DNA-dependent RNA-polymerase. RBT **1** has been useful against some isolates of RIF-resistant MDR-MTB, furthermore it is effective in prevention and treatment of disseminated atypical mycobacterial infection in AIDS patients.[7,8] With the present study we provide new insights for leads aiming TB and MDR-TB therapeutics based on the scaffold of RBT **1** that is

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modified to target *Mycobacterium avium* and *Mycobacterium tuberculosis*.

2. Results and discussion

2.1. Synthesis

From RBT **1** we prepared RBT analogs **2–11** aiming at better physicochemical properties as anti-mycobacterial drug candidates to overcome the biological barriers more efficiently than the lead. Few of them (**5–8**) may be further valuable as prodrugs *per se* thus requiring some form of molecular activation on site, for example, the activation by esterases or other hydrolytic cleavage of reactive bioreversible acyl esterified R^3 groups (Fig. 1) introduced to improve RBT **1** pharmacokinetics. The strategy developed has taken into account the well-known involvement of oxygenated functions in the interaction with the bacterial DNA-dependent RNA polymerase (DDRP).[9] Thus, we selected three sites which are known not to interfere in the active configurational requirements, and obtained new **5–11** structural analogs of RBT **1** by modifying through funcionalization and N′-acylation at the imidazolyl ring, deacylation at the ansa chain (C-25), reduction at the furanone ring, and N-oxidation at the *N*-piperidyl ring. Reaction conditions are summarized in Schemes 1 and 2.

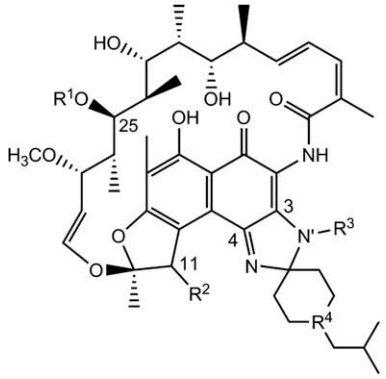
Stereoselective reduction of the furanone in RBT **1** afforded **2**, and the deacetylation (alkaline hydrolysis) of this gave derivatives **3** and **4**, respectively. Synthesis and conformational studies of compounds **2**, **3,** and **4** had already been described.[10,11] Both the 25-deacetyl derivative **3** and the N-oxide **11** were previously identified as urinary metabolites of RBT **1**.[12] Besides the N-oxide **11**, we report here for the first time the syntheses of N-oxides **9** and **10**.[13]

Lipophilicity as octanol solubility of a given compound in the presence of water, measured by CLog*P*, is also seen as one of the most important parameters that affect anti-mycobacterials. According to Barry et al., the majority of the anti-mycobacterial agents have a Log*P* comprised between 2.0 and 4.5. Using the programme ALOGPS2.1 http://146.107.217.178/lab/alogps/start.html, 08/04/2008) we have estimated the average CLog*P* for RBT **1** and its derivatives. The results are summarized in Table 1.

With the goal of creating feasible and economically viable processes, and starting from RBT **1** and rifabutinol **2**, four N′-acylated derivatives **5–8** were prepared in nearly quantitative yields using conventional techniques for selective acylation of the secondary amine (Scheme 1). Using a different synthetic route, alkyl and aryl derivatives at the same amine of the imidazolyl ring were previously described by others.[15]

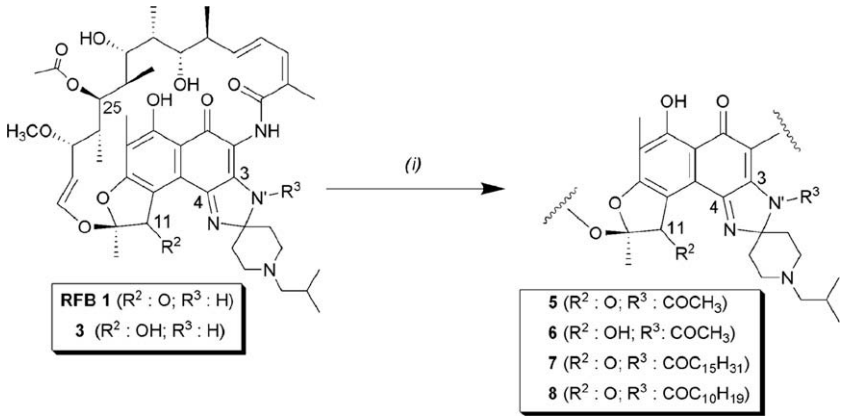
Owing to the presence of available electron pairs, heteroatoms are good acceptors of oxygen. Taking advantage of this knowledge, in situ-generated dimethyldioxirane, an electrophilic oxidant, was used to selectively oxidize the tertiary amine function[16–18] on RBT **1,** or its derivative rifabutinol **2** producing both N-oxides **9** and **10** in 51% yield and N-*o*xide **11**, obtained from 25-deacetyl-rifabutin **3**, in 19% yield (Scheme 2).

We have thus obtained compounds **2–11** as novel RBT **1** analogs, with a structural diversity where attendant differences in physicochemical variables such as, but not limited to, polarity, electronic field effects, lipophilicity, stereochemical bulk, and hydrogen bond acceptor and donor potentials are significant[14].

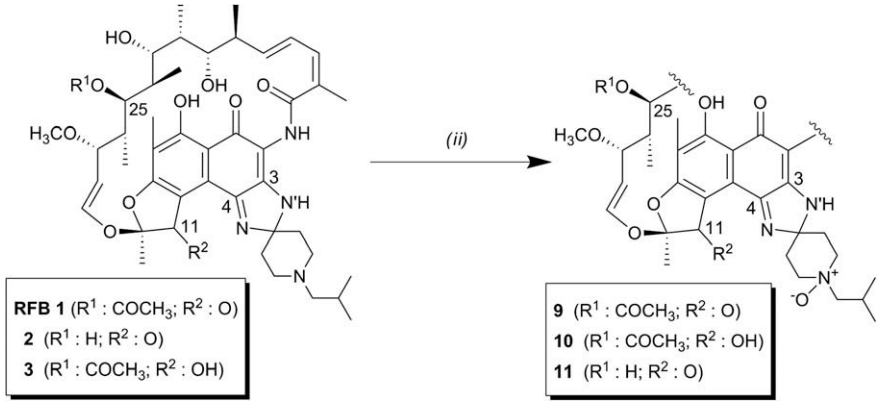


1: Rifabutin (R^1: $COCH_3$; R^2: O; R^3: H; R^4: N)
2: Rifabutinol (R^1: $COCH_3$; R^2: OH; R^3: H; R^4: N)
3: 25-deacetyl-rifabutin (R^1: H; R^2: O; R^3: H; R^4: N)
4: 25-deacetyl-rifabutinol (R^1: H; R^2: OH; R^3: H; R^4: N)
5: *N′*-acetyl-rifabutin (R^1: $COCH_3$; R^2: O; R^3: $COCH_3$; R^4: N)
6: *N′*-acetyl-rifabutinol (R^1: $COCH_3$; R^2: OH; R^3: $COCH_3$; R^4: N)
7: *N*′-palmitoyl-rifabutin (R^1: $COCH_3$; R^2: O; R^3: $COC_{15}H_{31}$; R^4: N)
8: *N′*-(undec-10′-enoyl)-rifabutin (R^1: $COCH_3$; R^2: O; R^3: $COC_{10}H_{19}$; R^4: N)
9: Rifabutin *N*-oxide (R^1: $COCH_3$; R^2: O; R^3: H; R^4: N^+-O^-)
10: Rifabutinol N-oxide (R^1: $COCH_3$; R^2: OH; R^3: H; R^4: N^+-O^-)
11: 25-deacetyl-rifabutin *N*-oxide (R^1: H; R^2: O; R^3: H; R^4: N^+-O^-)

Figure 1. Rifabutin (RBT **1**) and derivatives.



Scheme 1. Reagents and conditions: (i) ClR^3, cat.; THF.



Scheme 2. Reagents and conditions: (ii) oxone, acetone, BTEAC/$NaHCO_3$; CH_2Cl_2/H_2O.

Table 1
Computer estimated CLog*P* for RBT **1** and derivatives **2–11** (cf. Fig. 1)

Compound	CLog*P*s	Formula	*M* (g/mol)	R^1	R^2	R^3	R^4
RBT **1**	4.25	$C_{46}H_{62}N_4O_{11}$	847.02	$COCH_3$	O	H	N
2	4.23	$C_{46}H_{64}N_4O_{11}$	849.04	$COCH_3$	OH	H	N
3	3.68	$C_{44}H_{60}N_4O_{10}$	804.97	H	O	H	N
4	3.47	$C_{44}H_{62}N_4O_{10}$	806.98	H	OH	H	N
5	4.35	$C_{48}H_{64}N_4O_{12}$	889.04	$COCH_3$	O	$COCH_3$	N
6	4.34	$C_{48}H_{66}N_4O_{12}$	891.06	$COCH_3$	OH	$COCH_3$	N
7	7.50	$C_{62}H_{92}N_4O_{12}$	1085.51	$COCH_3$	O	$COC_{15}H_{31}$	N
8	7.33	$C_{57}H_{80}N_4O_{12}$	1013.58	$COCH_3$	O	$COC_{10}H_{19}$	N
9	3.14	$C_{46}H_{62}N_4O_{12}$	863.00	$COCH_3$	O	H	$N^+–O^-$
10	3.24	$C_{46}H_{64}N_4O_{12}$	865.02	$COCH_3$	OH	H	$N^+–O^-$
11	2.71	$C_{44}H_{60}N_4O_{11}$	820.97	OH	O	H	$N^+–O^-$

Their potential contribution to the anti-mycobacterial activities is studied herein.

2.2. Biological evaluation

The minimum inhibitory concentrations (MICs) of compounds **1–11** against *M. avium* and *M. tuberculosis* were evaluated.

Table 2
In vitro activity of RBT analogs against *Mycobacterium avium* 1581

Compound	*Mycobacterium avium*	
	Concentration (μg/mL)	Inibition (%)
RBT **1**	0.1	>99
	0.2	>99
2	0.2	86
	0.4	>99
3	0.2	91
	0.4	>99
4	0.2	n.t.[a]
	0.4	—[b]
5	0.2	>99
	0.4	>99
6	0.2	54
	0.4	>99
8	0.2	60
	0.4	>99
9	0.2	n.t.[a]
	0.4	—[b]
10	0.2	44
	0.4	53
11	0.2	n.t.[a]
	0.4	—[b]

[a] Not tested.
[b] Number of colonies comparable to that in plates without antibiotic.

2.2.1. Anti-*Mycobacterium avium* activity

Susceptibility of *M. avium* to compounds **1–11** was assayed (Table 2). *N*′-Acetyl-rifabutin **5** appears to be as active as RBT **1** against *M. avium*, at concentrations of 0.2 and 0.4 μg/mL, followed by 25-deacetyl-rifabutin **3**. The presence of a hydroxyl group, resulting from the hydrolysis of the acetyl moiety at the ansa chain, seems to have different impact on the *M. avium* activity depending on the presence of the furanone or furanol at C_{11}. Accordingly, compound **3** by comparison with RBT **1** is slightly less active against *M. avium* (with 91% inhibition at the lowest concentration tested). With respect to the derivatives obtained from the acylation of the imidazolyl secondary amine, from Table 2 it can be observed that *N*′-acetyl-rifabutin **5** is most active followed by *N*′-undec-10″-enoyl-rifabutin **8** and *N*′-acetyl-rifabutinol **6**.

With the exception of the N-oxide **10**, N-oxidation products are inactive against *M. avium* even at the highest concentration tested. The N-oxide **10** represents also the unique case where a C_{11}-furanol derivative shows higher activity than the homologous RBT **1** derivative. A possible explanation for this result may be related to hydrogen bonding between the C_{11}-hydroxyl and the oxygen of the N-oxide, balancing the global hydrophilicity of the molecule rifabutin N-oxide **9**. Apparently, electronic effects in the piperidyl branch of RBT **1** have little or no effect, but a predictable effect caused in lipophilicity measured by the drop of CLog*P* (Table 1) appears to be a potential factor associated to the lost activity of N-oxides.

2.2.2. Activities against *Mycobacterium tuberculosis* susceptible strain H_{37}Rv *and multidrug-resistant* clinical isolates

The activities of the compounds **1–11** against *M. tuberculosis* H_{37}Rv, which show good correlation with Bactec radiometric methods,[19] are reported in Table 3.

Table 3
In vitro activity of RBT analogs against *M. tuberculosis* H37Rv and MDR-MTB

Compound	MIC (μg/mL)		
	MTB	MDR-MTB	
	$H_{37}Rv$	Strain A[a]	Strain B[b]
RBT **1**	<0.013	>10.0	>10.0
2	<0.05	n.t.[c]	n.t.[c]
3	0.025	>10.0	>10.0
4	0.2	>10.0	>10.0
5	<0.013	>10.0	>10.0
6	0.1	8.0	8.0
7	0.1	>10.0	>10.0
8	0.1	6.0	6.0
9	0.2	>10.0	>10.0
10	0.6	>10.0	>10.0
11	0.6	>10.0	>10.0

[a] MTB-MDR strain A (Isoniazid—INH; Rifampicin—RIF, Streptomycin—SM resistance).
[b] MTB-MDR strain B (INH, RIF, Ethambutol—EMB resistance).
[c] Not tested.

Most RBT derivatives were active against *M. tuberculosis* $H_{37}R_v$ in the assay at concentrations as low as 0.1 μg/mL, with the more polar N-oxide derivatives **9**, **10,** and **11** being an exception. The compounds that showed higher activity were RBT **1** and *N*′-acetyl-rifabutin **5**. As for *M. avium*, *N*′-acetyl-rifabutin **5** appears to be as active as the lead compound (RBT **1**), at least at the lowest concentration tested (0.013 μg/mL).

From Table 3 it is also observed that the compounds *N*′-acetyl-rifabutinol **6** and *N*′-undec-10″-enoyl-rifabutin **8** exhibited higher activities against MDR clinical isolates having a MIC value of 8 and 6 μg/mL, respectively.

2.2.3. Activity against non-replicating *Mycobacterium tuberculosis*

The tendency for worse activity of C_{11}-furanol derivatives lead us to just consider the evaluation of RBT **1** and its analogs **3**, **5**, **7**, **8**, and **9** for their activity against non-replicating persistent tuberculosis (NRP-TB) by the luciferase-based low-oxygen recovery assay (LORA) (Table 4).

Both the luciferase and CFU endpoints indicated that RBT **1** and all its derivatives were more active against NRP-TB than even RIF, with *N*′-undec-10″-enoyl-rifabutin **8** demonstrating an activity similar to that of RBT **1**. However, *N*′-undec-10″-enoyl-rifabutin **8** also appears to be more cytotoxic than RBT **1**.

As shown in Table 4, RPT is less cytotoxic than any of the RBT derivatives, even the *N*-acetyl-rifabutin **5**. The latter appears to be, by far, the RBT structure analog that is less toxic toward Vero cells (higher IC_{50} value), despite presenting narrower therapeutic window than RBT **1**, similar to all the other RBT derivatives tested.

Table 4
Activity against non-replicating *M. tuberculosis* (NRP-MTB) and mammalian cell cytotoxicity of RBT **1** and its derivatives

Compound	Activity against non-replicating cultures		Toxicity for Vero Cells	SI[d]
	LORA MIC[a] (μM)	CFU MBC[b] (μM)	IC_{50} (μM)[c]	LORA
RBT **1**	0.08	0.05	>46.6	>582
3	0.29	0.18	>46.6	>161
5	0.28	0.18	>92.7	>331
7	1.08	0.14	>34.8	>32
8	0.08	0.08	36.9	462
9	0.44	0.28	>35.8	>82
RIF	1.41	0.39	138.3	98

[a] Minimum inhibitory concentration (luciferase readout).
[b] Minimum bactericidal concentration determined by quantification of colony-forming units (CFUs).
[c] Fifty percent inhibitory concentration (cytotoxicity toward Vero cells).
[d] Selectivity index (IC_{50}/MIC).

2.2.4. In vivo activity

The above described in vitro experiments showed that RBT analogs **5** and **8** were most effective of all RBT derivatives, so they were tested in a model of progressive pulmonary tuberculosis in Balb/c mice. Tuberculous mice were treated with either RBT **5** or RBT **8** and their efficiency was compared with animals treated with RBT **1**. Their efficacy was evaluated through the determination of pulmonary bacilli loads by counting colony-forming units (CFUs), and by quantifying the extension of tissue damage by the determination of the percentage of lung surface affected by pneumonia in animals infected with drug-sensitive $H_{37}Rv$ or a MDR clinical isolate. Mice were infected by intratracheal injection using a high dose (2.5×10^5 live bacilli), and the treatment was started after two months post-infection, when animals were suffering ongoing progressive disease with high amount of live bacilli and lung consolidation. Animals received daily administration of RBT **1**, or RBT analogs **5** or **8** in two different doses (5 or 15 mg/kg) by nasogastric tube, and one month after treatment, groups of five animals were sacrificed, and one lung (right or left) was used for determining lung bacilli loads and the other was used for histopathology.

The lowest dose (5 mg/Kg) produced better results than the higher dose (15 mg/kg). Mice infected with drug-sensitive strain $H_{37}Rv$ treated with RBT **5** showed a similar CFU reduction than those treated with RBT **1**, producing a significant threefold reduction of lung bacilli loads in comparison to the control non-treated animals, with non-significant lesser pneumonia (Fig. 2), while RBT **8**-treated animals showed a similar CFU and a lung surface affected by pneumonia in relation to the mice in control group.

In animals infected with the MDR strain, the treatment with RBT **1** or its derivatives **5** and **8** showed similar efficient activity, producing a significant fivefold lesser CFU than in control mice, and lesser but not significant reduction of pneumonia (Fig. 3).

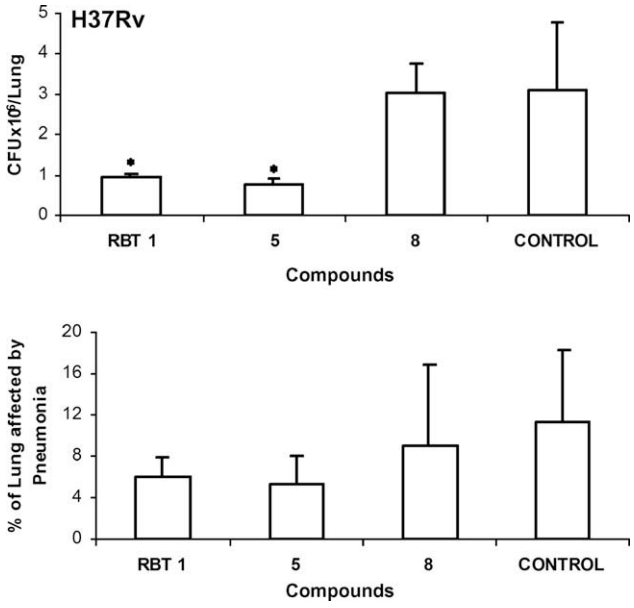


Figure 2. Effect of the administration of RBT **1** and its analogs **5** and **8** at the dosage of 5 mg/Kg on bacterial loads and pathology during advanced disease in the lungs from mice infected with MTB $H_{37}Rv$ strain. Each point corresponds to the mean and SD of four mice per group. Asterisks represent statistical significance between treated and control groups ($^{*}p \leqslant 0.05$).

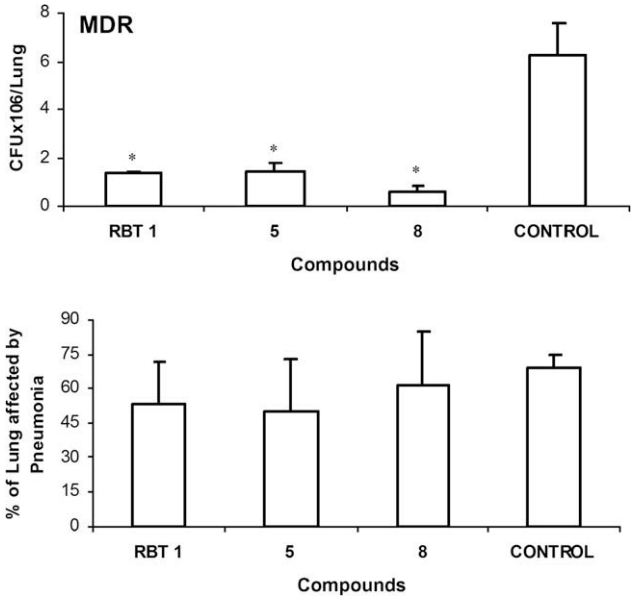


Figure 3. Effect of treatment with RBT **1** or its derivatives **5** and **8** at the dosage of 5 mg/Kg on the lung bacillary loads and pathology during late disease produced by MDR mycobacterial isolate. Each point corresponds to the mean and SD of four mice per group. Asterisks represent statistical significance between each treated group and the control group ($^{*}p \leqslant 0.05$).

3. Conclusions

As a contribution to a further understanding of the mechanisms involved in the action of current anti-mycobacterial therapeutics with RBT **1** and structurally related anti-mycobacterials, the present work examined the activities of a series of RBT analogs based on a concomitant prodrug design strategy able to take advantage of natural esterases and amidases for enhancement of intracellular uptake and efficacy.

With respect to the *M. avium* assay, we have noticed that furanone compounds were more active than the corresponding furanol derivatives, namely RBT **1** versus rifabutinol **2**, 25-deacetyl-rifabutin **3** versus 25-deacetyl-rifabutinol **4** and also with *N*′-acyl derivatives **5** and **6**. With the exception of rifabutinol N-oxide **10**, it appears that the N-oxides have no effect on the *M. avium* strain tested.

In vitro experiments with susceptible *M. tuberculosis* strain showed *N*′-acetyl-rifabutin **5** as the best drug candidate, with a MIC value below the lowest concentration tested as is the case of RBT **1.** *N*′-acetyl-rifabutin **5** is after RIF the least cytotoxic of all the compounds tested including RBT **1**, being also active against non-replicating MTB.

In the assays with MDR-MTB strains, two compounds proved to be better than RBT **1**, *N*′-acetyl-rifabutinol **6** and *N*′-undec-10″-enoyl-rifabutin **8**, with MICs of 8.0 μg/mL and 6.0 μg/mL, respectively. The latter exhibits activity equal to that of RBT **1,** with a LORA-based MIC of 0.08 μM, against dormant/non-growing/non-replicating MTB and the highest SI value after this. Small differences in MIC values may have reflection in future dosages of the medicinal formulation according to the clinical development.

In vivo assays were performed with the best in vitro candidates **5** and **8**. In conclusion, after one month of treatment, *N*′-acetyl-rifabutin **5** was most active against MTB H_{37}Rv strain, promoting a statistically significant reduction of lung bacilli load. This compound also showed good activity against *M. tuberculosis* MDR strain, while corresponding to the previously observed in vitro results, compound *N*′-undec-10″-enoyl-rifabutin **8** was more efficient against MDR strains, inducing lower bacilli loads than RBT **1** and RBT **5**.

In summary, compounds **5**, **6, 8,** and **9** were found to offer new perspectives as prodrug candidates for pharmacokinetics studies and as leads for the understanding of molecular structure particularities influencing the therapeutic potential for diseases where RBT **1** has been currently indicated and administered. These studies provided preclinical candidates with improved potency, the antibiotics **5** and **8,** which were selected for in vivo assessment of efficacy and showed potential to be further studied for use with conventional chemotherapy in synergy for shorter treatment against susceptible MTB, or in combination with other second-line antibiotics to treat MDR tuberculosis mice.

4. Materials and methods

4.1. General

All the reagents and the solvents used were analytical pure products. The reactions were followed by thin layer chromatography (TLC) on Merck 60 F254 (0.2 mm), silica gel pre-prepared plates. The isolation of the products was performed by column chromatography using silica gel Merck 60 (230–400 mesh, 0.04–0.063 mm), at normal atmospheric pressure or flash with nitrogen pressure, or also by preparative thin layer chromatography on silica gel Merck 60 F_{254} (0.5 mm) plates. In the case of 25-deacetyl-rifabutin N-oxide **11**, column chromatography purification was performed with RP-18 silica of Fluka (>400 mesh, 0.015–0.035 mm). Drug rifabutin (RBT **1**) was isolated from Micobutin® and purified by column chromatography on silica and was compared with an authentic sample supplied by UpJohn & and Pharmacia.

The mass spectra were obtained in a Bruker Daltonics Esquire 3000 spectrometer, with an ESI source/interface detecting negative ions; the concentration of the analyzed solutions was 1 μg of substrate/mL of 1:1 solution of 0.1% acetic acid/acetonitrile. 'M' is the molecular ion; 'Ch' is the chromophore (molecule core without the ansa chain); and a, b, and c are ions.[20]

With the exception of the rifabutin N-oxide **10** and 25-hydroxy-rifabutin N-oxide **12**, nuclear magnetic resonance spectra were registered in a Bruker AMX spectrometer with a proton frequency of 300.15 MHz and a carbon-13 frequency of 75.47 MHz, all the other spectra were registered in a Bruker spectrometer with a proton frequency of 400.13 MHz or 500.13 MHz and a carbon-13 frequency of 100.62 MHz or 125 MHz, respectively. All samples, but rifabutinol N-oxide **11** that was dissolved in deuterated methanol, were dissolved in 99% deuterated chloroform, with tetramethylsilane as internal standard. The obtained results are presented in the following order: solvent, chemical shift (δ in ppm); relative intensity (as number of protons, H); multiplicity (*s*-singlet, br s-broad singlet, d-duplet, dd-double duplet, br d-broad duplet, t-triplet, q-quartet, m-multiplet); coupling constant *J* in Hz; and location in the molecule structure. The infrared spectra were obtained in a Perkin–Elmer FT-IR 1725X spectrometer, as film. The dichloromethane used was previously purified.[21] The mass spectra were determined by mass spectrometry operating with a laser ionization–desorption source, at 70 eV and close to 200 °C, with negative charged ions detection or as HR EIMS recorded on a FT-MS APEX-Qe mass spectrometer.

Synthesis of compounds **2**–**4** is described elsewhere.[11]

4.2. Chemistry

4.2.1. General procedure for the preparation of *N*′-acyl derivatives (5–8)

To a round-bottomed flask containing a solution of the appropriate rifabutin (500 mg; 0.59 mmol) in tetrahydrofuran (15 mL),

triethylamine (248 μL; 1.9 mmol) was added dropwise, at 0 °C, with stirring, followed by a solution of the appropriate acyl chloride (129 μL; 1.9 mmol) in tetrahydrofuran (10 mL), and the resulting mixture was left with stirring till it reached room temperature (~5 min). The resulting precipitate, the triethylamine hydrochloride, was filtered and the solvent was removed under reduced pressure. Then the product was extracted with ethyl acetate (3 × 20 mL) and the ethyl acetate was washed with water (3 × 10 mL), dried with $MgSO_4$, filtered, and evaporated to dryness, to afford the corresponding *N′*-acyl products.

4.2.1.1. *N′*-Acetyl-rifabutin (5). The compound was prepared from RBT **1** as a chromatographic homogeneous orange-colored solid in 95% yield; IR film (NaCl) 3460 (O–H), 3385 (N–H), 2965, 2936 (C–H), 1732 (C_{11}=O), 1714 (C_{35}=O), 1667 (C_{15}=O), 1651 (C_{38}=O), 1644 (C=N), 1606, 1488, 1471 (C=C), 1417 (C_8–O) and δ(O–H), 1373 δ(C–H), 1296 δ(O–H), 1251 (C_{35}–O–C_{25}), 1186 (C–N), 1085 (N–O), 1060 (C–O) and (C_{35}–O–C_{25}), 973 γ(C–H), 786 (chromophore's vibration); ^{1}H NMR ($CDCl_3$) δ −0.07 (3H, br s, CH_3-34), 0.59 (3H, d, $J_{33/24}$ = 6.0 Hz, CH_3-33), 0.86 (3H, d, $J_{31/20}$ = 6.0 Hz, CH_3-31), 0.99 (6H, d, J11′,12′/10′ = 6.3 Hz, CH_3-11′ and CH_3-12′), 1.03 (3H, d, $J_{32/22}$ = 7.5 Hz, CH_3-32), 1.6 (1H, m, H-24), 1.6 (1H, m, H-26), 1.77 (3H, s, CH_3-13), 1.8 (1H, m, H-22), 1.91 (1H, m, $J_{11′,12′/10′}$ = 6.4 Hz, H-10′), 1.7 (2H, m, CH_2-8′), 1.7 (2H, m, CH_2-4′), 2.02 (3H, s, CH_3-36), 2.05 (3H, s, CH_3-30), 2.44 (3H, br s, CH_3-39), 2.4 (2H, m, CH_2-9′), 2.4 (1H, m, H-20), 2.44 (3H, s, CH_3-14), 2.9 (2H, m, CH_2-5′), 2.9 (2H, m, CH_2-7′), 2.9 (1H, m, H-23), 3.01 (1H, br s, H-21), 3.09 (3H, s, CH_3-37), 3.41 (1H, m, H-27), 3.77 (1H, br s, 23-OH), 3.87 (1H, d, 21-OH), 4.97 (1H, br d, $J_{25/26}$ = 10.2Hz, H-25), 5.19 (1H, br s, H-28), 6.07 (1H, br d, $J_{19/20}$ = 6.3 Hz, H-19), 6.09 (1H, m, H-29), 6.23 (1H, br d, $J_{17/18}$ = 10.8 Hz, H-17), 6.72 (1H, m, H-18), 8.21 (1H, s, NH), 13.89 (1H, s, 8-OH); ^{13}C NMR ($CDCl_3$) δ 7.4 (C-14), 8.4 (C-33), 9.4 (C-34), 10.8 (C-32), 17.4 (C-31), 20.5 (C-30), 20.7 (C-36), 20.9 (C-11′), 20.9 (C-12′), 21.2 (C-13), 25.2 (C-39), 25.9 (C-10′), 33.0 (C-22), 35.3 (C-4′), 35.3 (C-8′), 37.3 (C-24), 38.2 (C-26), 38.2 (C-20), 51.1 (C-7′), 51.3 (C-5′), 57.2 (C-37), 65.9 (C-9′), 72.9 (C-21), 73.8 (C-25), 77.1 (C-23), 79.1 (C-27), 96.4 (C-2′), 106.9 (C-3), 108.9 (C-12), 111.0 (C-10), 113.3 (C-9), 113.8 (C-7), 116.7 (C-28), 125.7 (C-18), 126.4 (C-5), 129.5 (C-16), 135.0 (C-17), 140.8 (C-19), 140.8 (C-2), 142.4 (C-29), 153.9 (C-4), 166.6 (C-8), 166.7 (C-38), 167.6 (C-15), 172.1 (C-6), 172.2 (C-35), 185.9 (C-1), 192.0 (C-11); HR-ESI-MS *m*/*z* calcd for $C_{48}H_{65}N_4O_{12}$ 889.45935 $[M+1]^+$; found 889.45975.

4.2.1.2. *N′*-Acetyl-rifabutinol (6). The compound was prepared from RBT **2** as a chromatographic homogeneous orange bright-colored solid in 95% yield; IR_{film} (NaCl) 3459 (O–H), 3385 (N–H), 2965, 2933 (C–H), 1711 (C_{35}=O), 1668 (C_{15}=O), 1651 (C_{38}=O), 1600, 1562 (C=C), 1524 δ(N–H), 1417 (C_8–O) and δ(O–H), 1385 δ(C–H), 1281 δ(O–H), 1256 (C_{35}–O–C_{25}), 1156 (C–N), 1062 (N–O), 1062 (C–O) and (C_{35}–O–C_{25}), 972 γ(C–H), 803 (chromophorés vibration); ^{1}H NMR ($CDCl_3$) δ 0.13 (3H, d, $J_{34/26}$ = 6.0 Hz, CH_3-34), 0.63 (3H, d, $J_{33/24}$ = 6.0 Hz, CH_3-33), 0.88 (3H, d, $J_{31/20}$ = 6.9 Hz, CH_3-31), 0.98 (6H, d, $J_{11′,12/10}$ = 6.0 Hz, CH_3-11′ and CH_3-12′), 1.02 (3H, d, $J_{32/22}$ = 6.6 Hz, CH_3-32), 1.50 (1H, m, H-26), 1.6 (1H, m, H-24), 1.78 (1H, m, H-10′), 1.80 (1H, m, H-22), 1.95 (2H, m, CH_2-4′), 1.95 (2H, m, CH_2-8′), 2.07 (3H, s, CH_3-13), 2.05 (2H, sl, CH_2-9′), 2.12 (3H, s, CH_3-30), 2.22 (3H, s, CH_3-14), 2.07 (3H, s, CH_3-36), 2.48 (3H, br s, CH_3-39), 2.7 (2H, m, CH_2-5′), 2.7 (2H, m, CH_2-7′), 3.01 (1H, m, H-23), 3.06 (3H, s, CH_3-37), 3.4 (1H, m, H-20), 3.47 (1H, m, H-27), 3.84 (1H, m, H-21), 3.60 (1H, m, 21-OH), 4,07 (1H, d, $J_{OH/23}$, 23-OH), 5.01 (1H, dl, $J_{28/29}$ = 9.9 Hz, H-28), 5.11 (1H, br d, $J_{25/26}$ = 9.9 Hz, H-25), 5.48 (1H, s, H-11), 6.01 (1H, sl, H-29), 6.15 (1H, dd, $J_{19/18}$ = 16.0 Hz, $J_{19/20}$ = 6.9 Hz, H-19), 6.26 (1H, br d, $J_{17/18}$ = 10.5 Hz, H-17), 6.73 (1H, m, H-18), 6.95 (1H, s, OH-11), 8.21 (1H, s, NH), 13.1 (1H, s, 8-OH).^{13}C NMR ($CDCl_3$) δ 8.0 (C-14), 8.4 (C-33), 8.5 (C-34), 10.7 (C-32), 17.3 (C-31), 20.6 (C-30), 20.7 (C-36), 20.7 (C-11′), 20.7 (C-12′), 24.8 (C-39), 25.2 (C-13), 25.4 (C-10′), 32.8 (C-22), 36.3 (C-4′), 36.3 (C-8′), 36.8 (C-24), 37.2 (C-20), 39.1 (C-26), 57.4 (C-7′), 57.4 (C-5′), 57.4 (C-37), 65.7 (C-9′), 73.3 (C-21), 74.2 (C-25), 77.1 (C-23), 77.0 (C-27), 95.0 (C-2′), 107.7 (C-3), 112.6 (C-12), 111.4 (C-9), 111.6 (C-28), 114.8 (C-7), 120.5 (C-5), 120.8 (C-10), 125.3 (C-18), 128.6 (C-16), 132.0 (C-17), 137.0 (C-19), 141.5 (C-29), 141.9 (C-2), 158.1 (C-4), 160.8 (C-6), 163.4 (C-8), 166.3 (C-15), 165.7 (C-38), 172.5 (C-35), 184.8 (C-1); ESI-MS 891 $[M+1]^+$.

4.2.1.3. *N′*-(Palmitoyl)-rifabutin (7). The compound was prepared from RBT **1** as a chromatographic homogeneous red oil, yield 63%; IR_{film} (NaCl) 3536 (OH-8), 3472 (NH), 3412 (OH-23), 3223 (OH-21), 2925 (CH_2), 1731 (C_{35}=O), 1709 ($C_{1″}$=O), 1660 (C_{15}=O), 1639 (C_1=O), 1606 (C=C_{ansa}), 1465 (C···$C_{st.aromatic}$), 1372 ($OH_{\delta ip}$), 1345 (C–N), 1248 (C-15–N), 1248 (C–N), 1093 (C-27–O), 1060 (C–O), 973 (C=C–C=O), 757 (chromophorés vibration); ^{1}H NMR ($CDCl_3$) δ *−0.08* (3H, sl, CH_3-34), 0.57 (3H, sl, CH_3-33), 0.88 (3H, d, *J* = 6.0 Hz, CH_3-31), 0.88 (3H, t, *J* = 7.5 Hz, CH_3-16″), 0.89 (6H, d, *J* = 6.0 Hz, CH_3-11′ and CH_3-12′), 1.03 (3H, d, *J* = 6.0 Hz, CH_3-32), 1.25 (24H, m, H-4″, H-5″, H-6″ to H-15″), 1.62 (4H, m, H-22, H-24 and H-3″), 1.77 (5H, sl, CH_3-13, H-26 and H-10′), 2.04 (10H, s, CH_3-30, CH_3-36, H-4′ and H-8′), 2.17 (2H, m, H-7′), 2.32 (4H, sl, CH_3-14 and H-20), 2.32 (4H, m, H-9′ and H-2″), 2.76 (2H, m, H-5′), 3.01 (1H, d, *J* = 9.0 Hz, H-23), 3.08 (3H, s, CH_3-37), 3.29 (1H, m, H-27), 3.41 (1H, sl, OH-21), 3.66 (1H, m, H-21), 3.77 (1H, sl, OH-23), 5.01 (1H, sl, H-28), 5.12 (1H, sl, H-25), 6.04 (1H, d, *J* = 12.0 Hz, H-19), 6.23 (1H, d, *J* = 12.0 Hz, H-17), 8.12 (1H, s, N–H), 13.87 (1H, s, OH-8); ^{13}C NMR ($CDCl_3$): δ 7.5 (C-14), 8.5 (C-33), 10.7 (C-34), 14.0 (C-16, C-31, C-32 and C-14″), 20.6 (C-30), 20.8 (C-36), 21.0 (C-13), 22.6 (C-11′, C-12′ and C-15″), 24.2 (C-10′), 28.8 (C-3″), 29.1 (C-4″), 29.3 (C-5″ and C-13″), 29.4 (C-6″), 29.5 (C-7″), 29.6 (C-22, C-8″, C-9″, C-10″, C-11″ and C-12″), 31.9 (C-2″ and C-14″), 35.2 (C-4′ and 8′), 37.3 (C-24), 37.4 (C-26), 38.1 (C-20), 51.3 (C-5′ and C-7′), 57.5 (C-37), 77.0 (C-23 and C-27), 93.6 (C-2′), 105.60 (C-12), 106.5 (C-10), 111.9 (C-9), 124.2 (C-18), 125.6 (C-5), 166.7 (C-8 and C-15), 169.6 (C-6), 172.3 (C-35), 175.5 (C-1″); ESI-MS *m*/*z* 1085 $[M-H]^{\cdot -}$, 847 $[(M-H)-R]^{\cdot -}$, 829 $[(M-H)-R-H_2O]^{\cdot -}$, 787 $[(M-H)-R-CH_3COOH]^{\cdot -}$, 643 $[(M-H)-iC_4H_9NC_5H_9-c]^{\cdot -}$, 545 $[(M-H)-R-c]^{\cdot -}$, 423 $[(Ch-H)]^{\cdot -}$, 323 $[(Ch-H)-iC_4H_9N(CH_3)=CH_2]^{\cdot -}$; HR-ESI-MS *m*/*z* calcd for $C_{62}H_{93}N_4O_{12}$ 1085.51448 $[M+1]^+$; found 1085.71348.

4.2.1.4. *N′*-(Undec-10″-enoyl)-rifabutin (8). The compound was prepared from RBT **1** as a chromatographic homogeneous orange oil (84% yield); IR_{film} (NaCl) 3500 (OH-8), 3439 (NH), 3250 (OH-21 and OH-23), 3076 (CH=$CH_{2st.}$), 2926 (CH_2), 1729 (C_{35}=O), 1716 ($C_{1″}$=O), 1660 (C_{15}=O), 1641 (C_1=O), 1607 (C=C_{ansa}), 1579 (C=C), 1461 (C···$C_{st.aromatic}$), 1373 ($OH_{\delta ip}$), 1340 (C–N), 1252 (C-15–N), 1244 (C–N), 1089 (C-27–O), 1063 (C–O), 972 (C=C–C=O), 757 (chromophorés vibration); ^{1}H NMR ($CDCl_3$) δ −0.09 (3H, sl, CH_3-34), 0.57 (3H, sl, CH_3-33), 0.85 (3H, d, *J* = 6.3 Hz, CH_3-31), 0.91 (6H, d, *J* = 6.5 Hz, CH_3-11′ and CH_3-12′), 1.03 (3H, d, *J* = 7.0, CH_3-32), 1.25 (10H, m, H-4″, H-5″, H-6″, H-7″ and H-8″), 1.45 (1H, m, H-24), 1.61 (2H, m, H-3″), 1.77 (4H, s, CH_3-13 and H-26), 1.82 (2H, m, H-10′), 1.95 (4H, m, H-4′ and H-8′), 2.01 (2H, m, H-9″), 2.04 (6H, s, CH_3-30 and CH_3-36), 2.17 (2H, m, H-5′), 2.31 (3H, s, CH_3-14), 2.47 (2H, d, *J* = 7.2 Hz, H-9′), 2.63 (2H, m, H-7′), 3.00 (1H, d, *J* = 9.7 Hz, H-23), 3.08 (3H, s, CH_3-37), 3.40 (1H, d, *J* = 4.7 Hz, OH-21), 3.45 (1H, m, H-21), 3.78 (1H, sl, OH-23), 4.95 (2H, ddd, *J* = 17.7, 10.8 and 1.2 Hz, H-11″), 5.20 (1H, m, H-28), 5.79 (2H, m, H-18 and H-10″), 6.03 (1H, dd, *J* = 15.6 and 5.7 Hz, H-19), 6.22 (1H, d, *J* = 10.2 Hz, H-17), 6.7 (1H, m, H-29), 8.16 (1H, s, NH), 13.89 (1H, s, OH-8); ^{13}C NMR ($CDCl_3$) δ 7.5 (C-14), 8.5 (C-33), 10.7 (C-34), 17.6 (C-31), 20.8 (C-36), 20.6

(C-30), 20.9 (C-11′ and C-12′), 25.5 (C-10′), 28.8 (C-8″), 29.0 (C-7″), 29.2 (C-6″), 29.3 (C-5″), 29.4 (C-4″), 29.4 (C-22), 29.6 (C-3″), 33.7 (C-9″), 35.7 (C-4′), 37.3 (C-8′), 38.0 (C-24 and C-26), 38.2 (C-20), 51.0 (C-5′ and C-7′), 57.5 (C-37), 65.4 (C-9′), 72.6 (C-21), 73.7 (C-25), 77.2 (C-23), 79.4 (C-27), 96.5 (C-2′), 106.7 (C-12), 108.6 (C-10), 110.9 (C-9), 113.6 (C-3), 113.9 (C-28), 114.0 (C-7), 114.1 (C-11″), 125.0 (C-5), 127.0 (C-18), 130.0 (C-16), 135.5 (C-17), 139.0 (C-19), 139.1 (C-10″), 141.0 (C-2), 142.5 (C-29), 154.1 (C-4), 166.5 (C-8), 167.6 (C-15), 170.4 (C-6), 172.3 (C-35), 178.3 (C-1″), 185.7 (C-1), 192.3 (C-11); ESI-MS *m*/*z* 1013 $[M-H]^-$, 987 $[(M-H)-CH{=}CH_2]^-$, 953 $[(M-H)-CH_3COOH]^-$, 927 $[(M-H)-CH{=}CH_2-CH_3COOH]^-$, 895 $[(M-H)-CH{=}CH_2-CH_3COOH-CH_3OH]^-$, 847 $[(M-H)-R]^-$, 829 $[(M-H)-R-H_2O]^-$, 815 $[(M-H)-R-CH_3OH]^-$, 787 $[(M-H)-R-CH_3COOH]^-$, 711 $[(M-H)-R-iC_4H_9N(CH_3){=}CH_2-2H_2O]^-$, 545 [(M-H)–R–c]$^-$, 423 [(Ch–H)]$^-$, 323 $[(Ch-H)-iC_4H_9N(CH_3){=}CH_2]^-$; HR-ESI-MS *m*/*z* calcd for $C_{57}H_{81}N_4O_{12}$ 1013.58455 $[M+1]^+$; found 1013.58628.

4.2.2. General procedure for the preparation of *N*-oxide derivatives (9–10)

To a round-bottomed flask containing RBT **1** or rifabutinol **2** (100.0 mg; 0.118 mmol) in dichloromethane (4 mL) were added benzyl triethylammonium chloride (40.9 mg; 0.180 mmol), sodium hydrogen carbonate (6.7 mg; 0.080 mmol), Oxone (145.5 mg; 0.237 mmol) in acetone (1.0 mL), distilled water (5 mL), and a Metrohm pH 7 buffer (7.0 mL), with permanent agitation at room temperature. The reaction was followed by TLC, using petroleum ether/ethyl acetate/methanol (55:35:10) as eluant; after ca. 17 h, 1 equiv more of Oxone (146.0 mg; 0.237 mmol) and buffer (0.5 mL) was progressively added to adjust the pH of the medium. The reaction was complete after ca. 26 h 30 min. The content in the flask was transferred to a separatory funnel, and it was extracted thrice with dichloromethane followed by two washings with water. The combined organic layers were collected in an Erlenmeyer flask, dried over anhydrous sodium sulfate, and filtered. The solvent was evaporated at reduced pressure, and the crude red-colored solid that was obtained was dried *in vacuum* and purified by flash column chromatography [eluting with petroleum ether/ethyl acetate/methanol (55:35:10) for **9** and with increasing polarity mixtures of petroleum ether/ethyl acetate/methanol starting from 65:25:10 for **10**, to afford the required products].

4.2.2.1. Rifabutin N-oxide (9). The compound was prepared from RBT **1** as a chromatographic homogeneous red-colored solid (51.0% yield); IR_{film} (NaCl) 3423 (O–H), 2965, 2931 (C–H), 1725 (C_{11}=O), 1672 (C_{15}=O), 1602, 1562 (C=C), 1524 δ(N–H), 1422 (C_8-O) and δ(O–H), 1376 δ(C–H), 1295 δ(O–H), 1248 (C_{35}–O–C_{25}), 1161 (C–N), 1085 (N–O), 1062 (C–O) and (C_{35}–O–C_{25}), 976 γ(C–H), 770 (chromophorés vibration); ^{1}H NMR ($CDCl_3$) δ −0.08 (3H, d, $J_{34/26}$ = 7.0 Hz, Me-34), 0.61 (3H, d, $J_{33/24}$ = 6.9 Hz, Me-33), 0.86 (3H, d, $J_{31/20}$ = 6.9 Hz, Me-31), 1.03 (3H, d, $J_{32/22}$ = 6.9 Hz, Me-32), 1.1–1.7 (m, CH_2-4′/CH_2-8′) 1.21 (6H, d, $J_{11'/10} = J_{12'/10}$ = 6.6 Hz, Me-11′ and Me-12′), 1.42 (1H, m, H-24), 1.68–1.82 (2H, m, H-22/H-26), 1.76 (3H, s, Me-13), 1.98 (3H, s, Me-36), 2.06 (3H, s, Me-30), 2.35 (3H, s, Me-14), 2.30–2.41 (1H, m, H-20), 2.50 (1H, m, H-10′), 3.01 (1H, m, H-23), 3.09 (3H, s, Me-37), 3.34 (1H, m, H-27), 3.40 (1H, br s, 21-OH), 3.48 (2H, m, CH_2-9′), 3.58 (1H, d, 23-OH), 3.69 (1H, br d, $J_{21/20}$ = 9.9 Hz, H-21), 3.2–4.2 (m, CH_2-7′/CH_2-5′), 4.81 (1H, br d, $J_{25/26}$ = 9.9 Hz, H-25), 5.12 (1H, dd, $J_{28/27}$ = 7.2 Hz/$J_{28/29}$ = 12.3 Hz, H-28),), 6.00 (1H, dd, $J_{19/18}$ = 15.3 Hz, $J_{19/20}$ = 6.9 Hz, H-19), 6.14 (1H, d, $J_{29/28}$ = 12.6 Hz, H-29), 6.29 (1H, br d, $J_{17/18}$ = 10.2 Hz, H-17), 6.39 (1H, dd, $J_{18/17}$ = 10.2 Hz, $J_{18/19}$ = 15.3 Hz, H-18), 8.43 (1H, s, NH), 8.74 (1H, s, NH′), 14.54 (1H, br s, 8-OH); ^{13}C NMR (MeOD) δ 7.6 (C-14), 8.8 (C-33), 10.7 (C-34), 11.1 (C-32), 17.3 (C-31), 20.3 (C-30), 21.0 (C-36), 21.8 (C-13), 23.2 (C-11′/C-12′), 23.7 (C-10′), 31.6 (C-4′), 31.9 (C-8′), 32.9 (C-22), 37.6 (C-24/C-26), 38.4 (C-20), 56.9 (C-37), 62.8 (C-5′/C-7′), 72.5 (C-21), 73.0 (C-25), 76.9 (C-23), 78.4 (C-9′), 80.5 (C-27), 91.3 (C-2′), 105.4 (C-3), 107.3 (C-12), 108.8 (C-10), 111.8 (C-9), 114.8 (C-7), 115.4 (C-28), 124.2 (C-18), 124.7 (C-5), 131.0 (C-16), 133.2 (C-17), 140.8 (C-19), 141.8 (C-2), 144.0 (C-29), 156.2 (C-4), 168.1 (C-8), 168.3 (C-15), 171.6 (C-6), 172.1 (C-35), 181.9 (C-1); ESI-MS *m*/*z* 861 $[M-H]^{\cdot-}$, 845 $[(M-H)-O]^{\cdot-}$, 785 $[(M-H)-O-CH_3COOH]^{\cdot-}$, 753 $[(M-H)-O-CH_3COOH-CH_3OH]^{\cdot-}$, 709 $[(M-H)-O-CH_3COOH-CH_3OH-C_3H_8]^{\cdot-}$, 651 $[(M-H)-O-CH_3COOH-CH_3OH-C_3H_8-C_3H_8N]^{\cdot-}$, 571 [(M–3H)–O–b)]$^{\cdot-}$, 543 [(M–3H)–c]$^{\cdot-}$, 421 [Ch–H] $^{\cdot-}$; HR-ESI-MS *m*/*z* calcd for $C_{46}H_{62}N_4O_{12}$ 846.44206 [(M–1)–16]$^-$; found 846.44133.

4.2.3. Rifabutinol N-oxide (10)

The compound was prepared from RBT **2** as a chromatographic homogeneous red-colored solid (51.0% yield); IR_{film} (NaCl) 3418 (N–H), 3252 (O–H), 2964. 2938. 2880 (C–H), 1730 (C_{11}=O), 1673 (C_{15}=O), 1645 (C_4=N), 1633 (C_1=O), 1616. 1605. 1582 (C=C), 1520 δ(N–H), 1418 (C_8–O) and δ(O–H), 1374 δ(C–H), 1285 δ(O–H), 1256 (C_{35}–O–C_{25}), 1149 (C–N), 1078 (N–O), 1062 (C–O) and (C_{35}–O–C_{25}), 974 γ(C–H), 768 (chromophorés vibration); ^{1}H NMR (MeOD) δ −0.04 (3H, d, $J_{34/26}$ = 6.6 Hz, Me-34), 0.56 (3H, d, $J_{33/24}$ = 6.6 Hz, Me-33), 0.84 (3H, d, $J_{31/20}$ = 6.6 Hz, Me-31), 0.95 (3H, d, $J_{32/22}$ = 6.8 Hz, Me-32), 1.13 (6H, d, $J_{11'/10} = J_{12'/10}$ = 6.6 Hz, Me-11′ and Me-12′), 1.21–1.28 (1H, m, H-26), 1.62 (1H, m, H-24), 1.72 (1H, m, H-22), 1.85 (3H, s, Me-13), 1.94 (3H, s, Me-36), 2.03 (3H, s, Me-30), 2.08 (3H, s, Me-14), 2.32 (1H, m, H-20) 2.40 (1H, m, H-10′), 2.98 (3H, s, Me-37), 3.04 (1. m, H-23), 3.32 (2H, m, CH_2-9′), 3.38 (1H, m, H-27) 1.30–3.80 (m, CH_2-7′/CH_2-5′/CH_2-4′/CH_2-8′), 3.85 (1H, br d, $J_{21/20}$ = 9.6 Hz, H-21), 4.89 (1H, m, H-28), 5.18 (1H, d, $J_{25/26}$ = 10.0 Hz, H-25), 5.59 (1H, s, H-11), 5.93 (1H, d, $J_{29/28}$ = 12.4 Hz, H-29), 6.02 (1H, dd, $J_{19/18}$ = 15.6 Hz, $J_{19/20}$ = 6.8 Hz, H-19), 6.27 (1H, br d, $J_{17/18}$ = 10.4 Hz, H-17), 6.59 (1H, dd, $J_{18/17}$ = 11.0 Hz, $J_{18/19}$ = 15.6 Hz, H-18); ^{13}C NMR (MeOD) δ 8.4 (C-14), 9.5 (C-33), 9.7 (C-34), 11.3 (C-32), 18.0 (C-31), 20.8 (C-30), 21.0 (C-36), 23.5 (C-11′/C-12′), 24.6 (C-10′), 25.3 (C-13), 31.7 (C-4′/C-8′), 34.3 (C-22), 39.0 (C-20/C-24), 40.7 (C-26), 57.5 (C-37), 63.7 (C-5′), 64.0 (C-7′), 74.6 (C-21), 74.9 (C-25), 77.6 (C-11), 78.1 (C-23), 78.2 (C-27), 79.5 (C-9′), 91.5 (C-2′), 105.4 (C-3), 110.2 (C-9), 113.0 (C-12), 114.2 (C-7), 114.8 (C-28), 121.3 (C-5), 121.9 (C-10), 127.1 (C-18), 132.0 (C-16), 134.6 (C-17), 140.9 (C-19), 143.4 (C-29), 145.1 (C-2), 160.8 (C-4), 161.9 (C-6), 164.1 (C-8), 169.3 (C-15), 172.8 (C-35), 184.9 (C-1); ESI-MS *m*/*z*, 864 $[M]^{\cdot-}$, 848[M–O]$^{\cdot-}$, 820 $[(M-H)-C_3H_8]^{\cdot-}$, 735 $[(M-H)-iC_4H_9N(CH_3){=}CH_2]^{\cdot-}$, 703 $[(Ch-H)-(iC_4H_9N(CH_3){=}CH_2)-CH_3OH]^{\cdot-}$, 543 [(M–3H)–O–c]$^{\cdot-}$, 422 [(Ch–2H)–O]$^{\cdot-}$; HR-ESI-MS *m*/*z* calcd for $C_{46}H_{64}N_4O_{12}$ 848.45715 [M–16]$^-$; found 848.45779.

4.2.4. 25-Deacetyl-rifabutin *N*-oxide (11)

In a round-bottomed flask containing RBT **3** (150.0 mg; 0.186 mmol) in dichloromethane (7.9 mL), the following reactants were added with stirring at room temperature: benzyl triethylammonium chloride (70.0 mg; 0.307 mmol), $NaHCO_3$ (13.5 mg; 0.161 mmol), distilled water (7.9 mL), pH 7 buffer (10 mL), and Oxone® (179.1 mg; 0.291 mmol). The Oxone was slowly added to the reaction mixture during a period ca. 3 h 15 min of the reaction.

The reaction was followed by TLC (petroleum ether/ethyl acetate/methanol 45:35:20); after ca. 19 h of reaction, more Oxone was added (282.0 mg; 0.458 mmol), as well as a buffer, pH 7, in order to maintain ca. pH 7. The reaction was complete after ca. 28 h 30 min. The aqueous and organic layers were separated, and the aqueous layer was extracted with dichloromethane. The collected organic layers were washed with water, dried over anhydrous sodium sulfate, and filtered, and the solvent was evaporated at reduced pressure. A crude product was produced after drying *in vacuum* (102.5 mg; 67.0%), which was further purified by flash

column chromatography, using reverse phase silica, eluting with petroleum ether/methanol (2:98), to give **11** (29.3 mg; 19.1% yield). IR_{film} (NaCl) 3402 (O–H), 2965, 2934 (C–H), 1724 (C_{11}=O), 1672 (C_{15}=O), 1602. 1568 (C=C), 1521 δ(N–H), 1422 (C_8-O) and δ(O–H), 1374 δ(C–H), 1328 (C–N), 1295 δ(O–H), 1160 (C–N), 1083 (N–O), 1059 (C–O), 978 γ(C–H), 770 (chromophorés vibration); ^{1}H NMR ($CDCl_3$) δ −0.16 (3H, d, $J_{34/26}$ = 6.8 Hz, Me-34), 0.53 (3H, d, $J_{33/24}$ = 6.8 Hz, Me-33), 0.82 (3H, d, $J_{31/20}$ = 6.8 Hz, Me-31), 1.16 (6H, d, $J_{11'/10}$ = $J_{12'/10}$ = 6.8 Hz, Me-11′ and Me-12′), 1.04 (3H, d, $J_{32/22}$ = 6.8 Hz, Me-32), 1.16 (1H, m, H-24), 1.24 (1H, m, H-10′), 1.64 (1H, m, H-26), 1.73 (3H, s, Me-13), 1.70–1.80 (1H, m, H-22), 2.06 (3H, br s, Me-30), 2.28 (3H, s, Me-14), 2.36 (1H, m, H-20), 3.16 (3H, s, Me-37), 3.32 (2H, m, CH_2-9′), 3.3–3.6 (3H, m, H-23/H-25/H-27), 3.73 (1H, d, $J_{21/20}$ = 9.3 Hz, H-21), 4.20 (1H, s, 25-OH), 5.11 (1H, dd, $J_{28/27}$ = 8.7 Hz, $J_{28/29}$ = 12.4 Hz, H-28), 5.91 (1H, dd, $J_{19/18}$ = 14.6 Hz, $J_{19/20}$ = 6.2 Hz, H-19), 6.22 (1H, br d, $J_{29/28}$ = 12.7 Hz, H-29), 6.29–6.36 (2H, m, H-18 and H-17); ^{13}C NMR ($CDCl_3$) δ 7.7 (C-14), 8.4 (C-33), 11.0 (C-32), 11.2 (C-34), 17.4 (C-31), 20.2 (C-30), 22.7 (C-13), 23.4 (C-11′ and C-12′), 23.6 (C-10′), 32.8 (C-22), 38.6 (C-24), 39.1 (C-20), 56.5 (C-37), 62.8 (C-5′ and C-7′), 70.4 (C-25), 72.0 (C-21), 77.3 (C-23), 79.2 (C-9′), 91.6 (C-2′), 105.1 (C-3), 107.8 (C-12), 109.2 (C-10), 111.6 (C-9), 114.7 (C-7/C-28), 123.9 (C-18), 124.8 (C-5), 132.2 (C-16), 132.7 (C-17), 140.5 (C-19), 156.1 (C-4), 168.1 (C-8), 168.3 (C-15), 171.2 (C-6), 181.8(C-1), 192.6 (C-11); ESI-MS, *m*/*z*: 819 $[M-H]^{\cdot -}$, 803 $[(M-H)-O]^{\cdot -}$, 323 $[Ch-iC_4H_9N(CH_3)=CH_2]^{\cdot -}$.

4.3. Bioassays

Strains *M. tuberculosis* H_{37}Rv (ATCC 27294) and *M. avium* 1581 were used. The organisms were grown in Middlebrook 7H9 broth to a density of 10^8 CFU/mL and were stored at −80 °C in 1.0 mL aliquots. For each experiment, a single vial was thawed and used, and the unused portion was discarded.

4.3.1. In vitro activity against *M. avium*

To the culture medium agar 7H9 OADC medium (4.7 g Middlebrook 7H9 base (Difco–Becton Dickinson) enriched with 10% (v/v) OADC (Oleic acid, Dextrose Albumin, Catalase-BBL), the RBT derivatives were added at the final concentrations of 0.2 and 0.4 μg/mL, and RBT **1** was added at final concentrations of 0.1 and 0.2 μg/mL. Plates containing RBT **1** or its derivatives were inoculated with a strain of *M. avium* 1581 at dilutions 10^{-5} and 10^{-4}. Plates without antibiotic were inoculated with a strain of *M. avium* at dilutions of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}. Plates were then incubated for 15 days at 37 °C in the presence of 5% CO_2. By comparing with control plates (inoculated with the strain *M. avium* but in the absence of antibiotic) the concentration of the rifabutin derivative which is related with the strain whose colonies number is smaller than 1% in relation to the colonies number in control plates.

4.3.2. In vitro activity against *M. tuberculosis*

The Resazurin Method[22] was used for the determination of the MIC. In brief, the assay is accomplished in microplates (96 wells) using resazurin as an indicator of cellular viability in 7H9 OADC medium with the RBT **1** and derivatives dissolved in DMSO. The MIC determination was carried out by twofold serial dilutions of the drugs (ranging from 100.0 to 0.2 μg/mL) dispensed into each well of a 96-well microtitre plate.

4.3.3. Activity against non-replicating *M. tuberculosis*

Activity against non-replicating cultures of *M. tuberculosis* was determined by using the low-oxygen recovery assay (LORA) with CFU confirmation as previously described,[5] except that in the present study the anaerobic incubation time with test compounds was eight days (vs 10) and the aerobic recovery time prior to determination of luminescence was 24 h (vs 28). Briefly, a culture of low-oxygen adapted, luciferase-expressing *M. tuberculosis* was exposed to test compounds for eight days. An aliquot was diluted and plated on Middlebrook 7H11 agar for determination of CFU. The remaining sample was incubated under aerobic conditions for 24 h and then luminescence was determined following the addition of *n*-decanal. The LORA MIC was defined as the lowest concentration resulting in a 90% reduction of luminescence relative to drug-free controls. The MBC was defined as the lowest concentration resulting in a 90% reduction in CFU relative to drug-free controls.

4.3.4. Cytotoxicity

Cytotoxicity against green monkey kidney cells (VERO) was determined by measuring the extent of reduction of MTS following 72 h exposure to test compounds as previously described.[23]

4.3.5. In vivo activity

4.3.5.1. Experimental model of tuberculosis infection in mice. All animal work was performed in conformity with the Local Ethical Committee for Experimentation in Animals in Mexico. The tuberculosis model has been described in detail elsewhere.[24] Male Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA), and were used at 6–8 weeks of age. Virulent *M. tuberculosis* H_{37}Rv and a MDR clinical isolate (code 900) resistant to all primary antibiotics were cultured in Youman's modification of the medium of Proskauer and Beck. Colonies were harvested after four 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 ratio of volume to area, and by counting ten 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 aliquoted at −70 °C Before use bacteria were recounted, and viability was checked as described.[25]

In order to achieve intra-tracheal infection, mice were anesthetized with 56 mg/kg of intraperitoneal pentothal. The trachea was exposed via a small midline incision, and 2.5×10^5 viable bacteria were injected in 100 μL of PBS. 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.

4.3.5.2. Drug administration. Animals surviving 60 days after infection were randomly allocated to the required treatment groups. Thus, treatment was started 60 days after infection, and groups of these animals were sacrificed after one month of treatment. The selection of the appropriate dose was calculated according to the MIC determined in vitro (drug concentration efficient to kill 1×10^6 bacilli) by adjusting the drug concentration with the estimated number of bacilli in mice lungs after two months of infection. For the treatment of animals, this drug amount was triplicated considering its dilution after absorption and systemic distribution after oral administration. We tested two different doses (5 and 15 mg/Kg of body weight). RBT **1** and its derivatives **5** and **8** were administered intragastrically as a suspension, first dissolving the drug in a small volume of ethanol and then further dissolving in 0.05% sodium carboxymethyl cellulose and 0.04% Tween 80, in a total of 0.1 mL with a canula every day until the end of the experiments.[26]

4.3.5.3. 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 histopathology 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). The time for incubation was 21 days. Four animals were sacrificed at each time point, in 2 different experiments, so that the data points are the means of eight animals.

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

4.3.5.4. Statistics. A one way analysis of variance (ANOVA) and Student's *t*-test were used to compare CFU and morphometry determinations in infected mice treated with RBT **5** or **8** and non-treated control animals. A difference of $P < 0.05$ was considered as significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.006.

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