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Strong *In Vitro* Activities of Two New Rifabutin Analogs against Multidrug-Resistant *Mycobacterium tuberculosis*▽†

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Two new rifabutin analogs, RFA-1 and RFA-2, show high *in vitro* antimycobacterial activities against *Mycobacterium tuberculosis*. MIC values of RFA-1 and RFA-2 were ≤0.02 μg/ml against rifamycin-susceptible strains and 0.5 μg/ml against a wide selection of multidrug-resistant strains, compared to ≥50 μg/ml for rifampin and 10 μg/ml for rifabutin. Molecular dynamic studies indicate that the compounds may exert tighter binding to mutants of RNA polymerase that have adapted to the rifamycins.

Each year, 8 million to 10 million new cases of tuberculosis (TB) are diagnosed, making *Mycobacterium tuberculosis* a leading cause of death in adults (2 million to 3 million/year) due to an infectious agent (16). Coinfection with human immunodeficiency virus is common (15), with 31% of AIDS deaths in Africa being attributed to TB infections (5). The World Health Organization (WHO) has declared TB a global public health emergency as it is approaching epidemic proportions in undeveloped regions of the world. The continuing rise in multidrug-resistant strains of *Mycobacterium tuberculosis* (MDR-TB) (6) has further contributed to the dire need for new TB antibiotics (no new TB drugs have been introduced into clinical use in the last three decades; however, fluoroquinolones have become more commonly used in the management of TB) (3, 11). The rifamycins are the most commonly used drugs for TB (13), and semisynthetic derivatives have been reported that show improved antimycobacterial activities. These include rifampin (RIF) and rifabutin (RBT), which have structural modifications at the C-3 and C-4 centers of the naphthoquinone core, respectively (9). RIF is the cornerstone of current short-course tuberculosis treatment, and RBT is used as an alternative for patients unable to tolerate RIF. The Barluenga laboratory has previously reported the synthesis of new rifabutin variants, which we call rifastures (RFA) (1). *In vitro* antimycobacterial activity against rifamycin-susceptible strains of *M. tuberculosis* was confirmed for seven of these compounds, the most active of which were RFA-1 and RFA-2 (compounds RFA-1 and RFA-2 in this paper are the same as compounds RFA3aM and RFA-3cM, respectively, in reference 2). Both of these compounds have the same stereochemical configuration at the spiranic carbon C-1′, which was determined to be *R* by 2D nuclear magnetic resonance (NMR), nuclear Overhauser effect spectroscopy (gNOESY), and rotational Overhauser effect spectroscopy (ROESY) experiments (Fig. 1) (12). In the present study, we report in greater detail the *in vitro* antimycobacterial properties of RFA-1 and RFA-2 against a wide panel of RIF-susceptible and multidrug-resistant strains of *M. tuberculosis* and study the basis for their enhanced bioactivity by molecular dynamics calculations.

In vitro antimycobacterial activities of RFA-1 and RFA-2 were evaluated against 79 strains of *M. tuberculosis*, including 63 clinical isolates from local hospitals (Hospital Universitario Central de Asturias, Oviedo, Spain, and Hospital Universitario Gregorio Marañón, Madrid, Spain) and 16 reference strains (11 of which were obtained from the Supranational Reference Laboratory belonging to the WHO/IUATLD network [N. M. Casabona, Servicio de Microbiología, Hospital Vall d'Hebron, Barcelona, Spain]). Among the 63 clinical strains, 38 were susceptible to conventional drugs, 8 were resistant to several drugs other than rifampin, and 17 were MDR-TB strains. Among the 16 reference strains, 2 were susceptible and 8 were resistant to several drugs other than RIF, and 6 were MDR-TB or RIF-resistant strains. MIC values of the compounds were determined by the agar dilution method as described in Table 1 (10). All strains were tested with several dilutions of RIF, RBT, RFA-1, and RFA-2 (0.02, 0.04, 0.08, 0.1, 0.3, 0.5, 0.7, 1, 3, 5, 10, 25, and 50 μg/ml). Each susceptibility test was repeated three times. Against all of the RIF-susceptible strains, RFA-1 and RFA-2 showed MICs of ≤0.02 μg/ml (Table 1, entries 1, 2, 4, and 5), similar to RIF and RBT, which were used as controls. With respect to RIF-resistant and MDR strains (Table 1, entries 3, 6, and 7), MIC values for RFA-1 (22 of the 23 strains) and RFA-2 (21 of the 23 strains) were ≤0.5 μg/ml, versus 50 μg/ml for RIF (23/23) and 10 μg/ml for RBT (20 of the 23 strains). These data indicate that RFA-1 and

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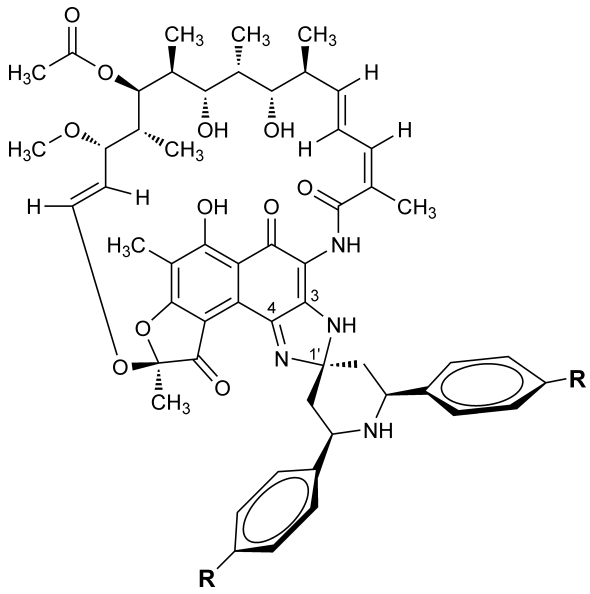

FIG. 1. Absolute configuration of RFA-1 (R = H [hydrogen]) and RFA-2 (R = F [fluorine]).

RFA-2 possess high levels of *in vitro* activity against both drug-sensitive and multidrug-resistant strains of *M. tuberculosis*. Additionally, in MDR-TB and RIF-resistant strains, RFA-1 and RFA-2 display MIC values 100 and 20 times lower than the reference antibiotics, RIF and RBT, respectively, and there is only a 25-fold difference in the ratio of MICs against RIF-susceptible strains compared to MDR strains (versus 2,500-fold for RIF and 500-fold for RBT). This dramatic difference may be attributed to the likely mechanism of action of the rifastures as inhibitors of bacterial RNA polymerase. Rifamycins act against mycobacteria by binding with the β-subunit of RNA polymerase protein to form a stable drug-enzyme complex that then blocks RNA biosynthesis (7). It has been reported that >97% of RIF-resistant *M. tuberculosis* strains acquire rifamycin resistance due to a nucleotide mutation in the gene encoding for the β-subunit of the RNA polymerase (*rpoB*) (14). We therefore investigated mutations in the *rpoB* gene for our RIF-resistant mutants (carried out by the reverse hybridization technique [INNO-LiPA Rif.TB; Innogenetics]). In all but one of the RIF-resistant strains analyzed, the following mutations in the *rpoB* gene were detected: S531L (11 strains), H526Y (7 strains), H516V (2 strains), and H526D (1 strain) as well as a mutation in probe region S5 (1 strain). We therefore hypothesize that the significantly lower MIC values of RFA-1 and RFA-2 toward RIF-resistant TB strains arise from an improved binding affinity for the mutant RNA polymerase enzyme. Molecular modeling calculations of RFA-1 binding to mutant RNA polymerase were performed, using the published crystal structure of RNA polymerase (RNAP) from *Thermus aquaticus* (*Taq*) (Fig. 2) (4). Formation of the complex between RFA-1 and the S411L mutant of the *Taq* RNAP enzyme was investigated using molecular dynamics simulations and free energy calculations (see the supplemental material).

The computed interaction energy between RFA-1 and the S411L mutant enzyme (analogous to the S531L mutant in *M. tuberculosis*) is 2.2 and 1.2 kcal/mol more stable than those of RIF and RBT, respectively. The molecular dynamics calculations also predict that RIF and RBT are bound in a similar orientation to the one observed in the initial crystal structure, whereas RFA-1 shifts within the binding pocket and establishes different enzyme/ligand contacts due to its enhanced hydrophobicity. This suggests that the enhanced antimicrobial activities for RFA-1 and RFA-2 compared to that for RIF and RBT is due to tighter binding to RNA polymerase in drug-resistant TB strains. Additional studies are being set up to confirm this experimentally using RNA polymerase from RIF-susceptible and MDR-TB strains. Further experiments are also planned to assess *in vivo* properties, including efficacy and toxicity, in animal models. Preliminary *in vitro* cytotoxicity testing of RFA-1 and RFA-2 against bovine endothelial cells showed no deleterious effects on the cells after 48 h at concentrations of 1 μg/ml up to 20 μg/ml (see the supplemental material for details).

In summary, these new results indicate that RFA-1 and RFA-2 are powerful antimycobacterial agents that are highly effective against multidrug-resistant *M. tuberculosis*. These

TABLE 1. *In vitro* activities of rifampin (RIF), rifabutin (RBT), and rifastures (RFA-1 and RFA-2) against 79 isolates of *Mycobacterium tuberculosis*

Type of strain (total no. of strains)	Entry no.	*M. tuberculosis* strain tested	MIC (μg/ml) (no. of strains with indicated MIC result)			
			RIF	RBT	RFA-1	RFA-2
Clinical (63)	1	*M. tuberculosis* S (38 strains susceptible to rifamycin)	≤0.02	≤0.02	≤0.02	≤0.02
	2	*M. tuberculosis* RDOTR (8 strains resistant to drugs other than rifampin)	≤0.02	≤0.02	≤0.02	≤0.02
	3	*M. tuberculosis* MDR-TB (17 strains resistant to at least isoniazid + rifampin)	50	10 (15), 50 (2)	0.5	0.5 (16), 3 (1)
Reference (16)	4	*M. tuberculosis* S (2 strains susceptible to rifamycin)	≤0.02	≤0.02	≤0.02	≤0.02
	5	*M. tuberculosis* RDOTR (8 strains resistant to drugs other than rifampin)	≤0.02	≤0.02	≤0.02	≤0.02
	6	*M. tuberculosis* RIF-R (5 strains resistant to rifampin)	50	10 (4), 50 (1)	0.3 (1), 0.5 (3), 1 (1)	0.3 (1), 0.5 (3), 0.7 (1)
	7	*M. tuberculosis* MDR-TB (1 strain resistant to at least isoniazid + rifampin)	50	10	0.5	0.5

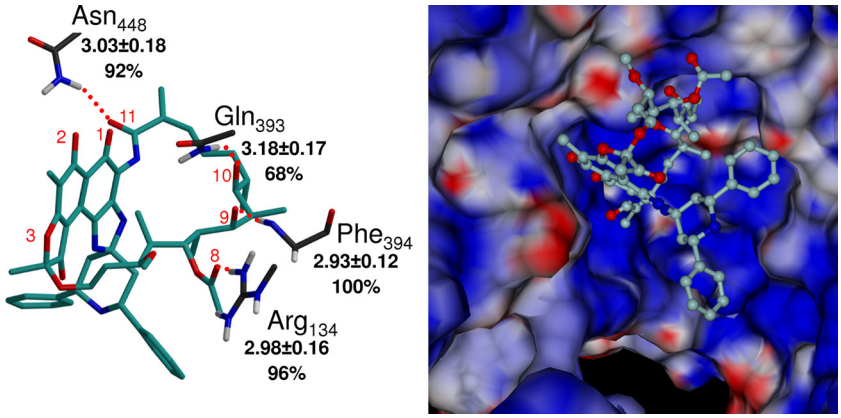

FIG. 2. Molecular dynamics-generated structure for the complexation of RFA-1 to *Taq* RNAP. (Left) Enzyme-ligand H-bond contacts observed for RFA-1 with average atomic distances (Å) and percentage of occurrence indicated. (Right) *Taq* RNAP molecular surface and electrostatic potential at the rifampin binding pocket with RFA-1 displayed in ball-and-stick format.

compounds provide outstanding opportunities for the development of new antibiotics to overcome MDR-TB infections and may be useful candidates for preclinical studies (8).

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Two patents have been issued to the University of Oviedo for use of rifastures as antimycobacterial agents (EP 1 783 129 A1; US 7,521,458 B2). None of the authors of this study hold financial interests in this intellectual property.

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