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Rifamycin derivatives active against pathogenic rapidly-growing mycobacteria

[2]Keith D. Combrink[a,#], Andrea Ramirez Ramos[a], Stephanie Spring[a], Sebastian Schmidl[a,d], Kira Elizondo[a], Petronilo Morin[a], Bryant De Jesus[a], and Florian P. Maurer[b,c]

[a] Department of Biology and Chemistry, Texas A&M International University, Laredo, USA

[b] National and WHO Supranational Reference Laboratory for Mycobacteria, Research Center Borstel, Leibniz Lung Center, Borstel, Germany

[c] University Medical Center Hamburg-Eppendorf, Hamburg, Germany

[d]Present address: RELLIS campus, Texas A&M University, Bryan, TX, USA

[#]Corresponding author:

Keith Combrink

Department of Biology and Chemistry

Texas A&M International University

Laredo, TX, 78045 USA

keith.combrink@gmail.com

Abstract

Infections due to rapidly growing mycobacteria (RGM), and in particular the RGM species *Mycobacterium abscessus* (Mab), are very difficult to treat and reports on novel therapeutic options are scarce. A hallmark of all pathogenic RGM species is their resistance to the four first-line drugs used to treat infections with *Mycobacterium tuberculosis* including rifampicin. This study demonstrates that modification of the rifampicin scaffold can restore rifampicin activity against the three most commonly isolated pathogenic

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RGM species including Mab. We also note that the structure-activity relationship for Mab is different as compared to the non-pathogenic RGM species *Mycobacterium smegmatis*.

Main text

Rapidly growing mycobacteria (RGM) are increasingly recognized as important bacterial pathogens in vulnerable patient populations.[1] RGM infections can affect almost all organs, but are most commonly linked to lung, skin and soft tissue.[2] 80% of lung disease caused by RGM are attributed to organisms belonging to the *Mycobacterium abscessus* (Mab) complex.[2] The two other most frequently encountered pathogenic RGM species are *M. fortuitum* (Mfo) and *M. chelonae* (Mch). Infections due to RGM, and in particular Mab, are very difficult to treat and antibiotic therapy recommended by the American Thoracic Society (ATS) gives response rates for treatment of Mab pulmonary disease below 60% as measured by negative sputum cultures for more than 12 months.[2] This unsatisfactory outcome is in part explained by the extensive natural resistance of Mab to a wide variety of antimicrobials.[2-3] In consequence, treatment regimens are several months in duration with multiple antibiotics often resulting in frequent adverse events.

While rifampicin (RMP, **1**) is a cornerstone drug for treatment of infections caused by slowly growing mycobacteria such as the *M. tuberculosis* (Mtb) complex, it has no activity against RGM.[2-3] In contrast, rifabutin (RBT), another member of the rifamycin class of antimicrobials, has been shown to have low micromolar MICs against Mab as compared to RMP.[4] Rifabutin does not appear to be a substrate for Arr modification and we were unable to detect ribosylation of the C23 hydroxy group of rifabutin with Mab. However, while there is less induction of hepatic microsomal enzymes with RBT than with RMP, elevated transaminases have been reported with high-dose (600 mg/day) rifabutin treatment in combination with macrolides and RBT. [5-6]

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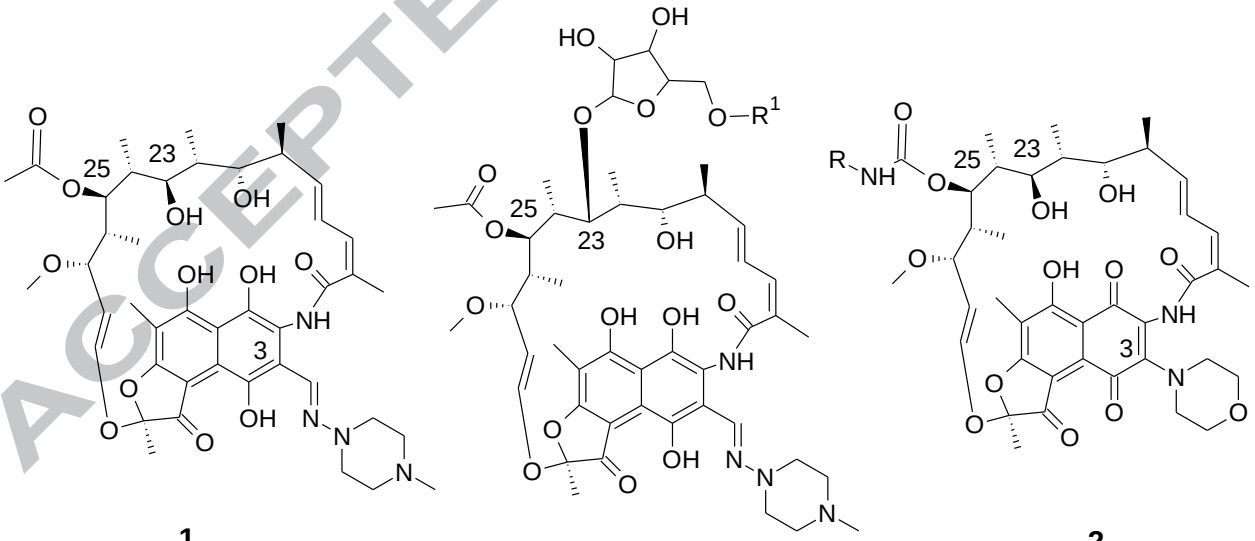
Two mechanisms conferring rifamycin resistance have been described in mycobacteria. Most commonly, resistance is conferred by mutations in the *rpoB* gene encoding the biochemical rifamycin target, the RNA dependent polymerase subunit B.[7-9] Genetic analysis of Mab type strain ATCC 19977 showed that the core region of the *rpoB* genetic sequence is identical to that of rifampicin susceptible Mtb strains.[10] The second mechanism is enzymatic drug modification due to the addition of an ADP-ribosyl group to the C23 hydroxyl group of the rifamycin scaffold (Fig. 1a-b). This is conferred by orthologues of the ADP-ribosyltransferase Arr, which was first described in *M. smegmatis* (Msm) and other RGM.[11-12] ADP-ribosylation is known to play an important role in signaling and disease in all kingdoms of life.[13] However, to our knowledge, ADP-ribosylation of rifamycins is i) the only known example for modification of non-peptide substrates and ii) the only example for ADP-ribosylation as a mechanism of antimicrobial resistance.

Figure 1: Structures of modified rifamycins. 1, rifampicin; **1a-b**, ADP-ribosyl-rifampicin; **2**, C25-carbamate derivatives**.**



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In a previous study with nonpathogenic RGM species *M. smegmatis* (Msm), we showed that addition of a carbamate linked group of sufficient size at the C25 position of a rifamycin derivative **2** was able to block enzymatic inactivation of RMP by Arr_{Msm}.[14] Recently, Rominski et al. prepared three of the compounds from this study (**2a**, **2d** and **2e**) and evaluated them against Mab.[10] The authors showed that the prepared rifamycin carbamates had modest activity against the Mab type strain ATCC 19977. Interestingly, the authors also showed that C25 modification not only increased rifamycin activity against the Mab wild type, but also against a Δarr_{Mab} mutant indicating that the increased activity of C25 rifamycin derivatives is only partially due to resistance to modification by Arr_{Mab}. Lastly, the authors concluded that the studied RMP derivatives were still partially inactivated by Arr_{Mab}. In our original work on Msm, we observed a similar effect with our less active compounds.[14] Here, we prepared seven compounds active against Msm from our previous study and two additional new carbamate derivatives, **2c** and **2f** and evaluated these compounds for activity against a panel of 39 clinical RGM isolates belonging to *M. abscessus* subsp. *abscessus* (n = 8), *M. abscessus* subsp. *bolletii* (n = 7), *M. abscessus* subsp. *massiliense* (n = 8), as well as Mch (n = 8), and Mfo (n = 8).

We synthesized compounds, **2a-b, 2d-e, 2g** as previously described[14] (Fig. 2, Table 1) as well as two new compounds, **2c** and **2f**. The 2-[bis(isopropyl)amino] ethanamine derivative, **2f**, was prepared using the commercially available amine. Compound **2c** was prepared from the [(3-phenyl-5-isoxazolyl)methyl]amine which was prepared from N-Boc-propargyl amine and N-hydroxybenzene-carboximidoyl chloride (Fig. 3). The [(3-phenyl-5-isoxazolyl)methyl]amine was coupled to the activated rifamycin carbamate as were all other derivatives shown in Figure 2. All compounds were characterized by ^{1}H NMR and LC/MS and were consistent with spectral data obtained previously. For the new compounds **2c** and **2f**, structures were confirmed by ^{1}H NMR and LC/MS data and molecular formulas were confirmed by high resolution mass spectrometry.

Figure 2: Synthesis of C25 rifamycin carbamate derivatives.

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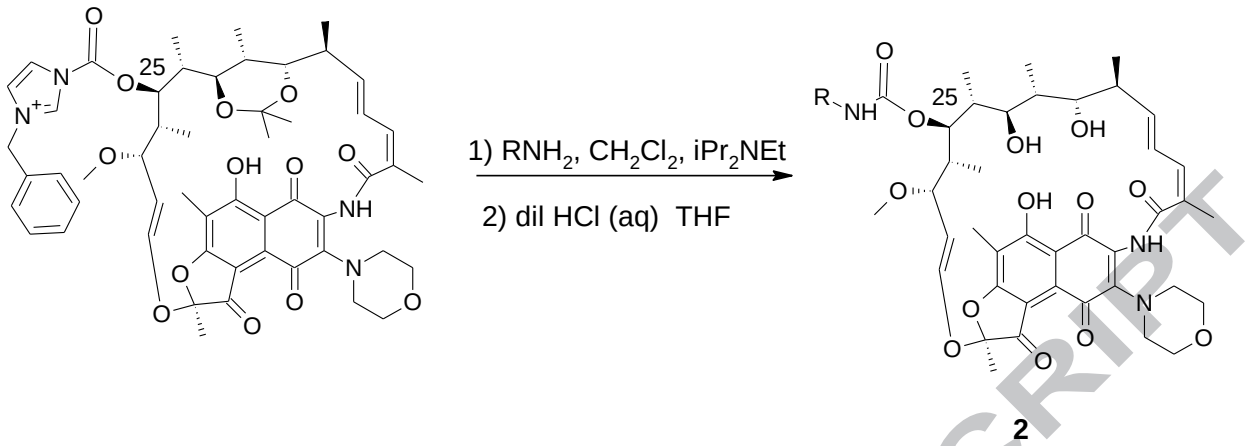
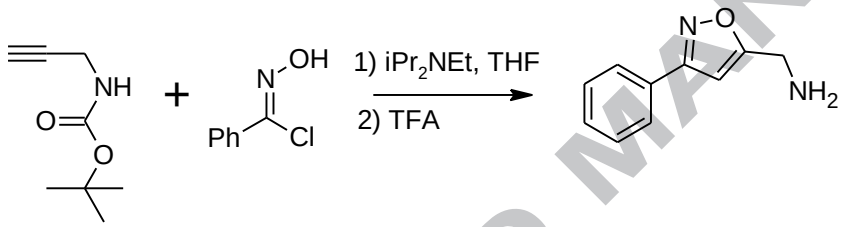


Figure 3: Synthesis of 5-phenyl-isoxazole



The compounds were evaluated in a minimum inhibitory concentration assay (MIC) using standard conditions as detailed in Clinical Laboratory and Standards Institute (CLSI) document M24-A2.[15-16] MIC values presented in Table 1 reflect the median value of the investigated clinical isolates for each (sub-) species (Table 1).

It is interesting to note that the previously reported structure activity relationships (SARs) for Msm differ significantly from the SARs observed in this study of RGM. For Msm, **2a** and **2b** were among the most active compounds with MIC values of 0.06 μg/ml.[14] In contrast, both **2a** and **2b** had only modest activity against Mab with MIC values of 8 and 64 μg/ml respectively. In this study, compound **2g** was the most potent compound tested thus far against Mab and was also a sub-micromolar inhibitor of Msm.

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Compound **2c** (MIC of 0.5 μg/ml) was surprisingly more potent against Mab than the other two isoxazole derivatives **2d** and **2e** which had MIC's of 8 and 64 μg/ml respectively. Both **2d** and **2e** were sub-micromolar inhibitors of Msm.[14] Compound **2f** had similar potency against Mab and Msm but was not uniformly potent across all investigated RGM species. In addition, **2f** was considerably less effective against Mfo with an MIC of 8 mg/ml compared to a range of <0.03 to 1 μg/ml for the other compounds. Mfo was more susceptible to all the C25 carbamates tested in this study than any of the other investigated *Mycobacterium* species with the exception of **2f**.

We further evaluated compounds **2c**, **2e** and **2g** in a zone of inhibition assay using Mab ATCC 19977 and a clinical strain FM0058. For this purpose, a bacterial inoculum corresponding to a McF 0.5 turbidity standard was streaked on a Muller Hinton II agar plate, and filter paper disks containing 25 μl of the investigated derivatives at various concentrations were placed on the agar plate using sterile forceps.[17] Inhibition zone diameters were obtained following 3 days of incubation at 37°C. Compounds **2c** and **2e** had similar zones of inhibition despite having considerably different MIC values. This may be reflected in the better diffusion of **2e** versus **2c** into the agar. Most importantly, compound **2g** showed an inhibitory effect on Mab growth at concentrations down to 6.25 ng and essentially cleared the plate at the highest doses. This observation corroborates the superior anti-mycobacterial activity of compound **2g** in the MIC assay.

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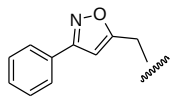
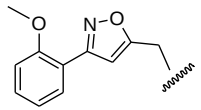
Table 1: Minimum inhibitory concentrations in μg/ml for *M. abscessus*, *M. chelonae* and *M. fortuitum*. Shown are median MIC values for 8 clinical isolates per (sub-)species. All concentrations are expressed in μg / ml.

Compound	Substituent	Species					
		M. abscessus* subsp. *abscessus	***M. abscessus* subsp. *bolletii***	***M. abscessus* subsp. *massiliense***	***M. chelonae***	***M. fortuitum***	***M. smegmatis*[14]**
1	Rifampicin	>64	>64	>64	>64	>64	32
2a	R = 4-CH_3OPh-	64	16	8	>64	1	0.06
2b	R = 4-$(CH_3)_2N$Ph-	8	8	8	16	1	0.06
2c	R = 	0.5	0.125	0.5	0.5	0.0625	NT
2d	R = 	64	16	64	64	0.25	0.12

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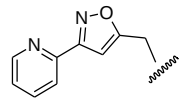
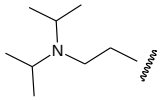
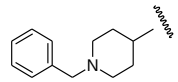
2e	R = 	8	8	8	64	1	0.5
2f	R = 	0.5	0.25	1	1	8	NT
2g	R = 	<0.03	0.0625	0.25	<0.03	<0.03	0.12

Table 2: Zones of inhibition for *M. abscessus* ATCC 19977 and clinical strain FM0058 with compounds 2c, 2e and 2g. All zones are reported in millimeters measured as the diameter across the cleared zone including the filter paper disc. Each disc was loaded with 25 µl of the respective antibiotic solution. The average of three independent measurements is reported. No inhibition was observed below 3 ng for any compounds.

	Inhibition Zone diameter (mm)							
Compound	rifampin		2e		2c		2g	
	Bacterial Strain							
Disk Load (ng)	ATCC1477	FM0058	ATCC1477	FM0058	ATCC1477	FM0058	ATCC1477	FM0058
800	0	0	27	>30	24	>30	>30	>30
400	0	0	24	30	20	26	>30	>30
200	0	0	20	25	16	21	>30	29
100	0	0	16	20	12	17	24	24
50	0	0	14	9	7	13	21	22
25	0	0	0	0	0	7	16	20
12.5	0	0	0	0	0	0	13	16

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6.25	0	0	0	0	0	0	0	11

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To explore the finding of Rominski et al. that some RMP C25 derivatives were still subject to ADP-ribosylation, we incubated the Mab type strain ATCC 19977 with compound **2g**. Using LC/MS, we were unable to find any C23 ribosylated or adenine ribosylated diphosphate derivatives. In contrast, when we incubated Mab with **1**, greater than 90% of **1** was converted to the C23 ribosylated rifampicin **1a** and we did not observe any adenine ribosylated diphosphate derivative **1b**, as described by Dabbs and Baysarowich.[11-12]

In the light of highly successful, rifampin-based therapies for tuberculosis, the unavailability of rifampin derivatives with strong activity against RGM presents a considerable therapeutic challenge.[3] This study shows that rifamycin-based activity can be restored by modification of the rifamycin structure to block Arr-mediated inactivation of rifamycin derivatives and that compounds such as **2g** can be generated that present a novel therapeutic option in antimycobacterial regimens targeting RGM. We are currently preparing additional analogs of this initial series to improve the promising Mab activity seen with this initial set. We are also trying to grow Mab resistant to these compounds by serial passage in media containing sub inhibitory drug concentrations. We expect to see *rpoB* mutations but to date have not yet observed any mutational resistance to these compounds. In *Staphylococcus aureus*, *rpoB* resistant mutants can be obtained after a single passage.[14]

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17. A diagram of the plate layout as well as pictures of the zones of inhibition for each compound can be found in the supplemental material.

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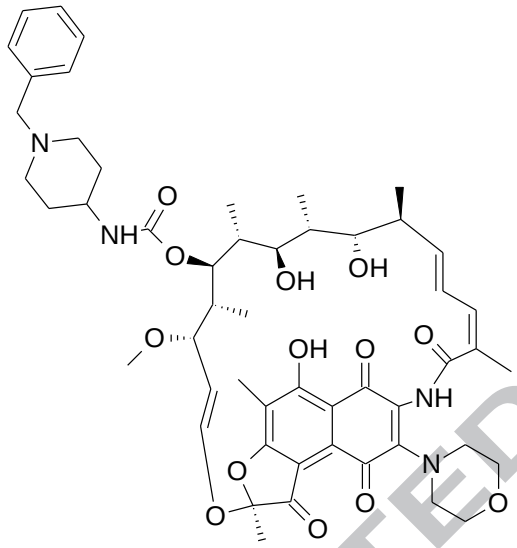
Graphical Abstract

C25 carbamate rifamycin derivatives show potent activity against rapidly growing mycobacterium, RGM, including *Mycobacterium abscessus* and are not substrates for the bacterial modifying enzyme Arr. Rifampin has no activity against RGM and is heavily modified by RGM.



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