

RESEARCH ARTICLE

Defining the minimum inhibitory concentration of 22 rifamycins in iron limited, physiologic medium against *Acinetobacter baumannii*, *Escherichia coli*, and *Klebsiella pneumoniae* clinical isolates

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Abstract

Recently, we reported rifabutin hyper-activity against *Acinetobacter baumannii*. We sought to characterize if any additional rifamycins (n = 22) would also display hyper-activity when tested in iron-limited media against *A. baumannii*, *K. pneumoniae*, and *E. coli*. MICs were determined against representative clinical isolates using the iron-limited media RPMI-1640. Only rifabutin was hyperactive against *A. baumannii*.

Introduction

We previously conducted a modified compound screen assay against *Acinetobacter baumannii* using RPMI-1640 broth, a medium that better models the *in vivo* physiologic blood environment as compared to the rich medium Mueller Hinton II broth, cation-adjusted (MHII). We found that rifabutin (RBT) possesses previously unrecognized hyper-activity against *A. baumannii*, but only in physiological media, and not in rich media [1–3]. RBT is able to rapidly enter the cell through the FhuE protein, an iron transport protein that is downregulated when the bacteria are replete with iron [1,4]. We have previously tested rifabutin, rifampin, rifaximin, rifapentine, FCE-22250, rifalazil, and rifamycin sv against *A. baumannii* and only rifabutin was able to traffic through the FhuE protein in iron limited conditions [1]. Here, we sought to determine if any additional, commercially available rifamycins would have increased activity in iron limited conditions when tested against representative isolates of *A. baumannii*, *Klebsiella pneumoniae*, and *Escherichia coli*.

Rifamycin MIC distributions

We determined rifamycin MICs (n = 185) against a larger panel of 21 rifamycin compounds against *A. baumannii* (n = 5), *K. pneumoniae* (n = 3), and *E. coli* (n = 1) clinical isolates using

Competing interests: Authors BL and BS are inventors on a patent for rifabutin therapy for *A. baumannii* infections and own equity in ExBaq, which has licensed the technology for development. The University of Southern California owns intellectual property related to these development efforts. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

the broth microdilution method per the Clinical & Laboratory Standards Institute (CLSI), but modified to use the iron-limited medium RPMI-1640 as previously described [1–3,5,6]. A summary of the compounds tested is listed in Table 1. Consistent with our previous data, we did observe hyper susceptible RBT phenotypes (MIC < 0.05 mg/L) for the *A. baumannii* HUMC1 and ATCC17978 isolates [1,2]. However, no isolate for any species tested was hyper-susceptible to any other compound tested (Table 1, Fig 1).

CGP-4832

Previous studies have shown that the rifamycin CGP-4832 is able to traffic through the *E. coli* iron transport protein FhuA [7–9]. CGP-4832 MICs against *E. coli* MG1655 were 8 mg/L and 0.25 mg/L in MHII and RPMI-1640 respectively. This 32-fold shift in MIC is consistent with the described mechanism of action for CGP-4832 against *E. coli*. As this mechanism is conceptually similar to the mechanism described for RBT entry, we tested if *A. baumannii* is hyper susceptible to CGP-4832. However, no benefit was observed for *A. baumannii*. CGP-4832 MICs against *A. baumannii* HUMC1 and LAC-4 were 2 mg/L and 4 mg/L respectively in MHII; and 8 mg/L and 2 mg/L respectively in RPMI-1640.

It was previously estimated that about 33% of clinical isolates were hypersusceptible to CGP-4832 [7]. However, this number was determined using only MHII for susceptibility testing because it was not yet known that transport of CGP-4832 through the FhuA protein depended on an iron-limited environment. CGP-4832 MICs were determined against a panel of 45 *E. coli* clinical isolates, obtained from the CDC and FDA AR Isolate Bank, in MHII or RPMI-1640 to test if a greater proportion of clinical isolates would be hypersusceptible to CGP-4832 if MICs were determined in iron-limited media. There was a significant difference

Table 1. MICs for rifamycin compounds determined in RPMI-1640.

Drug	AB HUMC1	AB ATCC 17978	Ab 5075	Ab 6049	AB LAC-4	EC JJ1886	KP KPC- KP1	KP KP3	KP KP4
Rifabutin	0.0625	0.0625	0.0625	0.0625	1	8	16	16	16
Rifampin	8	8	4	2	1	32	>32	32	32
Rifamycin AF	>32	32	ND	16	2	16	>32	32	32
Rifamycin AF-K4033	8	4	ND	8	8	8	>32	32	32
Rifamycin AF-K55517	16	8	16	32	8	32	>32	>32	>32
Rifamycin AF-K56035	32	32	16	32	32	32	>32	>32	>32
Rifamycin AF-K28259	4	16	8	32	4	32	>32	>32	>32
Rifamycin AF-API	16	16	8	8	1	8	>32	32	>32
Rifamycin AF-EPTAPI	>32	>32	32	ND	>32	16	>32	>32	>32
Rifamycin AF-K91725	16	8	16	4	8	16	>32	32	>32
Rifamycin AF-DA	>32	>32	>32	32	>32	>32	>32	>32	>32
Rifamycin AF-O13	>32	>32	>32	32	>32	>32	>32	>32	>32
Rifamycin AF-pNFI	32	>32	>32	16	>32	32	>32	>32	>32
Rifamycin AG	2	1	2	2	1	2	16	8	16
Rifamycin AMI-DA	>32	>32	4	32	>32	32	>32	>32	32
Rifamycin AMP-DA	>32	32	32	32	32	16	32	>32	>32
Rifamycin M14	>32	32	>32	32	>32	16	>32	>32	>32
Rifamycin O	16	16	8	8	32	16	>32	>32	>32
Rifamycin PR-14	>32	>32	16	32	>32	16	32	>32	32
Rifamycin PR-3	16	16	8	8	16	8	32	4	16
Rifamycin S, 8-methyl-	32	16	>32	32	32	16	>32	>32	>32

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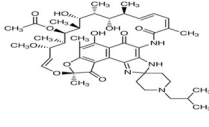
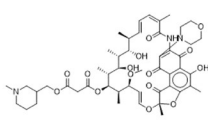
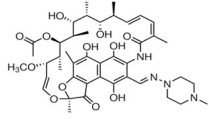
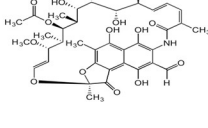
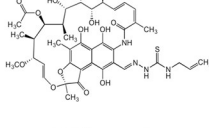
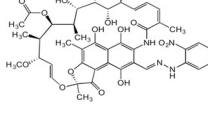
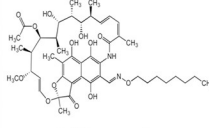
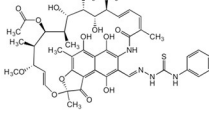
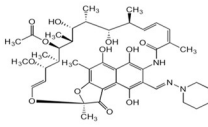
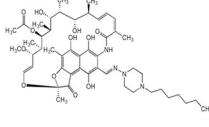
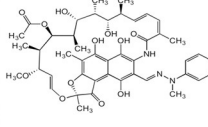
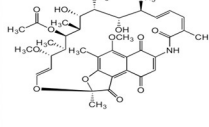
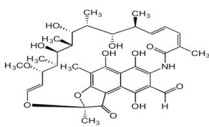
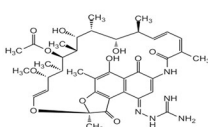
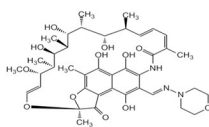
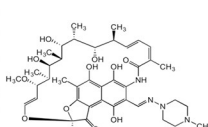
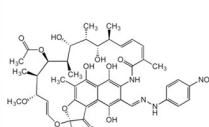
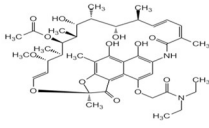
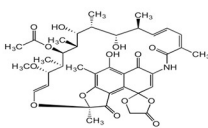
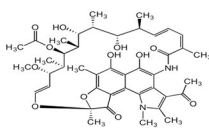
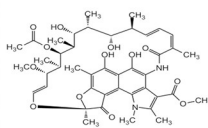
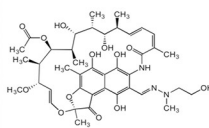
				
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Rifamycin AF-K28259 CAS 51707-27-8	Rifamycin AF-API CAS 13292-40-5	Rifamycin AF-EPTAPI CAS 41971-22-6	Rifamycin AF-K91725 CAS 13292-38-1	Rifamycin S, 8-Methyl CAS 42898-13-5
				
Rifamycin AF-DA CAS 16783-97-4	Rifamycin AG CAS 38128-51-7	Rifamycin AMI-DA CAS 17096-02-5	Rifamycin AMP-DA CAS 16783-99-6	Rifamycin AF-pNFI CAS 39976-91-5
				
Rifamycin M14 CAS 2750-76-7	Rifamycin O CAS 14487-05-9	Rifamycin PR-14 CAS 21240-38-0	Rifamycin PR-3 CAS 21288-98-2	Rifamycin AF-K43033 CAS 57369-86-5

Fig 1. Description of compounds tested.

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in CGP-4832 and RBT MICs determined in MHII ($p = 0.001$, Mann-Whitney), but no significant difference when MICs were determined in RPMI-1640. Additionally, there was not a statistically significant difference between the distribution of GCP-4832 MICs determined in the iron-rich MHII as compared to the iron-limited RPMI-1640 media (**Fig 2, S1 Table**). This result was somewhat surprising because sideromycin antibiotics such as albomycin, also dependent on transport by FhuA, displayed increased activity in iron-limited media [10].

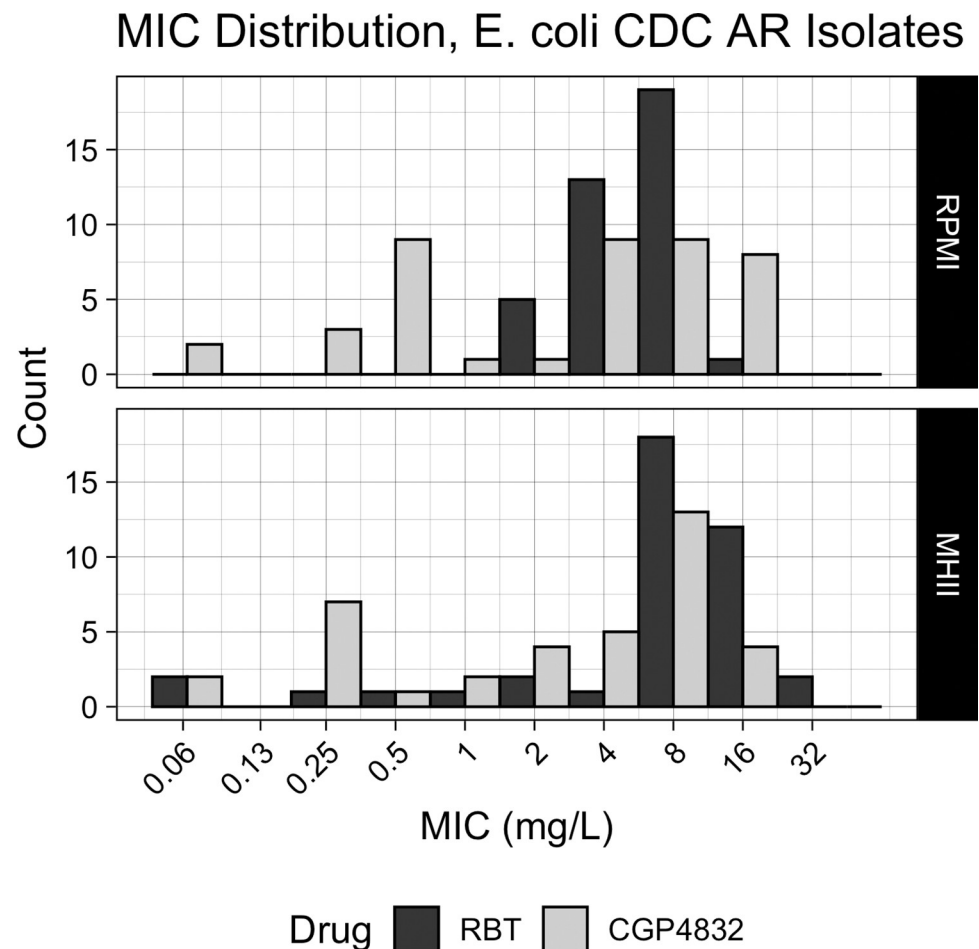


Fig 2. Distribution of CGP-4832 MICs determined in MHII or RPMI-1640 media against 45 *E. coli* clinical isolates.

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Conclusions

The use of culture media that mimics the *in vivo* physiologic environment during infection is a valuable tool for characterizing antibiotic activity *in vitro*. Rifabutin and CGP-4832 are the only rifamycin compounds that are able to traffic through the FhuE protein of *A. baumannii* or the FhuA protein of *E. coli* respectively. We have previously shown that rifabutin is not able to enter *E. coli* through FhuA of *E. coli* [2]. The library of compounds tested represents broad chemical diversity and will further guide the development of additional derivatives capable of being transported more broadly by both FhuA and FhuE proteins of diverse bacterial species.

Methods

MIC assay

Unless otherwise indicated, the standard broth microdilution method following CLSI methodology was used to determine MICs [5]. The media used for the minimum inhibitory concentration (MIC) assays performed in this study were MHII or RPMI-1640 as previously described [1–3,6,11,12].

Briefly, 100 μ L of media was added to the wells in columns 2–10. Column 11 served as a positive growth control and contained only bacteria and media. Column 12 served as the

sterility control and contained only culture media without bacteria. Next, 200 μ l of a 2X antibiotic working solution was added to the wells in column 1. Two-fold serial dilutions of the antibiotic were performed through column 10. Next, 100 μ l of a 1×10^6 CFU/mL working solution of bacteria were added to each of the wells in columns 1–11. The inoculum concentration was confirmed by plating serial dilutions on TSA plates. MIC plates were incubated at $35 \pm 2^\circ\text{C}$ and results were recorded at 24 hours.

Supporting information

S1 Table. MIC summary. Distribution of CGP-4832 MICs determined in MHII or RPMI-1640 media against 45 *E. coli* clinical isolates. (CSV)

Author Contributions

Conceptualization: Brad Spellberg, Brian Luna.

Data curation: Brian Luna.

Funding acquisition: Brad Spellberg, Brian Luna.

Investigation: Peggy Lu, Francesco Alletto, Bosul Lee, Elizabeth Burk, Jasmine Martinez, Fabio Prati, Emilia Caselli, Brad Spellberg, Brian Luna.

Methodology: Brian Luna.

Supervision: Brian Luna.

Writing – original draft: Peggy Lu, Brian Luna.

Writing – review & editing: Peggy Lu, Brian Luna.

References

1. Luna B, Trebosc V, Lee B, Bakowski M, Ulhaq A, Yan J, et al. A nutrient-limited screen unmasks rifabutin hyperactivity for extensively drug-resistant *Acinetobacter baumannii*. *Nat Microbiol.* 2020; 5: 1134–1143. <https://doi.org/10.1038/s41564-020-0737-6> PMID: 32514072
2. Lee B, Yan J, Ulhaq A, Miller S, Seo W, Lu P, et al. In Vitro Activity of Rifabutin and Rifampin against Antibiotic-Resistant *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. *mSphere.* 2021; 6: e0092021. <https://doi.org/10.1128/msphere.00920-21> PMID: 34817233
3. Cheng J, Yan J, Reyna Z, Slarve M, Lu P, Spellberg B, et al. Synergistic Rifabutin and Colistin Reduce Emergence of Resistance When Treating *Acinetobacter baumannii*. *Antimicrob Agents Chemother.* 2021;65. <https://doi.org/10.1128/AAC.02204-20> PMID: 33468472
4. Trebosc V, Schellhorn B, Schill J, Lucchini V, Bühler J, Bourotte M, et al. In vitro activity of rifabutin against 293 contemporary carbapenem-resistant *Acinetobacter baumannii* clinical isolates and characterization of rifabutin mode of action and resistance mechanisms. *J Antimicrob Chemother.* 2020. <https://doi.org/10.1093/jac/dkaa370> PMID: 32869081
5. Performance Standards for Antimicrobial Susceptibility Testing, 32nd edition. Clinical and Laboratory Standards Institute; 2022.
6. Miller S, Goy K, She R, Spellberg B, Luna B. Antimicrobial Susceptibility Testing Performed in RPMI 1640 Reveals Azithromycin Efficacy against Carbapenem-Resistant *Acinetobacter baumannii* and Predicts In Vivo Outcomes in *Galleria mellonella*. *Antimicrob Agents Chemother.* 2022; e0132022. <https://doi.org/10.1128/aac.01320-22> PMID: 36468875
7. Wehrli W, Zimmermann W, Kump W, Tosch W, Vischer W, Zak O. CGP 4832, a new semisynthetic rifamycin derivative highly active against some gram-negative bacteria. *J Antibiot.* 1987; 40: 1733–1739. <https://doi.org/10.7164/antibiotics.40.1733> PMID: 2448280

8. Pugsley AP, Zimmerman W, Wehrl W. Highly efficient uptake of a rifamycin derivative via the FhuA-TonB-dependent uptake route in *Escherichia coli*. *J Gen Microbiol*. 1987; 133: 3505–3511. <https://doi.org/10.1099/00221287-133-12-3505> PMID: 3332686
9. Ferguson AD, Ködding J, Walker G, Bös C, Coulton JW, Diederichs K, et al. Active transport of an antibiotic rifamycin derivative by the outer-membrane protein FhuA. *Structure*. 2001; 9: 707–716. [https://doi.org/10.1016/s0969-2126\(01\)00631-1](https://doi.org/10.1016/s0969-2126(01)00631-1) PMID: 11587645
10. Braun V. Active transport of siderophore-mimicking antibacterials across the outer membrane. *Drug Resist Updat*. 1999; 2: 363–369. <https://doi.org/10.1054/drup.1999.0107> PMID: 11498352
11. Luna BM, Ershova K, Yan J, Ulhaq A, Nielsen TB, Hsieh S, et al. Adjunctive transferrin to reduce the emergence of antibiotic resistance in Gram-negative bacteria. *J Antimicrob Chemother*. 2019. <https://doi.org/10.1093/jac/dkz225> PMID: 31170282
12. Ambrose PG, VanScoy BD, Luna BM, Yan J, Ulhaq A, Nielsen TB, et al. Apotransferrin in Combination with Ciprofloxacin Slows Bacterial Replication, Prevents Resistance Amplification, and Increases Antimicrobial Regimen Effect. *Antimicrob Agents Chemother*. 2019;63. <https://doi.org/10.1128/AAC.00112-19> PMID: 30782989