Synergistic rifabutin and colistin reduce emergence of resistance when treating *Acinetobacter baumannii*

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Running title: Rifabutin and Colistin Combo
Abstract

Recently, we reported rifabutin hyper-activity against *Acinetobacter baumannii*. We sought to characterize potential interactions between rifabutin and colistin, the last resort drug for carbapenem-resistant infections. Rifabutin and colistin were synergistic *in vitro* and *in vivo*, and low dose colistin significantly suppressed emergence of resistance to rifabutin. Thus, this combination is a promising therapeutic option for highly resistant *A. baumannii* infections.
Carbapenem-resistant *Acinetobacter baumannii* poses a significant problem for healthcare and has been identified by the World Health Organization (WHO) and the Centers for Disease Control (CDC) as a priority pathogen for which new antibiotics are needed (1–3). We recently conducted a modified compound screen assay using RPMI broth with serum, a medium that better models the *in vivo* physiologic environment as compared to rich media (e.g., Mueller-Hinton II broth). We found that rifabutin (RBT) possesses previously unrecognized hyper-activity (MIC < 0.05 mg/L) against *A. baumannii*, but only in physiological media, and not in rich media (4). Additionally, *A. baumannii* infected mice were treated with a 300-fold lower dose of RBT had significantly better survival as compared to mice treated with rifampin (RIF) (4).

In a randomized controlled trial, adjunctive RIF significantly improved microbiological eradication when added to colistin (COL) for treatment of extremely drug resistant *A. baumannii* infections, but it did not significantly improve mortality (5). Since RBT is much more potent than RIF *in vitro* and *in vivo*, we hypothesized that combination therapy with RBT and COL might be a more promising option to treat carbapenem-resistant *A. baumannii* infections. Here, we sought to determine the nature of any interaction between RBT and COL, and define the impact of COL on emergence of resistance to RBT.

We began by determining monotherapy MICs. Clinical isolates of *A. baumannii*, which were tested for susceptibility towards RBT, RIF, and COL in Mueller Hinton II Broth (MHII) and RPMI with serum. The ten selected isolates comprised a panel with different susceptibilities against RBT and COL, including antibiotic sensitive, single antibiotic resistant and double antibiotic resistant (Table 1). Consistent with what we previously reported (4), there was no difference between the MICs of RBT and RIF in MHII media. However, in RPMI with serum media, the MICs of RBT were lower than those of RIF for RBT hypersensitive (MIC < 0.05 mg/L) isolates. Of great interest was that COL resistant strains, as defined by MICs greater than 2 mg/L in MHII media, had significantly lower COL
MICs in RPMI with serum, again suggesting that more physiologically relevant culture media alters apparent susceptibility of antibiotics compared to rich broth in vitro.

Next, RIF and RBT MICs were determined in combination with COL. For most strains tested, we found both RIF and RBT were synergistic (fractional inhibition concentration < 0.5) with COL in vitro (Table 1). However, for some strains, synergy was not observed in both medias. The drugs did not demonstrate antagonism against any strain in either media.

One of the concerns with using rifamycins as monotherapy is that bacteria may rapidly acquire resistance. We sought to determine if the addition of COL could suppress the emergence of RBT resistance in A. baumannii (Fig. 1). By plating high bacterial inocula, we found that the combination of RBT + COL significantly reduced the emergence of resistance to RBT (Kruskal-Wallis, p<0.05) (Fig. 1).

To allow for the accumulation of low-resistance conferring mutations, we also conducted low inoculum serial passage of bacteria by serially passaging 20 times in sub-MIC conditions in MHII and RPMI without serum (Fig. 2). Antibiotic susceptibility was tested every five days and the antibiotic concentration used for culture was increased if possible. As expected, culturing bacteria with subinhibitory concentrations of single antibiotics fostered emergence of resistance. However, the combination of RBT + COL suppressed emergence of resistance to RBT for most strains in both media conditions (with the exception being HUMC1 in MHII media).

Next, we evaluated the effect of combination therapy in vivo. As suggested by prior results (4), we confirmed that administration of combination RBT + COL therapy to A. baumannii infected mice resulted in superior survival (Fig. 3A). We repeated the experiment to evaluate changes in bacterial density, which were not evaluated previously (Fig. 3B, 3C). At 24 hours after infection, administration
of combination therapy resulted in below detectable level bacterial density in blood and kidneys, while monotherapy groups had significantly higher bacterial density than the combination therapy group. Specifically, RBT + COL combination significantly reduced CFUs in the blood and kidneys as compared to PBS (Kruskal-Wallis, $P \leq 0.001$ for all comparisons, Fig 3B-3C).

Bacterial density was too low to enable selection for RBT-resistant mutants on selective plates directly. Therefore, blood and kidney homogenates were used to seed overnight cultures, and the outgrowth was plated on trypticase soy agar (TSA) plates supplemented with 8 mg/L RBT (Fig. 3D, E). In the blood outgrowth culture, there were significantly fewer emergent mutants from organs taken from mice treated with RBT + COL as compared to the PBS control (Kruskal-Wallis, $P = 0.008$) and RBT monotherapy (Kruskal-Wallis, $P = 0.026$) (Fig. 3D). There was a notable, but not significant, difference between the RBT + COL combination as compared to the PBS control group (Kruskal-Wallis, $P = 0.05$) (Fig. 3E).

We and others have recently described that RBT is hyperactive against *A. baumannii* because RBT, and not RIF, is able to rapidly traffic through the bacterial FhuE protein (4, 8). Furthermore, it has been postulated that COL can potentiate rifamycins by disrupting the membrane permeability of the bacteria and thus allow for increased intracellular trafficking of the rifamycin antibiotic (5–7). Thus, one potential explanation for diminished synergy in RPMI media is that the bacterial cell is already highly permeable to RBT in RPMI due to the upregulation of FhuE proteins.

In summary, combination RBT + COL is a promising strategy to improve survival and reduce the emergence of resistance to RBT during treatment of *A. baumannii* infections. Importantly, a subtherapeutic dose of COL, which likely would result in diminished toxicity compared to standard dosing, was able to reduce the emergence of resistance to RBT during treatment of *A. baumannii* infections.
bacteremia in mice. These results indicate the promise of a low dose COL + RBT combination regimen in the treatment of such infections.
Acknowledgements

Funding: This work was supported by National Institute of Allergy and Infectious Diseases (NIAID) grants (R01AI139052 to BL; R01AI130060, R01AI117211 to BS); the Food and Drug Administration (FDA) (BAA Contract HHSF223201710199C to BL).

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.
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Table 1. MICs of RBT, RIF and COL alone and drug-drug interactions of RBT + COL or RIF + COL for *A. baumannii*. Drug-drug interactions were evaluated by calculating the fractional inhibitory concentration index (FICI). Synergy was defined by FICI ≤0.5, No Interaction by FICI >0.5 to ≤4.0 and Antagonism by FICI >4.0.

| Strain      | Media     | COL (mg/L) | RBT (mg/L) | RIF (mg/L) |
|-------------|-----------|------------|------------|------------|
| HUMC1       | MHII      | 0.25       | 1.56 (S)   | 1.56 (N)   |
|             | RPMI+10% serum | 0.125   | 0.05 (N)   | 12.50 (N) |
| HUMC1 Δ *fhuE* | MHII      | 0.25       | 12.50 (S)  | 1.56 (S)   |
|             | RPMI+10% serum | 0.125   | 1.56 (N)   | 12.50 (N) |
| LAC-4       | MHII      | 0.125      | 3.13 (S)   | 0.78 (S)   |
|             | RPMI+10% serum | 0.125   | 0.78 (N)   | 1.56 (N)   |
| LAC-4 Col-R | MHII      | 64         | 3.13 (S)   | 0.78 (S)   |
|             | RPMI+10% serum | 0.125   | 0.78 (S)   | 3.13 (S)   |
| VA-AB41     | MHII      | 2          | 3.13 (S)   | 1.56 (S)   |
|             | RPMI+10% serum | 0.125   | 0.39 (S)   | 6.25 (S)   |
| AB5075      | MHII      | 0.25       | 1.56 (S)   | 1.56 (S)   |
|             | RPMI+10% serum | 0.25   | 0.05 (S)   | >25.00 (S) |
| AB5075 tn::fhuE | MHII | 0.5       | 3.13 (S)   | 1.56 (S)   |
|                | RPMI+10% serum |          |          |
|----------------|----------------|----------|----------|
|                | 0.25           | 0.39 (N) | 12.50 (N)|
| C8             | >64            | 12.50 (S)| 12.50 (S)|
|                | RPMI+10% serum | 0.125    | 0.05 (S) | 6.25 (S) |
| C14            | 32             | >25.00 (S)| 6.25 (S) |
|                | RPMI+10% serum | 0.5      | >25.00 (S)| 25.00 (S)|
| AR0299         | 1              | >25.00 (S)| >25.00 (S)|
|                | RPMI+10% serum | 1        | >25.00 (S)| >25.00 (S)|
**Figure 1. Selection of antibiotic resistant mutants by high inoculum plating.**

*baumannii* clinical isolates were cultured in MHII or RPMI media overnight, and mutants were selected by plating bacteria on MHII or RPMI drug plates containing 8 mg/L of RBT, 16 mg/L of COL or the combination of both antibiotics. No CFUs were observed in any of the combination treatment groups. **A)** For HUMC1, there was a significant difference between the combination group and RBT alone in MHII (Kruskal-Wallis, $P = 0.008$) and RPMI (Kruskal-Wallis, $P = 0.027$). **B)** For LAC-4, there was a significant difference between the combination group and RBT alone in MHII (Kruskal-Wallis, $P = 0.008$) and RPMI (Kruskal-Wallis, $P = 0.017$). N=3 for all groups. The median and interquartile range were plotted for all graphs.
Figure 2. Selection of antibiotic resistant mutant by sub-MIC 20 days serial passage. A. *baumannii* clinical isolates were cultured in MHII or RPMI media with 1/3X the MIC of RBT, COL, or both antibiotics. After each 5 day passage, the resistance of bacteria was determined by plating them on 3x and 10x the current passage antibiotic concentration. The starting concentration for the next five days is increased based on the plating result. For all groups cultured in RPMI, the combination treatment suppressed the emergence of resistance as compared to monotherapy.
Figure 3- Efficacy of rifabutin colistin combination treatment in vivo. A) C3HeB/FeJ mice (n=10 per group) were infected with 1.2-3.9E7 CFUs of the hyper-virulent (LD_{100} < 2E7 CFU) carbapenem-resistant A. baumannii HUMC1 (9, 10) and treated with PBS, 0.05 mg/kg RBT, 0.005 mg/kg COL, or a combination of RBT + COL. There was a significant difference comparing the RBT+COL group to PBS (<0.001, Log-Rank) and COL monotherapy (0.0113, Log-Rank). There was no significant difference between the combo treatment group and RBT monotherapy group. B) C3HeB/FeJ mice (n=6 per group) were infected with 5E7 CFUs of A. baumannii HUMC1. Mice were treated once after infection with PBS, 0.05 mg/kg RBT (subtherapeutic), 0.005 mg/kg COL (subtherapeutic) or RBT + COL. Blood and kidney samples were collected 24 hours post infection and kidneys were weighed and homogenized. Blood and C) kidney homogenates were enumerated on TSA plates and results were recorded. No CFUs were observed RBT + COL treatment group in the blood and kidney homogenate. In the blood, there was a significant difference between RBT + COL combination compared to PBS (Kruskal-Wallis, P = 0.001) and COL (Kruskal-Wallis, P = 0.001). In the kidneys, there was a significant difference between the RBT + COL compared to PBS (Kruskal-Wallis, P = 0.0006) and COL (Kruskal-Wallis, P = 0.001). D) 100 µL blood and E) 100 µL kidney homogenates were used to inoculate 10 mL of TSB and the outgrowth from the overnight cultures were serially diluted and plated on drug and non-drug TSA plates and the frequency of resistant mutants were enumerated. In the outgrowth from the blood sample, there was a significant difference between RBT + COL as compared to PBS (Kruskal-Wallis, P = 0.008) and RBT (Kruskal-Wallis, P = 0.026). In the outgrowth from the kidney sample, there was a notable but not significant difference between RBT + COL as compared to PBS (Kruskal-Wallis, P = 0.05). The median and interquartile range were plotted for all graphs.
**HUMC1**
8 mg/L RBT MHII
16 mg/L COL MHII
8 mg/L RBT + 16 mg/L COL MHII
8 mg/L RBT RPMI
16 mg/L COL RPMI
8 mg/L RBT + 16 mg/L COL RPMI

Mutants per 1E8 CFU

10^{-2}
10^{-1}
10^0
10^1
10^2

A)

**LAC4**
8 mg/L RBT MHII
16 mg/L COL MHII
8 mg/L RBT + 16 mg/L COL MHII
8 mg/L RBT RPMI
16 mg/L COL RPMI
8 mg/L RBT + 16 mg/L COL RPMI

B)
A) Monotherapy or combo treatment in C3H mice (n=10)

B) Blood Sample CFU

C) Kidney Sample CFU

D) Blood overnight mutants

E) Kidney overnight mutants

- **P = 0.05**
- **P = 0.001**
- ***P = 0.0001***
- ns = not significant

- PBS
- RBT, 0.05 mg/kg
- COL, 0.005 mg/kg
- RBT+COL

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Blood Sample CFU

PBS
- RBT, 0.05 mg/kg
- COL, 0.005 mg/kg
- RBT+COL

Kidney Sample CFU

PBS
- RBT, 0.05 mg/kg
- COL, 0.005 mg/kg
- RBT+COL

Blood overnight mutants

PBS
- RBT, 0.05 mg/kg
- COL, 0.005 mg/kg
- RBT+COL

Kidney overnight mutants

PBS
- RBT, 0.05 mg/kg
- COL, 0.005 mg/kg
- RBT+COL