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In Vitro Time-kill Activities of Rifalazil, Alone and in Combination with Vancomycin, against Logarithmic and Stationary Cultures of *Staphylococcus aureus*

Marcia S. Osburne[†], David M. Rothstein, Ronnie Farquhar[††], Christopher K. Murphy

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Abstract Rifalazil is a novel rifamycin that, like other members of this class, inhibits bacterial transcription by targeting the β subunit of prokaryotic DNA-dependent RNA polymerase. To address the high-frequency resistance seen with rifamycins, we assessed the ability of rifalazil, alone and in combination with vancomycin, to both kill cells and to suppress the appearance of resistant mutants in log and stationary phase *Staphylococcus aureus* cultures, using high cell densities in an *in vitro* kill curve model. We found that 1) rifalazil alone killed log-phase cultures more rapidly than rifampicin, but both drugs quickly selected for resistant mutants, 2) co-treatment of log phase cultures with rifalazil and vancomycin increased bacterial killing by about 3-Log_{10} over either drug used alone and delayed the appearance of rifamycin-resistant mutants, 3) rifalazil and vancomycin in combination killed stationary phase cultures by 3~4 Log_{10} by 48 hours.

Keywords rifampicin, vancomycin, time kill assay, combination treatment

Introduction

Rifamycins inhibit the β subunit of bacterial RNA polymerase encoded by the *rpoB* gene, and have excellent antibacterial activity against a wide range of Gram-positive and some Gram-negative organisms [1]. Rifalazil, also known as KRM-1648 and ABI-1648, represents the next generation of rifamycins, with improved potency against certain pathogens, and lacking the CYP450 isozyme induction that causes undesirable drug-drug interactions for rifampicin [1]. Although rifampicin and rifalazil are bactericidal *in vitro* and show efficacy in animal models of infectious disease [1], resistance to these drugs occurs at high frequency among Staphylococci and Streptococci (10^{-8}/cell/generation), precluding their use as monotherapy for many infections. Resistance mutations map to the the *rpoB* gene [2].

Combining rifalazil with another agent such as vancomycin is one potential strategy to obtain potent bactericidal activity while suppressing resistant mutants. Vancomycin, which has been used clinically in combination with rifampicin, is only slowly bactericidal and may benefit further from co-treatment with more rapidly bactericidal rifalazil. In prior *in vitro* time-kill studies using rifampicin and vancomycin against *Staphylococcus aureus* cells, vancomycin appeared to enhance the activity of rifampicin by suppressing the proliferation of rifampicin-resistant mutants [3]. Enhanced activity with this combination has also been seen in infection models [4, 5]. As rifalazil has improved properties over rifampicin, we tested the efficacy of the rifalazil/vancomycin combination against *S. aureus* cells, using a modification of *in vitro* time kill experiments in which high initial concentrations of log phase *S. aureus* were treated with drugs. This modification better reflects *in vivo* bioburdens, and, importantly, allowed the appearance of rifamycin-resistant mutants to be monitored. We also

M. S. Osburne (Corresponding author)**, D. M. Rothstein, R. Farquhar, C. K. Murphy:** ActivBiotics Inc., 128 Spring St., Lexington, MA 02421 USA, E-mail: mosburne@mit.edu

[†] Present address: Masschusetts Institute of Technology, Room 48-336B, 15 Vassar St., Cambridge, MA 02139. USA.

[††] Present address: Charles River Laboratories Inc, 57 Union St., Worcester, MA 01608 USA

tested the combination against stationary phase *S. aureus*, which are found in some infections, *e.g.*, indwelling medical devices and abscesses [5].

Materials and Methods

Time-kill Curves

We used *S. aureus* strain ATCC 29213, which has a rifalazil MIC higher than those of many methicillin-sensitive and -resistant *S. aureus* clinical isolates [6]. Colonies were grown on Mueller-Hinton agar (Becton Dickinson) at 35°C for 18 hours. 3～5 colonies were inoculated into 50～100 ml of cation-adjusted Mueller-Hinton broth in a 500 ml Erlenmeyer flask and grown at 37°C with shaking to an OD_{600} of 0.5, *i.e.*, $2～5\times10^8$ cfu/ml (log cells), or an OD_{600} of 2.2 to 2.4, approximately 5×10^9 cfu/ml (stationary cells). Drugs were added (time 0), and 1-ml aliquots were removed at various time points, centrifuged for 5 minutes at 14,000 rpm in a microcentrifuge, resuspended in a fresh medium without drugs, diluted and plated on Mueller Hinton agar without and with rifampicin (1 μg/ml), to determine total cfu and rifamycin-resistant cfu, respectively. Rifalazil-resistant cells are cross-resistant to rifampicin (data not shown). Plates were incubated at 35°C for 18～24 hours.

MIC Determinations

MICs for low-density (5.0×10^5 cfu/ml) *S. aureus* cultures were: rifampicin (Sigma), 0.015 μg/ml; rifalazil, 0.015 μg/ml; and vancomycin (Calbiochem), 1 μg/ml, determined by the microtiter broth dilution technique [7]. The MIC of vancomycin in shake flasks at higher cell density (2×10^8 cfu/ml) was 2 μg/ml . High-density MIC determinations were not possible for rifampicin and rifalazil due to the proliferation of resistant mutants at this high cell density by 24 hours.

MIC Checkerboard Experiments

Rifalazil and vancomycin were added to cation-adjusted Mueller-Hinton broth in 96-well microtiter plates to give two-fold dilutions in the horizontal and vertical directions, respectively. Cells ($1～8\times10^5$/ml) were added, and plates were incubated for 20 hours at 37°C. The sum of the fractional MICs defined synergy (=0.5) or additivity (>0.5 and <1) [8].

Rifalazil Mutant Selection Window

S. aureus 29213 was grown for 24 hours with rifalazil (0.05～666×MIC). All cultures were saturated (OD_{600} >2.5) by 24 hours, and rifamycin-resistant cfu were determined on Petri plates. The lower end of the mutant selection window was the lowest concentration of rifalazil for which the 24 hours culture consisted predominantly of rifamycin-resistant mutants (Fig. 3).

Results

Treatment of Log Phase *S. aureus* Cultures with Rifalazil and Vancomycin

Fig. 1A shows that rifalazil alone (6.6×MIC) caused a rapid decrease in cfu (up to 3.5-Log_{10} in 4 hours). By 24 hours, however, the culture recovered to approximately 1×10^8 cfu/ml. For comparison, rifampicin (6.6×MIC) showed a smaller initial decrease in cfu/ml (2.5-Log_{10}), and then recovered with the same kinetics as rifalazil (data not shown). Vancomycin treatment at 5×MIC caused only a slight decrease in cfu/ml, even after 24 hours. However, rifalazil and vancomycin combined showed a beneficial effect, killing cells (>3-Log_{10}) by 24 hours, with no recovery by 48 hours. Fig. 1B shows that whereas the rifalazil culture was populated by resistant mutants at 24 hours, cultures treated with the combination had few resistant mutants, even by 48 hours. Increasing the vancomycin concentration to 7.5×MIC did not further increase cell killing and suppression of mutants when combined with rifalazil (Figs. 1C and D), but the supply of vancomycin was possibly depleted by 48 hours, thereby potentially reducing the full effectiveness of the combination. Similar results were obtained for rifampicin/vancomycin combinations (not shown). Lower concentrations of rifalazil (1.7×, 0.8×, and 0.4×MIC) with vancomycin (7.5×MIC) were as effective as higher concentrations with regard to both bactericidal activity and the suppression of rifamycin-resistant mutants (Figs. 1E and F).

With regard to the mechanism of action of the rifamycin-vancomycin combination, according to one scenario, the drugs may act independently rather than synergistically in that rifalazil could rapidly kill the abundant rifampicin-sensitive cells, and vancomycin could prevent growth of the small minority of resistant mutants. If this reasoning is correct, the beneficial effect would only be observed at cell densities high enough to give rise to resistant mutants. We carried out checkerboard experiments at low cell density, precluding the appearance of rifamycin-resistant mutants. The presence of rifalazil and vancomyin in combination did not alter the MIC of either drug. This result indicates that rifalazil and vancomycin acted independently, with no apparent benefit or synergy between the two (Fig. 2), consistent with independent action of the two drugs. We

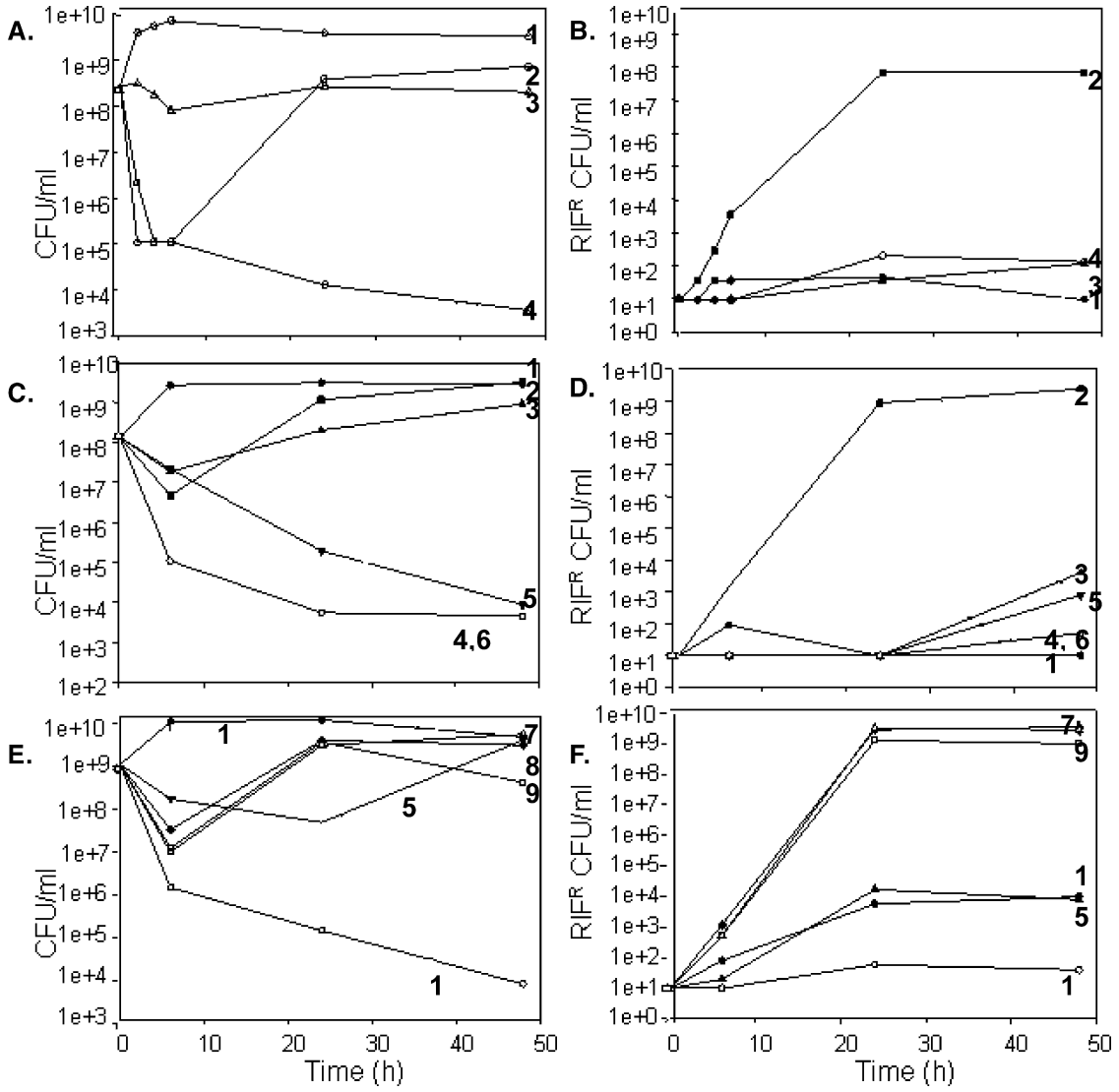

Fig. 1 Time-kill activity of rifalazil (RFZ) and vancomycin (VAN) *versus* log phase *S. aureus* 29213.

Cells were grown as described in the text. **1**: No drug; **2**: RFZ 6.6×MIC; **3**: VAN 5×MIC; **4**: RFZ 6.6×MIC, VAN 5×MIC; **5**: VAN 7.5×MIC; **6**: RFZ 6.6×MIC, VAN 7.5×MIC; **7**: RFZ 0.4×MIC; **8**: RFZ 0.8×MIC; **9**: RFZ 1.7×MIC; **10**: RFZ 0.4×, RFZ 0.8×, RFZ 1.7×MIC, VAN 7.5×MIC. A, C, and E: time-kill experiments. B, D, and F: appearance of rifamycin-resistant mutants corresponding to time-kill experiments in A, C, and E, respectively. For simplicity, data is combined into one curve when results were substantially equivalent.

subsequently determined that the lowest rifalazil concentration that gave a beneficial effect in combination with vancomycin against log phase cells was the same concentration as the lower end of the mutant selection window (Materials and Methods), *i.e.*, the minimum concentration at which resistant mutants can be selected [9] (0.4×MIC, Fig. 3). Thus the benefit of the combination could be detected only at rifalazil concentrations sufficient to select for resistant mutants.

Treatment of Stationary Phase *S. aureus* Cultures with Rifalazil and Vancomycin

Stationary phase cultures of *S. aureus* 29213 were treated with rifalazil alone (6.6×MIC), and in combination with up to 15×MIC vancomycin. Culture viability did not decrease significantly with vancomycin or rifalazil alone (Fig. 4A). However, rifalazil combined with either concentration of vancomycin, or with 2 doses of vancomycin at 7.5×MIC (at time 0 and at 24 hours), resulted in a drop in cell viability of approximately 3-Log_{10} at 48 hours. Fig. 4B shows that some mutants did arise in the culture treated with rifalazil (6.6×MIC) and vancomycin (7.5×MIC).

However, the high cell density of stationary cells may have exhausted the vancomycin, allowing some cell growth using nutrients made available by killed cells. In fact, when a higher level of vancomycin was used at the start of the experiment, or a second dose of vancomycin was given at 24 hours, proliferation of rifamycin-resistant cells was almost completely prevented.

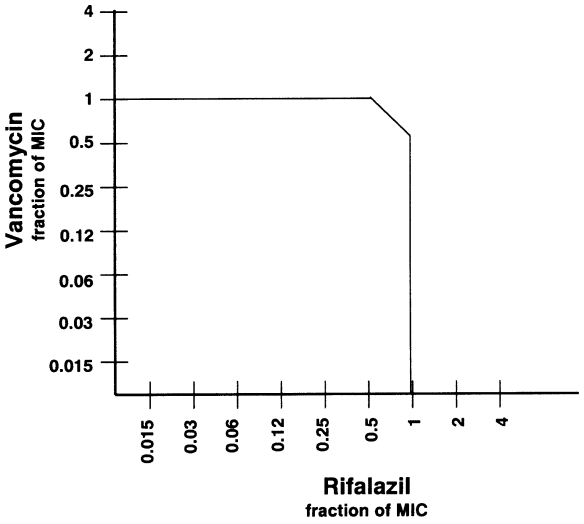

Fig. 2 Isobologram representation of MIC checkerboard experiments with rifalazil and vancomycin.

Discussion

Rifalazil treatment of log phase cells resulted in rapid bactericidal activity, 10～50-fold greater than that seen with rifampicin, followed by a recovery over a 6～48 hours period due to the proliferation of rifamycin-resistant mutants. For log phase cultures, the rifalazil/vancomycin combination was beneficial, resulting in significantly improved bactericidal activity and rifamycin-resistant mutant suppression compared with each drug used alone. Rifalazil and vancomycin were complementary rather than synergistic, with rifalazil providing rapid killing of rifamycin-sensitive cells and vancomycin inhibiting the outgrowth of the small rifamycin-resistant sub-population. This complementary effect was seen only when cell densities were high enough to select for resistant mutants, and when the rifalazil concentration was above the lower end of the mutant selection window. These findings may explain why previous studies reported indifference or even antagonistic effects for the rifampicin/vancomycin combination *in vitro*, but apparent synergy in some animal models of infection [10]: our kill curve protocol encompassed sufficient cell numbers to allow rifamycin-resistant mutants to appear and proliferate, uncovering the beneficial effect of this drug combination.

The rifalazil/vancomycin combination showed significantly enhanced activity against stationary cultures, important because stationary cells are present in *in vivo* infection and infection models [5], thus providing an

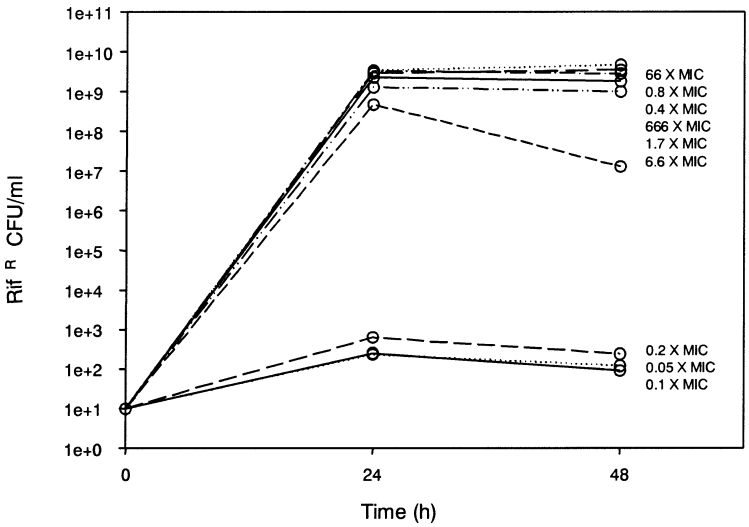

Fig. 3 Determination of the rifalazil mutant selection window.

S. aureus 29213 was grown with rifalazil at various concentrations (0.05～666×MIC). The number of rifamycin-resistant cfu present in each culture is shown. The lower end of the mutant selection window (0.4×MIC) was the lowest concentration of rifalazil for which the culture consisted predominantly of rifamycin-resistant mutants.

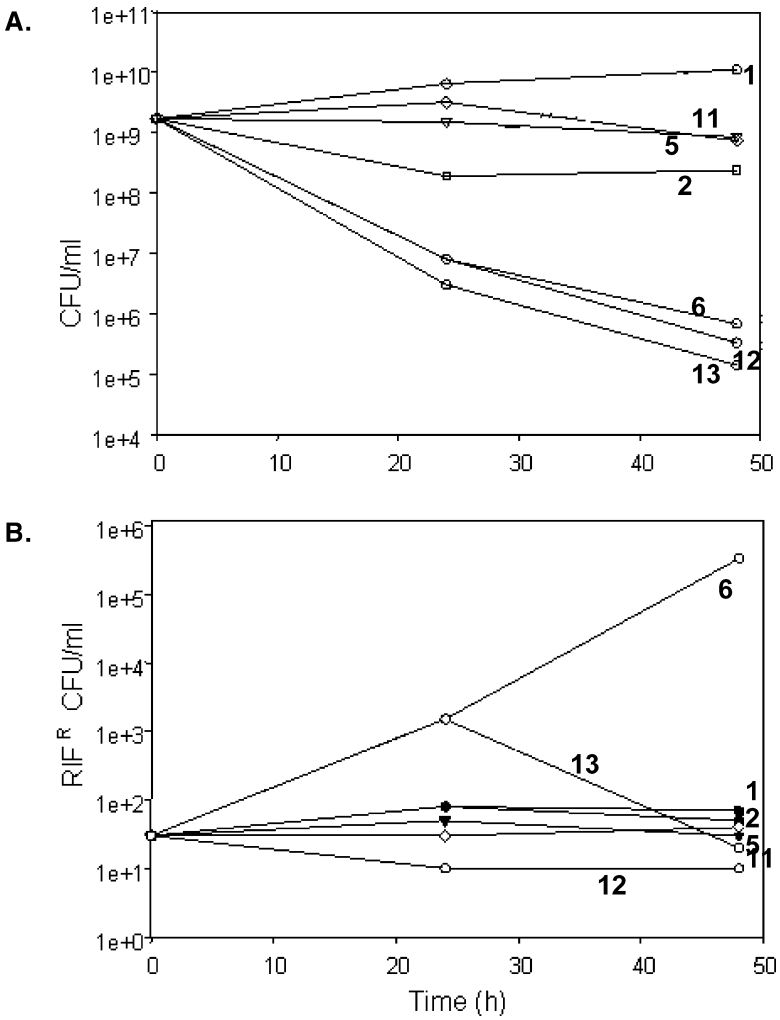

Fig. 4 Time-kill activity of rifalazil, vancomycin, and combinations *versus* stationary *S. aureus* 29213.

Cells were grown to stationary phase as described in the text. **1**: No drug; **2**: RFZ 6.6×MIC; **5**: VAN 7.5×MIC; **6**: RFZ 6.6×MIC, VAN 7.5×MIC; **11**: VAN 15×MIC; **12**: RFZ 6.6×MIC, VAN 15×MIC; **13**: RFZ 6.6×MIC, VAN 7.5×MIC, second dose of VAN at 24 hours. A: time-kill experiments. B: appearance of rifamycin-resistant mutants for time-kill experiments in panel A.

additional benefit in antibacterial treatment.

Our results suggest that the rifalazil/vancomycin combination should be explored further in *in vivo* infection models, and may have clinical applications.

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