J. Antibiot. 59(5): 303–308, 2006

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Enhanced Activity of Rifalazil in Combination with Levofloxacin, Linezolid, or Mupirocin against *Staphylococcus aureus In Vitro*

Marcia S. Osburne†, Christopher K. Murphy, David M. Rothstein

Received: March 24, 2006 / Accepted: May 13, 2006

Abstract Rifalazil is a potent second-generation ansamycin that kills bacterial cells by inhibiting the β subunit of RNA polymerase. Rifalazil has several improved properties compared with rifampicin, but retains rifampicin's propensity to develop resistant mutants at high frequency. To explore strategies to overcome resistance development, we studied the effects of rifalazil in combination with several different antibiotics in an *in vitro* time-kill model, against both log phase and stationary phase *Staphylococcus aureus* cells. Experiments were carried out at high initial cell density so that the frequency and proliferation of resistant mutants could be monitored. We found that each combination was advantageous in terms of enhanced killing and the suppression of mutants, compared with each drug used alone. None of the three combinations was effective against stationary phase cells.

Keywords rifalazil, mupirocin, linezolid, combination treatment, time kill assay

Introduction

Rifampicin and other rifamycins inhibit bacteria by targeting the β subunit of RNA polymerase, and are active against a wide range of Gram-positive and certain Gram-negative organisms [1]. *In vitro* experiments have demonstrated the bactericidal nature of this class of drugs [1]. A new generation of rifamycins, represented by rifalazil, also known as KRM-1648 and more recently, ABI-1648, has improved properties, including increased potency and lack of P450 induction [1]. However, resistance to both rifampicin and rifalazil occurs at high frequency (approximately 10^{-8}/bacterial generation) and precludes their use as a monotherapy for infections with high bacterial cell density. Mutations responsible for resistance to both drugs map to the bacterial *rpoB* gene, which encodes the β subunit of RNA polymerase [2].

We investigated previously whether rifalazil could be used in combination with vancomycin to produce both potent bactericidal activity and the suppression of resistant mutants, and found that co-treatment of either log or stationary phase *Staphylococcus aureus* cells with rifalazil and vancomycin increased the bactericidal activity over that obtained using either drug alone and delayed the appearance of resistant mutants [3]. To continue our study of strategies to address drug resistance, we assessed the effects of rifalazil co-treatment with the antibiotics linezolid, mupirocin, or levofloxacin on *S. aureus* by means of *in vitro* time kill curves, as previously described [3].

We wished to determine whether the bacteriostatic agents linezolid and mupirocin [4, 5], in combination with rifalazil, would show improved potency *i.e.*, suppression of resistance development while retaining cidality at least equivalent to rifalazil alone. Linezolid is the first in a new class of drugs, the oxazolidinones, which are synthetic antimicrobials with potent activity against Gram-positive pathogens. Prior work has shown that the combination of rifampicin and linezolid had more activity than either drug alone in a time-kill model against MSSA and MRSA [5, 6] and inhibited mutant proliferation [6]. The spontaneous resistance frequency to linezolid is low in *S. aureus* ($<8\times10^{-11}$), and resistance is slow to develop [7]. Mupirocin, a drug used topically to treat *S. aureus* and

M. S. Osburne (Corresponding author)**, C. K. Murphy, D. M. Rothstein:** ActivBiotics Inc., 110 Hartwell Ave., Lexington, MA 02421 USA, E-mail: mosburne@mit.edu

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

Streptococcus pyogenes skin infections [8, 9] and *S. aureus* nasal colonization [10], was found recently to show synergistic activity with amoxicillin-clavulanate against MSSA and MRSA in an *in vitro* time kill model [11]. We therefore wished to investigate its potential to interact favorably with rifalazil.

Earlier work showed that co-treatment of bacterial cultures with rifampicin and ciprofloxacin, both bactericidal compounds, prevented the emergence of *S. aureus* mutants resistant to either drug [12]. Levofloxacin, a related quinolone, was previously shown to be a very potent bactericidal agent in time-kill studies similar to ours [13], more potent against MSSA than ciprofloxacin, and did not lead to the emergence of resistance [13]. Therefore it was of interest to test the rifalazil/levofloxacin combination in our system, and as a control combination of two bactericidal compounds.

Our data comprise *in vitro* time-kill curves generated following treatment of high-density cultures of *S. aureus* with these agents, separately or in combination. Bactericidal activity was assessed over 24 hours using agar plate counts to determine CFU. The high initial density allowed us to monitor the appearance of rifamycin-resistant mutants, as described previously [3]. We found that for log phase (growing) cells, when compared with each drug used alone, rifalazil co-treatment with levofloxacin, linezolid, or mupirocin showed enhanced killing of *S. aureus* and suppression of rifamycin-resistant mutants.

Materials and Methods

Time-kill Curves

S. aureus strain ATCC 29213, which was used in this study, is a good representative for *S. aureus* species because the MIC of rifalazil for this strain is somewhat higher than the MIC_{50} for MSSA and MRSA clinical isolates [14]. Time-kill curves were carried out as described previously for high density cultures [3]. Briefly, 3∼5 colonies of *S. aureus* strain ATCC 29213, grown on a Mueller-Hinton agar (Becton Dickinson) plate at 35°C for 18 hours, were inoculated into 50∼100 ml of cation-adjusted Mueller-Hinton broth in a 500 ml flask and grown at 37°C with shaking. For studies of growing cells, cultures were grown to an optical density of 0.5 at 600 nm (time zero), corresponding to approximately $2\sim5\times10^8$ CFU/ml. Note that initial cell density was substantially higher than that typically used (2×10^6 to 1×10^7 CFU/ml) in time-kill experiments [15]. For stationary phase, cultures were grown to approximately 5×10^9 CFU/ml, corresponding to an optical density of ∼2.2 to 2.4 at 600 nm (time zero). In all experiments drugs were added to the cultures at time zero, 1-ml aliquots were removed at various time points and centrifuged for 5 minutes at 14,000 rpm in a microfuge. Cells were then washed in 1 ml of fresh medium without drug to eliminate drug carryover. Cells were serially diluted and plated on Mueller Hinton agar to determine total CFU, and on Mueller Hinton agar containing rifampicin (1 μg/ml) to determine rifampicin-resistant (Rif^R) CFU. We previously determined that cells resistant to rifampicin are cross-resistant to rifalazil and other ansamycins (data not shown). Plates were incubated at 35°C for 18∼24 hours, and colonies were counted.

MIC Determinations

The MICs of various drugs for *S. aureus* 29213 are shown in Table 1. MICs of all drugs were determined at low initial cell density (2×10^6 CFU/ml) by the microtiter broth dilution technique [16]. MICs of levofloxacin, linezolid, mupirocin, and vancomycin were also determined at high initial cell density (2×10^8 CFU/ml): for these experiments cells were grown in shake flasks at 37°C for 24 hours. The minimal inhibitory concentration was the lowest dilution of compound that resulted in no detectable growth, as evaluated by visual inspection. MICs of rifalazil were determined only at low density. Antibiotics were either purchased from commercial sources or synthesized and purified at ActivBiotics.

MIC Checkerboard Combination Experiments

To characterize the nature of the interaction between rifalazil and other drugs, rifalazil and the second drug were added to cation-adjusted Mueller-Hinton broth in 96-well microtiter plates to give two-fold dilutions of rifalazil in the horizontal direction and two-fold dilutions of the second drug, levofloxacin, linezolid, or mupirocin, in the vertical direction. Cells were inoculated at a concentration of $1\sim8\times10^5$/ml, and plates were incubated for 20 hours at 37°C. For each MIC obtained in combination, the fractional MIC (the fraction of the MIC of that drug alone needed to obtain an MIC in combination) of each compound was determined, and the sum of the fractional MICs defined synergy (fractional MIC=0.5) or additivity (fractional MIC>0.5 but <1) [2]. Each drug combination was tested in duplicate or triplicate.

Results

Treatment of Log Phase *S. aureus* with Rifalazil Alone and in Combination with Levofloxacin, Linezolid, or Mupirocin

The MICs of each of the drugs used are presented in Table 1. The MICs for levofloxacin, linezolid, and mupirocin were determined at high initial cell density, as described in Materials and Methods, both because this density (2×10^8 CFU/ml) is more reflective of a potential bioburden in an infection and because it allowed us to monitor the proliferation of rifalazil-resistant mutants in co-treatment experiments. Resistant mutants normally occur at a frequency of approximately 10^{-8} per cell per generation for rifalazil. MIC determination at high initial cell density is not possible for rifalazil because of the proliferation of resistant mutants by 24 hours.

Fig. 1A shows that treatment of *S. aureus* with rifalazil at about 6.6×the MIC resulted in an initial rapid drop in CFU/ml by 4 hours, followed by a recovery of the culture by 24 hours due to the proliferation of resistant mutants (Fig. 1B). Treatment with levofloxacin alone at 6×the MIC resulted in a 2-Log_{10} drop in CFU/ml, which persisted through 24 hours. The combination of rifalazil and levofloxacin showed cidality, with an approximately 3-Log_{10} decrease in CFU/ml over 24 hours, an enhancement over the killing seen with either drug alone. Furthermore, the combination resulted in a 6-Log_{10} suppression of the appearance of resistant mutants at 24 hours.

Linezolid, a bacteriostatic drug, had an MIC of 2 μg/ml against *S. aureus* at high initial cell concentration (Table 1), and poor cidal activity against this strain in log phase at 1, 2.5, and even 5×the MIC (Fig. 1C). However, at all of these concentrations the combination of linezolid and rifalazil resulted in a decrease in CFU/ml of 2.5-Log_{10} at 24 hours, at least 2-Log_{10} greater than that seen with the highest concentration of linezolid alone, in addition to a dramatic suppression of the appearance of Rif^R mutants by 6- to 9-Log_{10} at 24 hours (Fig. 1D). As substantially equivalent killing and suppression of mutants were observed using rifalazil combined with all three linezolid concentrations, for simplicity these combinations are represented as one curve.

Mupirocin, also known to be bacteriostatic, showed only poor bactericidal activity (Fig. 1E) when used alone. Recently, however, mupirocin was shown to exhibit synergy in combination with amoxicillin-clavulanate against 9 out of 49 MSSA and MRSA strains in *in vitro* time-kill studies with initial cell concentrations of 1.0×10^6 CFU/ml [11]. Synergy was defined as an additional reduction of the initial inoculum of greater than 2-Log_{10} CFU/ml at 24 hours, as compared with that of the more active of the two compounds [17]. Interestingly, using this less conventional definition, and with our high initial cell density protocol, we found apparent synergy between rifalazil and mupirocin against log phase *S. aureus* when mupirocin was used at either 1.5 or 5×the MIC (again, these curves were combined for simplicity because of substantially equivalent results). It has been shown previously that the frequency of spontaneous mupirocin-resistant *S. aureus* mutants *in vitro* was less than or equal to 1.0×10^{-9} [9]. We found that the combination of rifalazil and mupirocin suppressed the proliferation of Rif^R mutants by approximately 6-Log_{10} at 24 hours. These results suggest that the favorable drug interactions described above may result from the ability of the second drug to prevent the proliferation of Rif^R mutants.

Table 1 *In vitro* activities of rifalazil, levofloxacin, linezolid, mupirocin, and vancomycin against *S. aureus* 29213

Antimicrobial agent	MIC (μg/ml) Low density[a]	MIC (μg/ml) High density[b]
Rifalazil	0.015	—
Levofloxacin	0.5	0.25
Linezolid	2.5	2.0
Mupirocin	0.2	0.2
Vancomycin	2.0	2.0

[a] Low-density inoculum: 2×10^6 cells/ml, [b] high-density inoculum: 2×10^8 cells/ml.

MIC Checkerboard Combination Testing

To help determine the nature of the interactions between rifalazil and a second drug against growing *S. aureus* cells, MIC testing was conducted in a checkerboard array in combination with either levofloxacin, linezolid, or mupirocin. Note that these experiments were conducted at low cell density, as described in the Materials and Methods section, precluding the appearance of Rif^R mutants, as opposed to the high cell density time-kill experiments outlined above. Nonetheless, we found a positive interaction between each compound and rifalazil (Table 2). The positive interaction was particularly apparent for the combination of rifalazil and linezolid, where the fractional MIC sum for one concentration combination was 0.5. These experiments suggest that there may be a favorable interaction between these combinations beyond the suppression of resistant mutants.

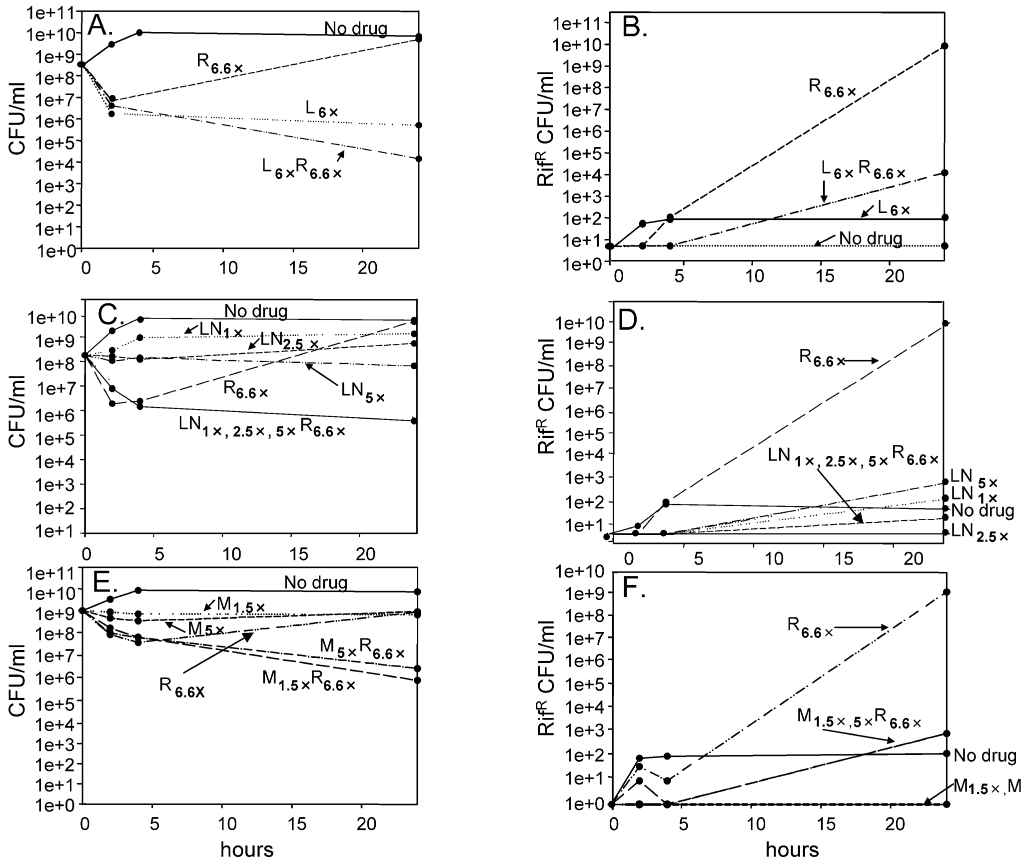

Fig. 1 Time-kill activity of rifalazil, and rifalazil plus levofloxacin, linezolid, or mupirocin *versus* log phase *S. aureus* 29213.

Cells were grown as described in the text. A: rifalazil (R), 6.6×MIC; levofloxacin (L), 6.0×MIC; and combinations; C: rifalazil (R), 6.6×MIC; linzolid (LN), 1×MIC, 2.5×MIC, 5×MIC; and combinations; E: rifalazil (R), 6.6×MIC; mupirocin (M), 1.5×MIC, 5×MIC; and combinations; B, D, and F: appearance of Rif^R mutants corresponding to time-kill experiments in A, C, and E, respectively.

Treatment of Stationary Phase (Non-growing) *S. aureus* Cultures with Rifalazil Alone and in Combination with Levofloxacin, Linezolid, or Mupirocin

As shown in Fig. 2A, the combinations of rifalazil with levofloxacin, linezolid, or mupirocin were ineffective for killing stationary cultures of *S. aureus*. In this set of experiments, cultures were followed for at least 24 hours, and those that showed any trend toward decreased viability at 24 hours were followed for an additional 24 hours. Rifalazil plus vancomycin, previously shown to have activity against stationary phase cells [3] and therefore used as a positive control, again showed effective bactericidal activity. Our data indicate that the rifalazil-vancomycin combination appears to be thus far uniquely effective against non-growing *S. aureus*.

In this experiment some proliferation of Rif^R cells occurred in the culture treated with rifalazil alone (Fig. 2B). We assume that the small amount of initial killing by rifalazil provided nutrients that resulted in some culture growth, allowing some resistant mutants to emerge.

Discussion

The modified *in vitro* time-kill model was an effective method for capturing the advantages of rifalazil in combination with levofloxacin, linezolid, or mupirocin against *S. aureus* 29213. Log phase cultures grown to high density (2~5×10^8 CFU/ml) provided a basis for evaluating the appearance and suppression of Rif^R mutants. For all combinations tested, we found both enhanced killing and suppression of Rif^R mutant proliferation. Checkerboard experiments, the conventional method of determining synergy, were carried out at low cell density (at which resistance development was not likely to be a factor) and showed positive interactions but no clear synergies between rifalazil and each of the other drugs. The more positive interactions in the high cell density experiments were thus likely to be due to inhibition of the proliferation of Rif^R mutants by the second drug, although the checkerboard assay data for rifalazil and linezolid suggests there may be

Table 2 MICs[a] of rifalazil in combination with levofloxacin, linezolid, or mupirocin in checkerboard assay[b]

R	L	R+L
0.5	0.5	1.0
0.25	0.5	0.75
0.125	0.5	0.625
0.063	0.5	0.563
R	**LN**	**R+LN**
0.5	0.125	0.625
0.25	0.25	0.5
0.125	0.5	0.625
0.063	0.5	0.563
R	**M**	**R+M**
0.25	0.5	0.75
0.5	0.25	0.75
0.5	0.125	0.625
0.5	0.063	0.563

[a] MICs of each drug singly were: rifalazil (R), 0.015 μg/ml; levofloxacin (L), 0.5 μg/ml; linezolid (LN), 2.5 μg/ml; mupirocin (M), 0.2 μg/ml.
[b] Synergy is defined by a fractional MIC=0.5, and additivity by a fractional MIC>0.5 and <1 (Materials and Methods).

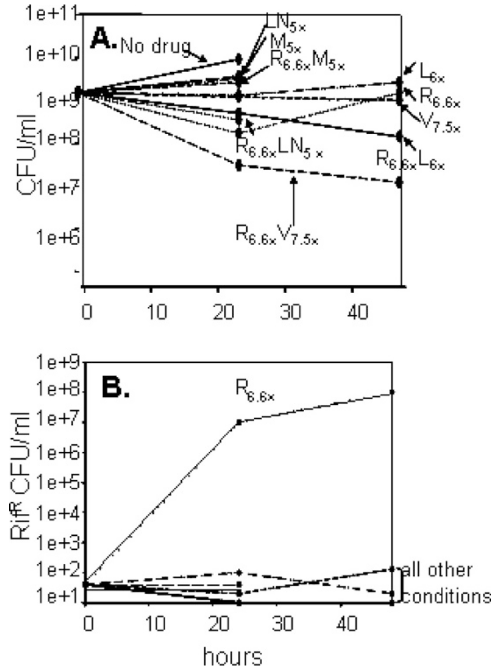

Fig. 2 Time-kill activity of rifalazil, levofloxacin, linezolid, mupirocin, vancomycin, and combinations *versus* stationary *S. aureus* 29213.

Cells were grown to stationary phase as described in the text. A: rifalazil (R), 6.6×MIC; vancomycin (V), 7.5×MIC; levofloxacin (L), 6×MIC; linezolid (LN), 5×MIC; mupirocin (M), 5×MIC; and combinations; B: appearance of Rif^R mutants for time-kill experiments in panel A.

other contributing factors beyond the suppression of resistant mutants.

Although none of these combinations was effective against stationary cells, their effectiveness against log phase cells is striking. The enhanced activity observed when rifalazil was combined with the weakly cidal or bacteriostatic drugs linezolid and mupirocin is especially intriguing as it suggests a possibility for extending the clinical applications of these drugs. For example, one potential benefit might be a shorter course of therapy with a combination of rifalazil and linezolid, as opposed to the long course of therapy with linezolid alone that is currently prescribed. Our results suggest that these drug combinations should be tested further in *in vivo* infection models.

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