

Rifalazil Retains Activity Against Rifampin-resistant Mutants of *Chlamydia pneumoniae*

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Abstract Rifampin-resistant mutants of the obligate intracellular pathogen *Chlamydia pneumoniae* were isolated and characterized, including strains that contained multiple mutations in the *rpoB* gene encoding the rifampin binding site. The highest MIC of rifampin against a mutant strain exceeded 100 µg/ml, whereas the highest MIC of rifalazil was 0.125 µg/ml. Derivatives of rifalazil (new chemical entities; NCEs) showed from 2~4 fold lower MICs, as well as 2~8 fold lower bactericidal concentrations against both wild type and mutant strains when compared with rifalazil. These results suggest that rifalazil and NCEs are appropriate therapeutic agents for the treatment of *C. pneumoniae* infections from the point of view of potency and resistance development.

Keywords rifalazil, Chlamydia, rifampin, resistance, *Chlamydia pneumoniae*

Introduction

Rifalazil [3'-hydroxy-5'-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin] represents a new generation of rifamycins, compounds which inhibit bacterial RNA polymerases isolated from a broad spectrum of microorganisms [1]. Clinically the rifamycins rifampin, rifabutin, and rifapentine have been used predominantly to treat tuberculosis, an indication for which rifalazil (also

known as KRM-1648 and as ABI-1648), was originally developed by Kaneka Corporation, Osaka, Japan, through phase II clinical trials [2, 3].

More recently it has been found that rifalazil was 50~500 times more potent against the obligate intracellular pathogens *Chlamydia trachomatis* [4, 5], and *Chlamydia pneumoniae* [4, 6], than azithromycin, the standard of care in treating chlamydial infections. *Chlamydia trachomatis* is a leading cause of sexually transmitted disease and of preventable blindness (trachoma) worldwide [8]. *Chlamydia pneumoniae* is a frequent cause of respiratory infections [9]. Potency may be important to overcome Chlamydia, which can enter the persistent state, which may be more refractory to many antibiotics [10, 11]. Rifalazil has a long half-life and large volume of distribution, properties that may enhance efficacy against these obligate intracellular pathogens *in vivo* [3, 12, 13]. Animal studies support this hypothesis. In the mouse pneumonitis model of infection with *C. pneumoniae*, rifalazil administered as 3 daily doses of 1 mg/kg was effective in clearing lung infection [6, 7].

In a Phase II clinical trial, a single 25 mg oral dose of rifalazil was shown to eradicate *C. trachomatis* from the majority of male patients infected with this bacterium who presented with nongonococcal urethritis [14]. More recently rifalazil has been evaluated for treatment of peripheral arterial disease, based on an association of atherosclerosis and *C. pneumoniae* infection, supported by animal model studies, and investigations showing the presence of *C. pneumoniae* in diseased vasculature [13, 15].

In this paper we address concerns about the potential for resistance development during rifalazil monotherapy. Both rifalazil and rifampin have been shown to share the property of frequent selection of resistant strains following

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exposure of these drugs to free-living bacteria such as *Mycobacterium tuberculosis* [16~19], *Staphylococcus aureus* [20~22], and *Streptococcus pyogenes* [23]. Mutations in the *rpoB* genes of these microorganisms account for the development of resistance. However, rifalazil was shown previously to be less prone to resistance selection when *C. trachomatis* was tested in cell culture, even though rifampin resistance was high [24]. The eradication of *C. trachomatis* among patients treated with rifalazil also suggests that resistance development among Chlamydia is not a high frequency consequence of rifalazil monotherapy [14].

In a previous study, mutants of *C. pneumoniae* having low-level resistance to rifampin and rifalazil were isolated, although the same mutants were not tested for susceptibility of both rifampin and rifalazil [25]. To advance these results, an extensive effort was undertaken to isolate high-level rifampin-resistant mutants of *C. pneumoniae*, and to test whether rifalazil and derivative compounds retained activity against these isolates.

Materials and Methods

Chemicals

Rifampin, obtained from Sigma, was prepared in DMSO to a concentration of 10 mg/ml and subsequently diluted in DMSO if necessary, to achieve concentrations ranging from 0.000032 to 0.5 $\mu\text{g/ml}$. Rifalazil and NCEs were provided by ActivBiotics.

Organisms

Hep-2 mammalian cells were the host. *C. pneumoniae* strains TW-183 and CWL029 were the susceptible wild type parent strains.

Susceptibility Tests

The MIC test was as previously described [26]. Briefly, Hep-2 mammalian monolayers were formed following incubation of 100 μl /well in 96-well plates in antimicrobial-free growth medium consisting of minimal essential medium with 10% fetal bovine serum and 220 μg of L-glutamine/liter added. Cells were centrifuged (1,200 $\times g$ for 1 hour at 37°C), and were then exposed to growth medium containing 1.0 $\mu\text{g/ml}$ cycloheximide and two-fold dilutions of rifamycins. After incubation for 72 hours at 37°C in 4.0% CO₂, cells were incubated and fixed with MeOH. Chlamydial inclusions were detected by fluorescence microscopy by using a genus-specific monoclonal antibody CF-2 (Washington Research Foundation, Seattle). MCC3 results were determined as described [26], utilizing the

same initial procedure as in MIC test, except that three antibiotic-free passages were carried out prior to assaying for inclusions.

Protection Experiments

The determination of protective concentrations after exposure to rifamycins was as described [27]. Briefly, mammalian cells were treated as in the MIC testing, exposed to rifamycins for 24 hours, centrifuged, washed, and incubated in fresh antibacterial-free medium, until elementary bodies of *C. pneumoniae* were added either 2 or 7 days of incubation in antibacterial-free medium. Medium was changed every two days when protection was tested after incubation for 7 intervening days.

Selection of Rifampin-resistant Mutants

If one Chlamydia preparation were the source of all inoculations during mutant selection, it might contain predominantly one rifampin-resistant mutant genotype as a sub-population. To avoid the possibility of selecting repeatedly for the same mutant, and to assure the best chance of isolating mutants from independent genetic events, it was necessary to make several preparations of Chlamydia, each preparation seeded with 1 or a limited number of homogeneous wild type, rifampin-susceptible bacteria (cloning by limiting dilution) [24]. Therefore, as a first step, *C. pneumoniae* serovar TW-183 was microbiologically cloned by limiting dilution to make 4 preparations. In addition a fifth preparation of another *C. pneumoniae* variant, CWL-029, was also made.

Chlamydia from each preparation were inoculated at a multiplicity of 5 onto Hep-2 mammalian cell monolayers in 25-cm flasks, and grown in the presence two-thirds the MIC of rifampin, in a method similar to that used to obtain mutants of *C. trachomatis* [24]. After 72 hours of incubation, the monolayer was lysed by sonication, and debris removed by slow-speed centrifugation. Aliquots of the supernatant were then inoculated onto fresh monolayers of Hep-2 cells in 8~12 mm shell vials, containing increasing amounts of rifampin, starting with a low concentration of 0.0053 $\mu\text{g/ml}$ of rifampin (2/3 the MIC) up to the high concentration 0.5 $\mu\text{g/ml}$, as depicted in Fig. 1.

Growth was monitored on corresponding wells of a 48-well plate to observe emerging resistance by routine microscopic immunofluorescent techniques described previously [24]. Plates were stained and read at 72 hours, and vials were passed onto fresh shell vial monolayers and overlaid with previous corresponding rifampin concentrations. After three passages of Chlamydia in shell vials maintaining the same concentration of rifampin in each case, the MIC was determined for both rifampin and

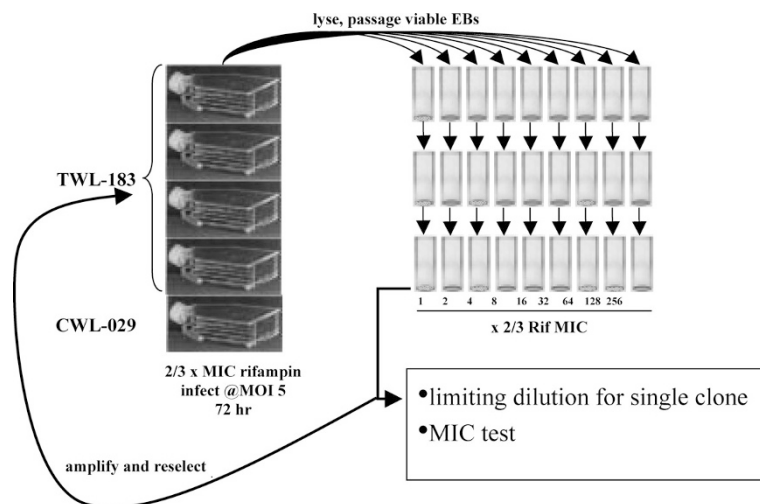


Fig. 1 Illustration of the method of selection of *C. pneumoniae* mutants resistant to rifampin.

C. pneumoniae were inoculated onto Hep-2 mammalian cells in flasks containing rifampin, and inoculated in subsequent passages in shell vials containing varying concentrations of rifampin as shown. Inclusion bodies observed in some vials indicated the presence of mutants. (EB=elementary body)

rifalazil by testing for inclusion body formation in the presence of 2-fold dilutions of these rifamycins. If the observed MIC exceeded the starting MIC, the mutant was purified and isolated by cloning by limiting dilution.

A portion of the sample containing the mutant(s) was also subjected to selection a second time. This enabled the isolation of a new derivative containing not only the original mutation, but also a second mutation. It was thus possible that these strains with multiple point mutations would be even more resistant to rifampin as seen in a previous study [24]. The method of passage in shell vials was identical this second time through, starting with 2/3 the mutant MIC.

If no emergence of resistance was observed in a particular sample after three passages, the selection regimen was repeated, in order to provide a second opportunity of recovering mutants from the wild type preparations.

DNA Sequencing

To define mutations in the *C. pneumoniae rpoB*, we sequenced PCR products covering nucleotides 1235 through 1785, corresponding to amino acid 412 through 595 for mutants and control strains. PCR products of this segment were amplified with primers designed based on the *rpoB* (Cpn0081) sequence of reference strain CWL029 using high fidelity DNA polymerase (PfuUltra, Stratagene). Both the sense and anti-sense strands were sequenced three times using forward primer CPrpoBF1: 5'-ATCTTGCTAA-TCGACGTGTCC and reverse primer CPrpoBR: 5'-

ACATTCTAATCCAGTGCCAAC. Consensus sequences were derived from assembled contigs and aligned with the Sequencher program.

Results

Characterization of Mutants by DNA Sequencing

From two of the four preparations of TW-183, no mutants were isolated. From the other two preparations of TW-183, as well as one preparation of CWL029, four mutants were isolated containing different single mutations, and 3 different mutants defined by having two mutations, all within the RNA polymerase β subunit gene, encoding the rifampin binding site. Therefore, rifampin resistance was explained in each mutant phenotype by an alteration in the subunit of RNA polymerase containing the rifampin binding site.

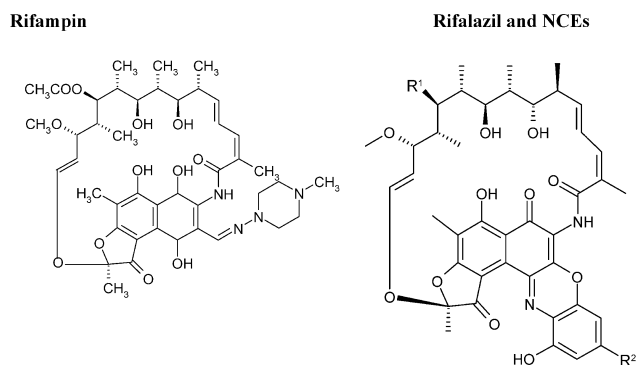
The locations of mutations leading to rifampin resistance, defined by DNA sequencing, are shown relative to mutations found in other species (Fig. 2). In the four cases of single mutations, all alterations were in residues at or near known contact points between rifampin and RNA polymerase from crystal structure studies with the RNA polymerase purified from the bacterium *Thermos aquaticus* [28]. The mutation at position 456, in fact, is in the same position as the mutation described in the previous study [25]. Codon 478 may also be a hotspot for mutations in *C. pneumoniae*, because mutants were independently isolated containing the same mutation at 478 from two

mutants were considerably more resistant to rifampin than to rifalazil. Thus, the MIC for rifampin was very high (above 100 $\mu\text{g/ml}$ for the most resistant mutant), a concentration that is unachievable in therapy under normal physiological conditions. The rifalazil MIC for even the highest mutant was 0.125 $\mu\text{g/ml}$. Considering the high intracellular level of rifalazil in mammalian cells [13], an inhibitory concentration of rifalazil may be achievable during clinical use. Thus the physiological concentrations of rifalazil, but not of rifampin, may be sufficient to reduce the selection of mutants.

The second method of comparison between rifampin and rifalazil is calculated by considering the MICs of wild type cells as the baseline level of potency, and to determine the increase in MICs of mutants relative to the MIC against the wild type parent strain. By this measure, rifalazil had a considerably lower ratio of mutant to wild type MIC (512 times the wild type MIC for the most resistant mutant) than did rifampin (16,384 times the wild type MIC) (Table 1).

Activity of NCEs

The potency of NCEs ABI-0043, ABI-0369, ABI-0597, and ABI-0699, depicted in Fig. 3, are shown in Table 2. The MIC of all the NCEs was 2~4 fold lower than that of rifalazil. This difference was also noted in the determination of the minimal bactericidal concentration (MCC₃; the concentration sufficient to suppress any growth following exposure and 3 subsequent passages in antibacterial-free medium). The NCEs had 2~8 fold lower



Compound	R ¹	R ²
Rifalazil	OH	
ABI-0043	OH	
ABI-0369	O-Ac	
ABI-0597	O-Ac	
ABI-0699	O-Ac	

*OH = hydroxyl; O-Ac = O-acetyl

Fig. 3 Structures of rifamycins.

Table 2 Activities of NCEs compared with rifalazil in cell culture

Strain	Test*	Rifalazil	ABI-0043	ABI-0369	ABI-0597	ABI-0699
TW-183 (wild type)	MIC	0.00025	0.000125	0.000125	0.000064	0.000064
	MCC ₃	0.008	0.002	0.004	0.001	0.001
	PD2	0.016	0.008	0.008	0.002	0.002
	PD7	0.032	0.016	0.016	0.004	0.004
L456I	MIC	0.0005	0.0005	0.00025	0.00025	0.00025
	MCC ₃	0.016	0.008	0.004	0.002	0.002
	PD2	0.032	0.016	0.002	0.004	0.008
	PD7	0.064	0.032	0.008	0.016	0.064
L478S	MIC	0.008	0.002	0.001	0.001	0.001
	MCC ₃	0.125	0.016	0.016	0.008	0.016
	PD2	0.125	0.064	0.016	0.032	0.064
	PD7	0.25	0.125	0.016	0.064	0.25
S476F	MIC	0.125	0.032	0.032	0.032	0.032
	MCC ₃	>5	1	1	0.5	0.5
	PD2	5	0.5	0.5	0.25	0.5
	PD7	>5	5	0.5	0.5	5

* MIC=Minimal inhibitory concentration, MCC₃=minimal bactericidal concentration, PD2 and PD5=Protective Dose after 2 and 5 days, respectively.

MCC₃s compared with rifalazil. The protective concentration was determined by exposing mammalian cells to the indicated compounds, and then growing the mammalian cells for either two or seven subsequent days of incubation in antibacterial-free medium prior to infection, as previously described [27]. The antibacterial concentration affording protection was again 2~8 fold lower for NCEs than for rifalazil.

Discussion

The selection and characterization of mutants in this paper that are highly resistant to rifampin demonstrates that rifalazil is less prone to development of resistance. The highest MIC against *C. pneumoniae* mutants was only 0.125 µg/ml, compared to an MIC of >100 µg/ml for rifampin. Thus it is possible that rifalazil would not select for resistant mutants of *C. pneumoniae* during monotherapy. These results are consistent with those found previously when the same question was examined in the case of the related obligate intracellular pathogen, *C. trachomatis*, in which the highest rifalazil MIC was 0.0064 µg/ml, despite the isolation of mutants fully resistant to rifampin [24]. In considering these results, together with those obtained for *C. trachomatis* [24, 25], it is clear that Chlamydia presents a special case in which rifalazil is not only potent against wild type bacteria, but also retains activity against mutant strains having chromosomal mutations in the *rpoB* gene which result in strong resistance to rifampin. The fact that rifalazil as a monotherapeutic agent was at least as effective as azithromycin, the standard of care, in the microbiological cure of *C. trachomatis* among men having nongonococcal urethritis in a clinical trial further supports the idea that development of resistance to rifalazil by Chlamydia is not a frequent event [14].

A total of 7 distinct rifampin-resistant mutants of *C. pneumoniae*, four strains having unique single mutations, and 3 strains having a unique second mutation in combination with the primary mutation at codon position 456, were isolated. It is interesting that the identical C→A transversion resulting in the codon change, L478S, was found in several samples derived from TW-183 preparation 1, and in TW-183 preparation 3. In addition, one mutant isolated from strain CPL029 contained an identical mutation to an allele reported previously [25], conferring the L456I change in RpoB. Even with the occurrence of these repeated genetic events, which might suggest mutational hotspots in the *rpoB* gene, rifampin-resistant mutants of *C. pneumoniae* were less frequent than for

mutants derived from *C. trachomatis* characterized previously [24]. It is possible that *C. pneumoniae* is less prone to rifampin-resistance development than *C. trachomatis*. Alternatively, it is also possible that less growth occurred in the case of the slower growing *C. pneumoniae*, and that fewer elementary bodies were transferred after each passage, which could account for the lower frequency of mutants isolated.

The NCEs that were tested were even more active than rifalazil in cell culture tests. The lower MICs of NCEs ABI-0043, ABI-0369, ABI-0597, and ABI-0699 (Table 2) is in keeping with previous findings that some NCEs are more active than rifalazil against *C. trachomatis* against both wild type and rifampin-resistant strains [29]. The NCEs also had lower MCC₃s (bactericidal concentrations) against *C. pneumoniae* than rifalazil by 2~8 fold (Table 2), further suggesting that NCEs have potential that could even surpass that of rifalazil in treating chlamydial infections. The NCEs also had a greater protective effect than rifalazil, showing that these derivative compounds share with rifalazil the propensity to concentrate and remain inside mammalian cells, and to exert their antichlamydial effect days after exposure. This attribute of rifalazil and the NCEs may prove to be valuable in eradicating Chlamydia and preventing it from persisting inside of host cells.

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