Efficacy of Benzoxazinorifamycins in a Mouse Model of *Chlamydia pneumoniae* Lung Infection

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The efficacy of rifalazil and other benzoxazinorifamycins was tested in a mouse model of lung infection against *Chlamydia pneumoniae*. Rifalazil and six related new chemical entities all showed efficacy after one dose per day for 3 days at either 0.1 or 1 mg/kg of body weight.

*Chlamydia pneumoniae* is a bacterial pathogen which causes acute respiratory disease and is the third leading cause of community-acquired pneumonia (7). *C. pneumoniae* has also been implicated in atherosclerosis based on seroepidemiological evidence of high antibody titers directed against *C. pneumoniae*; the presence of *C. pneumoniae* in diseased, rather than undiseased arteries; and animal studies which show that *C. pneumoniae* infection can accelerate the progression of atherosclerosis. Based on these findings and its potent antichlamydial activity, rifalazil, a benzoxazinorifamycin, is under investigation as a potential therapeutic for peripheral arterial disease (12).

The fact that *C. pneumoniae* is an obligate intracellular pathogen may enhance its ability to establish chronic infection, covertly replicating within its infection. It is remodeled to contain *Chlamydia*-encoded products. Furthermore, *C. pneumoniae* can respond to stress-inducing conditions, including antibacterial agents, by entering a persistent, more quiescent state, in which no replication or production of infectious particles, called elementary bodies, occurs (1, 9).

The most effective antichlamydial agents, therefore, might inhibit chlamydial functions necessary in both the replicative and persistent states. The ability to concentrate within mammalian cells might afford an additional advantage. Rifalazil is such a compound; it is a strong inhibitor of bacterial RNA polymerase (5), which has an essential function during both the vegetative and persistent states. Rifalazil’s high volume of distribution ensures that a high proportion of the compound is found inside cells (6, 12). Its MIC against *Chlamydia trachomatis* (12, 13) and *C. pneumoniae* (8, 12), determined in cell culture, is 0.00025 μg/ml, with one report of 0.00125 μg/ml (10). To date, rifalazil is the most potent antichlamydial compound that has been administered to humans.

Rifalazil has also been shown in a mouse model of *C. pneumoniae* lung infection to have efficacy against *C. pneumoniae* when administered by once-daily intraperitoneal injection at 1 mg/kg of body weight for 3 days, as determined by titrating the lungs 14 days after infection (8). The purpose of this study was to determine if other benzoxazinorifamycins (new chemical entities [NCEs]), which have equivalent or better potency against *C. pneumoniae* in terms of MIC testing against strain TW-183 in cell culture as shown in Table 1, are also efficacious in vivo in the mouse model.

The efficacy of the NCEs was analyzed utilizing the methods described previously (7) and by determining lung burden (2), enabling a more quantitative analysis of in vivo efficacy. In brief, *C. pneumoniae* strain AR-39 was prepared by growing in HL cells, purified by density gradient centrifugation using diatrizoate meglumine (Hypaque-76; Winthrop-Breon Laboratories, NY), resuspended in sucrose phosphate-glutamic acid chlamydial transport (SPG) medium, and frozen at −70°C until use. Strain C57BL/6J male mice 8 weeks of age (Jackson Laboratory, Bar Harbor, ME) were acclimated for 5 to 7 days, inoculated intranasally under light anesthesia (8.8 mg/kg xylazine and 130 mg/g ketamine i.p.) with *C. pneumoniae* (1 × 10^7 inclusion-forming units [IFU] per inoculum), and treated with antichlamydial agents. Compounds were administered by i.p. injection on a schedule depicted in Fig. 1, after initial preparation of a 10 mg/ml solution in dimethyl sulfoxide and dilution in dissolution solution (5% Etocas 35NF [Croda, Inc., Edison, NJ], 0.9% NaCl, and 0.1 mM Na_2HPO_4 [pH 7.4]) (11), to a final concentration in the dosing solution of 0.6 mg/ml. Animals were sacrificed on day 10. Lung tissue was homogenized to make a 10% suspension in SPG medium, and coarse debris was removed by centrifugation at 500 × g for 10 min at 4°C. This study was approved by the University of Washington Animal Care and Use Committee.

Titers of lung homogenates were determined by applying 0.1 ml of homogenate sample to HL cells grown on coverslips in 1-dram vials in triplicate. After incubation, infected cells of one vial were fixed and stained with a genus-specific monoclonal antibody conjugated to fluorescein isothiocyanate. The number of inclusions per gram of lung tissue was calculated after determination of the titer on the entire coverslip. The number of inclusions per gram of lung tissue was calculated after determination of the titer on the entire coverslip.

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Results of an initial range-finding experiment, in which the efficacy of rifalazil was determined as a function of dose, are shown in Table 2. Efficacy was measured as the proportion of...
animals whose lungs were culture positive following doses of 0.01, 0.1, and 1 mg/kg of rifalazil (Table 2). Consistent with previous results (8), the majority of animals were not culture positive when treated with 1 mg/kg of rifalazil. Efficacy was also detected when mice were administered doses containing 0.1 mg/kg of rifalazil, but at a reduced frequency. All animals were found to be culture positive when administered the lowest dose of 0.01 mg/kg.

In order to determine if chlamydiae were eradicated by drug treatment, the lung homogenates also were subjected to PCR, as described previously, using the HL-1 and HR-1 primer set (3). This method is one of the assays recommended by the Centers for Disease Control and Prevention for detection of *C. pneumoniae* DNA (4). The presence of *Chlamydia* DNA was verified by the positive PCR results from most of the lung homogenates. It should be noted that the presence of DNA does not prove the presence of live chlamydiae, but the PCR results suggest the possibility that *C. pneumoniae* may not have been eradicated in samples that tested negative by culture.

Based on the range-finding experiment (Table 2), infected mice were treated with rifalazil and NCEs at doses of 1 and 3 mg/kg, in order to evaluate their efficacies against *C. pneumoniae*. These experiments were carried out in three groups of mice: experiment 1 with rifalazil, ABI-0043, ABI-0094, and ABI-0299 at 1 mg/kg; experiment 2 with the same compounds at 3 mg/kg; and experiment 3 with ABI-0043, ABI-0369, ABI-0597, and ABI-0699 at both 1- and 3-mg/kg dosing regimens. Each experiment included an infected control, which was sacrificed at the same time as the treatment groups, so that the reduction in lung titer could be measured. Two measurements were made to determine efficacy. As in Table 2, the number of culture-positive animals was determined from the initial titer, and any negative results were confirmed by the second passage (Table 3). In addition, the number of inclusions was counted: titers for each animal are shown in Fig. 2, and mean titers are summarized in Table 3. Because the lowest detectable titer is $\times 500$ IFU (the number of inclusions when 1 IFU is observed from a tissue sample of 0.2 g), a conservative approach was adopted in which a “zero” result (less than 500 inclusions) is assigned a value of 100 IFU, rather than 0 IFU.

All NCEs showed efficacy against *C. pneumoniae*, in that there was a significant difference when considering individual animal titers (Fig. 2) and when comparing the mean titers (Table 3). In addition, for all NCEs tested, there were culture-negative animals, whereas no culture-negative animals were found among the untreated mice in this study (Tables 2 and 3) or from the previous study (8). In comparing NCEs tested in experiments 1 and 2, it is difficult to rank their potential solely on the basis of efficacy. Although the groups treated with ABI-0043 had the most culture-negative animals, the mean titers of mice treated with ABI-0043, ABI-0299, and rifalazil

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th><em>C. pneumoniae</em> MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifalazil</td>
<td>OH</td>
<td></td>
<td>0.00025</td>
</tr>
<tr>
<td>ABI-0043</td>
<td>OH</td>
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<td>0.000125</td>
</tr>
<tr>
<td>ABI-0094</td>
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</tr>
<tr>
<td>ABI-0299</td>
<td>O-Ac</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td>ABI-0369</td>
<td>O-Ac</td>
<td>0.000125</td>
<td></td>
</tr>
<tr>
<td>ABI-0597</td>
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<tr>
<td>ABI-0699</td>
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<td>0.000064</td>
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* OH, hydroxyl; O-Ac, O-acetyl.
* MIC against *C. pneumoniae* strain TW-183 determined as described in reference 14.
* ND, not done.
were very similar. ABI-0094 may have been slightly less efficacious when both lung Chlamydia titers and culture-free animals were examined. Experiment 3 showed that compounds ABI-0369, ABI-0597, and ABI-0699 had better efficacy than ABI-0043 when mean titers or proportion of culture-negative animals were considered (Fig. 2 and Table 3). The analysis of data from experiment 3 suggests a dose response, in that for each compound, greater efficacy occurred when mice were dosed with 3 mg/kg than with 1 mg/kg (Fig. 2 and Table 3).

The mouse model of lung infection has been proven to be an efficient and reproducible approach for evaluating efficacy of antibiotics against C. pneumoniae. Results were consistent in that all infected, untreated animals of this study (25 in all), as well as 5 animals from the previously reported study (8), were culture positive when lung homogenates were assessed. The proportion of animals which were culture positive within each treatment group varied to some extent. The inclusion of a quantitative measure (IFU/g lung) provides a more textured measure for evaluating the efficacies of drug candidates. For example, the titer of bacteria from lung homogenates of 18 of 20 mice treated with ABI-0043 was below the titer of every animal of the control group (Fig. 2). In only 1 of 20 animals treated with ABI-0043 was the titer approaching that of the mean for its corresponding untreated control group. On the basis of these results, we conclude that the NCEs, and especially ABI-0369, ABI-0597, and ABI-0699, are attractive anti-chlamydial candidates that could be further developed as treatments for peripheral arterial disease (12) or other indications in which Chlamydia may play a role.

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REFERENCES


