Short-course treatment regimen to identify potential antituberculous agents in a murine model of tuberculosis

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Introduction

Tuberculosis continues to be a serious health problem worldwide. It is among the leading causes of death due to an infectious agent.¹ Chemotherapy against drug-susceptible tuberculosis consists of a three- or four-drug regimen including isoniazid, rifampicin, pyrazinamide, and ethambutol or streptomycin, and is administered for 6 months or longer. The emergence of multidrug-resistant strains of Mycobacterium tuberculosis highlights the need for developing new, more effective chemotherapeutic agents.² Without this effort, controlling tuberculosis will be a difficult task.

There are many hurdles in developing new antimycobacterial agents. Once these agents are demonstrated to have in vitro activity against M. tuberculosis, the major hurdle is testing these potential agents in the murine model. Typically, testing in this model consists of a 4 week treatment regimen.²³ Following treatment, the mice are euthanized and organ cell counts are measured to determine the efficacy of the test compound. Owing to the long cultivation time required to detect M. tuberculosis on solid media (minimum incubation time of 3 weeks), the total experimental time is ~2 months. In addition, traditional treatment requires a large amount of test drug, which may limit the ability to perform in vivo testing of new compounds.

The purpose of this study was to devise a short-course in vivo testing method that would require fewer treatment days as well as reducing the total amount of test drug needed to evaluate its efficacy. Such a model could then be used to screen antituberculous agents and determine whether further evaluation is warranted. A short-course treatment model described by Sizemore et al.⁶ to test the efficacy of chemotherapeutic agents against Helicobacter pylori was adapted to evaluate the efficacy of agents against M. tuberculosis.

Initially, antimycobacterial agents that had been tested extensively in the standard 4 week regimen³⁷–³⁹ were evaluated in a 2 day treatment regimen. Additional studies were performed on other well-documented agents to confirm and characterize the effectiveness of this 2 day drug treatment regimen.³⁷,¹¹,¹²

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Materials and methods

Mice
Female C57BL/6j mice (Jackson Laboratories, Bar Harbor, ME, USA) were purchased at 5 weeks of age and allowed to acclimatize in the facility for 1 week. Animals were housed in microisolator cages (Lab Products, Inc., Maywood, NJ, USA) and maintained with water and Prolab RMH 3000 rodent chow (Purina, St Louis, MO, USA) in the ABSL-3 located in the Veterinary Medical Unit at the Veterans Affairs Medical Center (VAMC; Syracuse, NY, USA). Animal protocols were approved by the Subcommittee for Animal Studies of VAMC.

Drugs
Rifampicin and isoniazid were purchased from Sigma Chemical Co. (St Louis, MO, USA). Gatifloxacin was provided by Bristol-Myers Squibb (Princeton, NJ, USA) and PNU-100480 was provided by Pharmacia & Upjohn, Inc. (Kalamazoo, MI, USA). Sparfloxacin was provided by Parke Davis (Ann Arbor, MI, USA) and levofloxacin was provided by the RW Johnson Pharmaceutical Research Institute (Spring House, PA, USA). All drugs, with the exception of isoniazid, were dissolved in dimethyl sulfoxide (DMSO) with subsequent dilutions in distilled water.

M. tuberculosis isolate
M. tuberculosis ATCC 35801 (strain Erdman) was obtained from the ATCC (Manassas, VA, USA). The organism was grown in 20 tubes of modified 7H10 broth (pH 6.6; 7H10 agar formulation with agar and malachite green omitted) with 10% OADC (oleic acid, albumin, dextrose, catalase) enrichment (BBL Microbiology Systems, Cockeysville, MD, USA) and 0.05% Tween 80 for 5–10 days on a rotary shaker at 37°C. The cultures were pooled and diluted to 100 Klett units (equivalent to 5 × 10^4 colony forming units (cfu)/mL) (Photoelectric Colorimeter; Manostat Corp., New York, NY, USA). The culture was aliquotted and frozen at −70°C. On the day of infection, the culture was thawed and the final inoculum was determined. Owing to bacterial clumping problems (evidenced by the bacterial recovery 24 h post-infection) in the third experiment only, the culture was thawed and sonicated for ~10 min. The final inoculum size was determined by diluting to 5 × 10^2 and plating 0.1 mL, in triplicate, on 7H10 agar plates (BBL Microbiology Systems) supplemented with 10% OADC enrichment. The plates were incubated at 37°C in ambient air for 4 weeks.

Infection studies
These studies were performed in three separate experiments. Mice were infected intranasally using a modification of the technique described previously by Gray & Mattison. Specifically, groups of six mice were anaesthetized by intramuscular delivery of a telazol (45 mg/kg) / xylazine (7.5 mg/kg) cocktail (Lederle Parenterals, Carolina, Puerto Rico and Bayer Corp., Shawnee Mission, KS, USA, respectively) and subsequently infected intranasally with ~10^6 viable M. tuberculosis in a 20 μL volume.

Drugs were administered daily for 2 days by oral gavage in a 0.2 mL volume. The timetable for each experiment was as follows: day 0, intranasal infection; days 1 and 2, drug administration; and day 3, sacrifice. At the beginning of therapy, one group of mice, designated early controls, was sacrificed to determine the level of infection before drug treatment. In all experiments, but one, another group of mice, designated late controls, was sacrificed to determine the level of infection in the absence of therapy. As a means to validate this 2 day treatment model it was essential to use drug doses previously tested and documented in the standard 4 week treatment model. In the first experiment rifampicin, isoniazid, gatifloxacin and PNU-100480 were administered once daily at 20, 25, 100 and 100 mg/kg, respectively. In the second experiment, gatifloxacin was administered at 4, 20, 100 or 500 mg/kg and isoniazid was given at 25 mg/kg once daily. In the last experiment, gatifloxacin, sparfloxacin or levofloxacin were administered at 50, 100 or 300 mg/kg once daily.

Mice were sacrificed by CO2 inhalation 1 day after treatment (day 3 post-infection). Right lungs were aseptically removed and ground in a sealed tissue homogenizer (IdeaWorks! Laboratory Devices, Syracuse, NY, USA). The number of viable organisms was determined by serial dilution and titration on 7H10 agar plates. Plates were incubated at 37°C in ambient air for 4 weeks prior to counting.

Statistical evaluation
To compare the viable cell counts recovered from the right lungs of mice, the numbers were first converted into log cfu (log_{10}). Owing to the small sample size and the consequent need to protect against deviations from normality, the Mann–Whitney test was performed to determine statistical differences between control and treatment groups. Statistical significance was accepted with P values <0.05. In the second and third experiments, the two-sided exact Jonckheere–Terpstra test was performed to determine the dose–response relationship with respect to log cfu. P values <0.05 indicate a trend where the highest dose of each drug was significantly better than the intermediate dose, which was significantly better than the lowest dose.

Results
Efficiency of a 2 day treatment regimen to determine in vivo antimycobacterial activity

A broad range of antimycobacterial agents were evaluated in the 2 day treatment regimen to determine whether antimycobacterial activity could be observed within such a short treatment time. Mice were randomly assigned to the following groups: early controls, late controls, rifampicin at 20 mg/kg, isoniazid at 25 mg/kg, gatifloxacin at 100 mg/kg and PNU-100480 at 100 mg/kg once daily. Mice were infected intranasally with ~5.2 × 10^4 viable M. tuberculosis.

There was no statistical difference between counts recovered in the lungs from the early and late control mice (Table 1). Irrespective of the drug class used, 2 days of treatment significantly reduced the mycobacterial load recovered from the lungs compared with the early

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean log_{10} cfu ± S.D.</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early control</td>
<td>6.11 ± 0.25</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Late control</td>
<td>6.21 ± 0.10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Isoniazid 25 mg/kg</td>
<td>4.91 ± 0.24</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rifampicin 20 mg/kg</td>
<td>4.95 ± 0.47</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PNU-100480 100 mg/kg</td>
<td>5.19 ± 0.15</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gatifloxacin 100 mg/kg</td>
<td>4.47 ± 0.71</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*P value with respect to the mean log cfu recovered from the early control group. All P values relate to Mann–Whitney tests.
Table 2. Log₁₀ cfu recovered from the right lungs of mice infected intranasally with 9.6 × 10⁵ cfu of M. tuberculosis and treated once daily with isoniazid or gatifloxacin at various doses (n = 6 mice per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean log₁₀ cfu ± S.D.</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early control</td>
<td>6.85 ± 0.35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Isoniazid 25 mg/kg</td>
<td>5.65 ± 0.24</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gatifloxacin 4 mg/kg</td>
<td>7.20 ± 0.34</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Gatifloxacin 20 mg/kg</td>
<td>6.85 ± 0.17</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Gatifloxacin 100 mg/kg</td>
<td>5.83 ± 0.19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gatifloxacin 500 mg/kg</td>
<td>4.54 ± 0.29</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*P value with respect to the mean log cfu recovered from the early control group. All P values relate to Mann–Whitney tests.

and late control groups, but there was no significant difference in bacterial burden between the treatment groups (P > 0.05).

Gatifloxacin dose–response following 2 days treatment

Previous experiments using two different doses of gatifloxacin in the standard 4 week treatment regime have demonstrated a dose–response where the highest dose of gatifloxacin (300 mg/kg) was significantly better than the lower dose of gatifloxacin (100 mg/kg) at reducing mycobacterial infection in mice.14 In order to determine whether a dose–response could be observed with only 2 days of gatifloxacin treatment, mice were assigned to the following groups: early controls, gatifloxacin administered daily at either 4, 20, 100 or 500 mg/kg and isoniazid at 25 mg/kg (as a drug control). In the previous study, early and late control groups demonstrated statistical similarity; therefore, the late control group was omitted for this experiment. Mice were infected intranasally with 9.6 × 10⁵ cfu of M. tuberculosis.

Gatifloxacin administered at 4 and 20 mg/kg did not reduce the bacterial load compared with the early control group, but isoniazid and all other doses of gatifloxacin did significantly reduce the log cfu recovered (Table 2). A dose–response was observed with gatifloxacin in the 2 day treatment regimen, where gatifloxacin administered at 500 mg/kg was significantly better than 100 mg/kg, which was significantly better than 20 mg/kg (P < 0.05). There was no statistical difference between the 20 and 4 mg/kg groups (P > 0.05). Isoniazid at 25 mg/kg was significantly more active than 4 and 20 mg/kg of gatifloxacin (P < 0.05), but was no different from 100 mg/kg of gatifloxacin (P > 0.05). The highest dose of gatifloxacin (500 mg/kg) was significantly more active than isoniazid (P < 0.05).

Activity of other quinolones in the 2 day treatment regimen

The ability to demonstrate a dose–response in the 2 day treatment regimen using gatifloxacin prompted the question of whether other quinolones would behave similarly. Mice were randomly assigned to the following groups: early controls, late controls, gatifloxacin at 50, 100 or 300 mg/kg, levofloxacin at 50, 100 or 300 mg/kg, or sparfloxacin at 50, 100 or 300 mg/kg. Mice were infected intranasally with 1.8 × 10⁵ cfu of M. tuberculosis.

There was no statistical difference in log cfu recovered from the lungs of the early and late control mice. Treatment with all doses of the quinolones significantly reduced the bacterial load compared with the early and late control groups (Table 3).

Consistent with the previous study, gatifloxacin did show a trend towards a dose–response after 2 days of treatment (P < 0.05). Gatifloxacin administered at a dose of 300 mg/kg was better than 100 mg/kg, and 100 mg/kg was better than 50 mg/kg. Levofloxacin similarly showed a trend towards a dose–response, where 300 mg/kg was better than 100 mg/kg, which was better than 50 mg/kg (P < 0.05). Sparfloxacin failed to show a trend towards a dose–response (P > 0.05). Although 300 mg/kg of sparfloxacin was significantly better than 100 mg/kg (P < 0.05), there was no difference between 100 and 50 mg/kg of sparfloxacin (P > 0.05).

The bacterial reduction observed in the lungs of mice receiving gatifloxacin was no different from the reduction achieved with equal doses of sparfloxacin (P > 0.05). Sparfloxacin and gatifloxacin administered for 2 days were significantly better than equal doses of levofloxacin (P < 0.05).

Discussion

The need for new effective antimycobacterial agents, as well as the difficulties in developing them, is well documented.15,16 Agents that display superior in vitro activity must be evaluated in an animal model. This can be very expensive and time consuming. As previously noted, the standard in vivo murine model for testing antimycobacterial agents requires 4 weeks of treatment (5 days per week for a total of 20 treatment days) and 3–4 weeks of incubation following organ harvesting. Our laboratory, along with others, has been focusing efforts on improving the testing process by shortening the murine test methodology.17

Using several different antimycobacterial compounds, a 2 day treatment regimen was designed and evaluated. In this treatment regimen mice were infected intranasally, and 1 day later, therapy was started and administered for 2 days. The lungs were then processed to measure the ability of the compounds to limit mycobacterial growth. This method provides results in ~1 month, compared with 2 months using the standard in vivo model.
Rifampicin and isoniazid, standard drugs used in humans, have been shown to limit M. tuberculosis growth in the murine model after 4 weeks of therapy. These drugs were used initially to validate the 2 day treatment model. Rifampicin or isoniazid administered for 2 days was able to reduce mycobacteria in the lungs by 1.26 and 1.3 logs, respectively. Testing of other antimycobacterial compounds confirmed the usefulness of the 2 day treatment regimen. Similar to the results demonstrated with rifampicin and isoniazid, the oxazolidinone PNU-104480 and the quinolone gatifloxacin were effective at reducing mycobacterial growth compared to untreated mice in this 2 day treatment model. In a recent paper demonstrating the antimycobacterial activity of moxifloxacin in a murine model, Yoshimatsu et al. referred to similar results obtained from three consecutive days of therapy. In their model, mice were infected intravenously and the mycobacterial counts were measured in the spleens, which is in contrast to our model, where mice were inoculated intranasally to establish a respiratory infection and the mycobacterial counts were measured in the lungs.

In the standard 4 week in vivo model, our laboratory has demonstrated a dose–response using increasing doses of gatifloxacin. Our laboratory and others have demonstrated a similar dose–response using quinolones such as sparfloxacin, levofloxacin and moxifloxacin in a murine model of tuberculosis, as well as differences in the efficacy of each quinolone.

To determine whether it was possible to observe a dose–response and be able to rank-order drugs using the 2 day treatment regimen, gatifloxacin, sparfloxacin and levofloxacin were compared at varying doses. After only 2 days of therapy, a dose–response of gatifloxacin was observed, which was similar to the results in the 4 week in vivo model. Levofoxacin and sparfloxacin similarly demonstrated the greatest efficacy at higher doses. In the 4 week treatment regimen, Klemens et al. and Ji et al. both demonstrated the superior activity of sparfloxacin compared with levofloxacin. Using the 2 day treatment regimen, sparfloxacin was similarly superior to levofloxacin at equal doses. There was no significant difference between equal doses of gatifloxacin and sparfloxacin after 2 days of treatment.

The 2 day treatment regimen in the murine model has similarities to the well-documented early bactericidal activity (EBA) studies in humans. In this model, compounds that have been approved for clinical testing are administered to human volunteers for two consecutive days. Sputum samples are collected and assessed for M. tuberculosis bioburden just before treatment and 2 days post-treatment. The reduction in M. tuberculosis levels recovered between these days demonstrates the potential efficacy of the agents. Obviously, the greatest advantage of this 2 day treatment regimen in the murine model is that drugs do not need to go through extensive studies prior to use in animals, which is not the case for human studies.

Although 2 days of therapy in the murine model has been shown to be effective, it is unlikely that the 2 day treatment regimen will be able to predict the relative clinical efficacy of test agents. In the 4 week in vivo murine model, rifalazil is superior to rifampicin and rifampicin is superior to isoniazid, but in the 2 day treatment regimen there is no difference between these antimycobacterial agents (data for rifalazil not shown). Mice are able to mount a strong immune response and control M. tuberculosis growth until 10–14 days into the infection, and therefore, the reduction in mycobacteria using the 2 day treatment regimen is entirely drug related. The lack of immune-mediated mycobacterial reduction might account for the inability to separate drugs by their efficacy in the 2 day treatment regimen.

In the EBA studies by Sirgel et al., the possibility is discussed of the value of testing mycobacterial reduction in sputum samples after both 2 and 5 days of therapy. The initial drop in mycobacteria in the first 2 days represents killing of actively growing bacteria, whereas the drop from day 3 to day 5 represents killing of the slower-growing persisting organisms. Sirgel et al. suggest that measuring the EBA between day 3 and 5 might enable assessment of the sterilizing activity of a drug. Whether this phenomenon can be observed in mice needs to be determined, but if it can, it will be a very important finding.

This model has many advantages when information about the test compound is limited. Test drugs go through initial in vitro analysis, which requires very little compound, and then enter the in vivo portion of testing. Our model provides three very important pieces of information. If the test drug has activity in our 2 day treatment model, then the drug must: (i) have reached the lung after oral delivery (following intranasal delivery of M. tuberculosis there is no detectable circulation of the organism within the first 4 days of infection, indicating that bacterial reduction observed in the lung after treatment is due to mycobacterial killing in the lung); (ii) have entered the macrophage in which the bacilli resides; and (iii) kill the bacilli within the phagosome of the macrophage. In our testing of the model, an experimental drug provided by Janssen Research Foundation in Beerse, Belgium, with good in vitro activity (MIC 0.06 mg/L) failed to demonstrate any in vivo activity in the 2 day treatment regimen (data not shown). This indicates that this drug either did not reach the intracellular compartments of the macrophage within the lung, or was inactivated by some unknown mechanism. Agents that display favourable in vitro activity, but fail to demonstrate activity in the 2 day treatment regimen, may require further manipulation to enhance absorption or macrophage delivery.

Developing antimycobacterial agents requires patience and long-term commitment. For this reason, very few new compounds are being developed and tested. Test methods that can provide useful in vivo results in a shorter time period while using minimal material (and therefore with minimal expense) might be an attractive means to gain the commitment that is required. Agents that are found to be active in the 2 day treatment model should progress to a 4 or 12 week treatment study.

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References

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