Rapid Microbiologic and Pharmacologic Evaluation of Experimental Compounds against *Mycobacterium tuberculosis*

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The assessment of physiochemical and pharmacological properties at early stages of drug discovery can accelerate the conversion of hits and leads into candidates for further development. A strategy for streamlined evaluation of compounds against *Mycobacterium tuberculosis* in the early preclinical stage is presented in this report. As a primary assay to rapidly select experimental compounds with sufficient in vitro activity, the growth inhibition microtiter plate assay was devised as an alternative to current methods. This microdilution plate assay is a liquid culture method based on spectrophotometric readings of the bacillary growth. The performance of this method was compared to the performance of two established susceptibility methods using clinical available tuberculosis (TB) drugs. Data generated from all three assays were similar for all of the tested compounds. A second simple bioassay was devised to assess the oral bioavailability of compounds prior to extensive in vivo efficacy testing. The bioassay estimates drug concentrations in collected serum samples by a microdilution MIC plate method using *M. tuberculosis*. In the same assay, the MIC of the compound is also determined in the presence of 10% mouse serum as an indication of protein binding. The method was validated using different clinically available TB drugs, and results are discussed in this report. With these methodological advances, screening of compounds against tuberculosis in the preclinical phase will be rapid, can be adapted to semi-high-throughput screening, and will add relevant physicochemical and basic pharmacological criteria to the decision process of drug discovery.

The pharmaceutical process to develop a therapeutically useful drug requires an enormous budget and amount of time, due to high attrition rates of experimental compounds in preclinical and clinical development (2, 10). Pharmaceutical companies nowadays are focusing on reducing these preclinical-development attrition rates by attempting to accurately evaluate efficacy, safety, and drug manufacturing costs much earlier in the drug discovery process. A published survey on the causes of failure in drug development indicated that inappropriate pharmacokinetics were a major cause (19). This observation has led to an increased emphasis on pharmacokinetic input to the drug discovery process throughout the pharmaceutical industry (9, 20, 21).

Traditional assays and models for early preclinical screening of experimental compounds against *Mycobacterium tuberculosis* are lengthy due to the slow-growing nature of the bacteria. In addition, many of the current in vitro and in vivo assays for drug testing against TB (NT) are not well adapted for a higher throughput approach, which is necessary for rapid screening of compound series from different drug classes. Generally, compounds are first evaluated in vitro for activity against *M. tuberculosis* by determination of their MICs with either the BACTEC system (4, 16), the agar proportion method (7, 13, 25), or a microdilution assay microplate (3, 18). Subsequently, the potential cytotoxicity of the compounds is assessed in cultures of Vero or HepG2 cells to determine a concentration that inhibits 50% of eukaryotic cell growth. A selectivity index can then be calculated by dividing this concentration by the MIC. Selection criteria then determine which compounds will advance further in lengthy and labor-intensive mouse models for tuberculosis infection (e.g., compounds with a selectivity index of >10) (16).

In an attempt to shorten the screening process, we previously described a novel short-term in vivo model for the rapid screening of experimental compounds against *M. tuberculosis* (11). This model uses the highly susceptible, gamma interferon gene-disrupted C57BL/6 mouse strain and requires only 8 days of treatment to obtain highly significant statistical results. In this report, we propose two additional rapid screens which can be included in the early preclinical assessment of compounds in order to gain additional information earlier. The assays described below are (i) a rapid in vitro assay for activity against *M. tuberculosis* and (ii) a bioassay evaluating the in vivo bioavailability of experimental compounds.

**MATERIALS AND METHODS**

Determination of the 50% growth inhibitory concentration (GIC50) of a compound against *M. tuberculosis*. A culture of *M. tuberculosis* (strain H37Rv, from Trudeau Institute, Saranac Lake, NY) was grown under well-defined conditions, divided into aliquots, and served as a dependable frozen stock for the in vitro assays. Briefly, *M. tuberculosis* was grown in 50 ml of 7H9 broth (Difco) containing oleic acid-albumin-dextrose-catalase (OADC) enrichment (7H9-OADC) and 0.05% Tween 80. The cultures were incubated at 37°C with rotary agitation, grown to mid-exponential phase (optical density at 600 nm (OD600) of approximately 0.6 to 0.8, at 14 to 21 days), and harvested by centrifugation. The cell pellets were resuspended in a small amount of the enriched 7H9-OADC medium containing 10% sterile glycerol, transferred to cryogenic vials, and stored at −70°C as starter stocks for further use. To start a new culture, a thawed starter

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stock was added to 50 ml 7H9-OADC containing 0.1% Tween 80 and incubated at 37°C with agitation. The starter culture was grown to an OD600 of 0.3 to 0.5 and then diluted to an OD600 of approximately 0.1 by using 7H9-OADC containing 0.1% Tween 80 (resulting in ~3 × 10^5 CFU per well).

The concentration of compound that inhibits 50% of *M. tuberculosis* growth (GIC50) was determined in 96-well microtiter plates. Compounds were prepared at 100× stocks in appropriate solvents (typically water, dimethyl sulfoxide [DMSO], or ethanol) depending on solubility. Ten serial dilutions (1:2) of the compounds were prepared in the same solvent and added to the wells in a 2-μl volume, generally resulting in a final concentration ranging from 10 μg/ml to 0.019 μg/ml. Rifampin (RIF) and isoniazid (INH) were used as positive controls on every plate. Negative-control wells had only the carrier solvent added. Each well received 198 μl of the diluted bacterial culture. The plate was incubated at 37°C with gentle agitation, and the OD600 was read at intervals of 24 h by use of a Bio-Rad Benchmark Plus plate reader for up to 8 to 10 days. The readings provided kinetic data that were easily converted to percent inhibition at any time point for calculation of a GIC50 value for the compound.

**Determination of in vivo bioavailability of compounds after oral administration.** Briefly, 8- to 10-week-old, female, specific-pathogen-free, immunocompetent C57BL/6 mice (Charles River, Wilmington, MA) were dosed via oral gavage (at a dose lower than the maximum tolerated dose of the compound used, generally 300 mg/kg of body weight). At specific times after dosing (in this study, at 20 min, 1 h, 2 h, and 4 h), three mice were bled from the tail vein. Blood samples were collected aseptically, stored on ice, and centrifuged to collect serum. Serum samples were subsequently frozen at −70 degrees until further use (generally less than 1 week). The drug concentration in the collected serum samples was generally determined by a microdilution MIC method (23) using *M. tuberculosis* H37Rv, determined by spectrophotometric readings.

**RESULTS**

**Validation of the GIC50 assay.** In order to validate the GIC50 microtiter plate assay, the in vitro activities of clinically available TB drugs were evaluated and compared to in vitro activity data obtained by two established methods. In the GIC50 plate assay, serial dilutions of INH, RIF, streptomycin, ciprofloxacin, gatifloxacin, and moxifloxacin (from 0.5 ng/ml to 10 μg/ml) were tested against an actively growing *M. tuberculosis* suspension. Bacterial growth was measured by spectrophotometric analysis at 600 nm every 24 h up to 7 days (Fig. 1). The OD600 readings provide kinetic data which were converted to percent inhibition at 6 days for calculation of a GIC50 value for the compounds (Fig. 2). The GIC50 and the concentration of compound leading to >99% inhibition of *M. tuberculosis* growth (GIC99) were determined for all clinically available drugs tested (Table 1). The results of the GIC50 assay were compared to those obtained by use of two different reference methods.
using the same compounds. As a first reference method, the defined quantitative agar plate proportion test was performed, based on plating of the bacterial inoculum on 7H11 solid medium supplemented with OADC and containing the drugs tested (7, 13, 25). The second reference method used was the broth dilution-based microplate assay, which uses a visual readout to determine growth inhibition (3, 18) (Table 1). The results from the GIC assay were similar to those from the reference methods for all tested compounds (Table 1). The GIC50 results for the compounds appeared similar to the broth dilution plate assay results, whereas the GIC99 values were closer to the results obtained by the agar drop dilution method (represented in Table 1 by the ratio of the MIC obtained with the reference method to the GIC obtained by the GIC assay).

Validation of the bioavailability assay. To validate results obtained from the in vivo bioavailability assay, several clinically available compounds were tested first in order to evaluate serum drug levels in mice after oral administration of the compounds. The bioavailability study described here is representative of three repeats which all showed similar results. Mice were given a single dose of INH (25 mg/kg), RIF (20 mg/kg), ciprofloxacin (300 mg/kg), gatifloxacin (300 mg/kg), or moxifloxacin (300 mg/kg) by oral gavage or streptomycin (200 mg/kg) by intraperitoneal injection. Mice were bled from the tail vein at 30 min and 1, 2, and 4 h after dosing. Presence of drug in the collected serum samples was determined by a microtiter plate bioassay using M. tuberculosis H37Rv. The results of the OD600 readings and visual inspection of the microtiter plates showed that all six compounds were bioavailable, as expected. Inhibition of bacterial growth was observed for all compounds for most time points, which indicates the presence of sufficiently high concentrations of free, bioactive compound in the bloodstream.

TABLE 1. Comparative performance study of three in vitro susceptibility testing assays for six established drugs against M. tuberculosisa

<table>
<thead>
<tr>
<th>Drug</th>
<th>Growth inhibition assay</th>
<th>Agar drop dilution methodb</th>
<th>Microdilution plate assayc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GIC50 (µg/ml)</td>
<td>GIC99 (µg/ml)</td>
<td>MIC100 (µg/ml)</td>
</tr>
<tr>
<td>INH</td>
<td>0.065</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>RIF</td>
<td>0.02</td>
<td>0.25</td>
<td>0.1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.2</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.31</td>
<td>5.0</td>
<td>1</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>0.072</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.116</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a Results are data from one experiment of at least two independent repeats.

b See references 7, 13, and 25.

c See references 3 and 18.
TABLE 2. Basic pharmacokinetic data generated by testing serum samples of drug-treated mice with a bioassay using *M. tuberculosis*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Standard</th>
<th>Mouse data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (no serum)</td>
<td>MIC (10% serum)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>RIF</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

$^a$ i.p., intraperitoneal.
$^b$ BD, below detection limit of the bioassay. The detection limit is 10 times the MIC$_{50}$ of the tested compound in the presence of serum (due to the restriction of using a maximum of 10% serum in the *M. tuberculosis* bioassay).

Approximate drug levels of the compounds were calculated using the MIC$_{50}$ values obtained in the standard compound lanes. Except for ciprofloxacin, a detectable drug level in serum could be found until 2 h after drug administration. Below detection refers here to drug levels that are lower than 10 times the MIC$_{50}$ of the tested compound, which is due to the restriction of using a maximum of 10% serum in the *M. tuberculosis* bioassay. At early time points after drug administration, the estimated active-product levels for INH, streptomycin, and gatifloxacin in serum were high, and sera were not diluted enough to estimate the drug concentration by using the described method (using serum dilutions up to 1:320). This effect with the clinical drugs was as expected, since the range of drug dilutions is more appropriate for experimental, less active compounds. Peak drug levels (approximate maximum concentrations of drug in serum [C$_{max}$]) were estimated for INH after 20 min to 1 h at >19.2 µg/ml, RIF after 1 h at 40 µg/ml, streptomycin after 20 min at >118 µg/ml, ciprofloxacin after 20 min at 7.4 µg/ml, gatifloxacin after 20 min to 1 h at >13.44 µg/ml, and moxifloxacin after 1 h at 19.2 µg/ml. Although this dilution range was not optimal for clinically available compounds, the standard protocol as described above has proven to be very effective for testing the bioavailability of experimental compounds generally administered at 300 mg/kg.

The standards of the tested compounds were evaluated on the same 96-well microtiter plate by using dilution series of the compound ranging from 30 µg/ml down to 0.51 ng/ml in the presence of and without 10% mouse serum. The obtained MIC of the compound in 10% mouse serum indicates whether a compound has significant protein binding. The MIC of RIF increased twofold in the presence of 10% serum (from 0.06 to 0.12 µg/ml), which indicates significant protein binding of the drug (Table 2). For the other clinical compounds, no difference between the MICs with or without serum could be observed (Table 2).

**DISCUSSION**

In order to achieve a significant decrease in the attrition rates for drugs, the recent trend in drug discovery has been to access physicochemical and pharmacological information earlier in the drug discovery process (1, 9, 20, 21). In an attempt to parallel drug discovery and early preclinical development efforts against tuberculosis, we report here two simple assays that can facilitate and improve testing of experimental compounds in the early preclinical phase.

As a primary assay to evaluate the in vitro activity of experimental compounds in a rapid manner, a growth inhibition microtiter plate assay was optimized to select for in vitro activity. This microplate assay can be used as an alternative to the existing reference methods and is an adaptation of the microdilution broth-based approach (3, 18). The microdilution plate assay (also called the microscopic observation drug susceptibility [MODS] assay) is a liquid culture method based on the microscopic detection of characteristic *M. tuberculosis* growth morphology. The drug susceptibility testing performance of a similar broth-based method with microscopic reading of bacillary growth was compared extensively by Park et al. (18) to that of the reference 7H10 agar proportion method. The MODS assay is, however, a qualitative test in which the observer determines the MIC of a compound only by visualizing growth. We slightly modified the MODS method by introducing spectrophotometric readings of the bacillary growth in order to generate quantitative data. Daily OD measurements were converted to percent growth inhibition versus levels for untreated control wells and used to calculate the GIC$_{50}$ of a compound. In this report, we validated the GIC assay by testing several clinically available TB drugs from different drug classes and compared the results to those obtained by two other in vitro susceptibility methods. Results from the GIC assay were very similar to these of the reference methods. The format of the GIC assay has proven to be sensitive and rapid, often providing data within 3 to 4 days of culture. This method is a direct measure for growth based on optical density and does not rely on the addition of a dye or a substrate or measure indirect processes such as luminescence. In addition, this assay provides multiple datum points, whereas the frequently used MIC method has the disadvantage of being an endpoint assay. Since there is no interference of dyes or substrates, the minimum bactericidal concentration of the compound can be determined by using the same assay plate. To this purpose, bacteria from every well without visual growth can be plated at the completion of the experiment (after 10 days) on 7H9 plates. The lowest compound concentration without any growth on the agar plate determines the minimum bactericidal concentration of that compound. Of significance, we need to emphasize that readings by spectrophotometer are to be interpreted with care, since optical density may not always be proportional to viable
bacterial numbers (24). For more advanced evaluation of the antimicrobial properties, such as concentration-dependent killing or time-dependent killing, a method based on actual counting of discrete bacterial colonies is still the recommended approach (8).

One of the difficulties encountered by using mouse models for initial efficacy testing against tuberculosis is the uncertainty of drug absorption across the intestinal tract after oral administration. Since usually little is known about the biological and chemical characteristics of novel compound series, it was necessary to develop a simple bioassay to determine the oral bioavailability of a compound prior to lengthy and expensive efficacy drug trials with animals. Mice were given a single dose of compound by oral gavage, and drug levels were determined by testing serum samples with a microdilution plate method using *M. tuberculosis*. This bioassay is a variation of existing assays such as (i) the established serum inhibitory assay (also known as the Schlichter test) which determines the serum static titer of drugs in patients (15) and (ii) bioassays developed for the same purpose but using other bacterial strains, such as *Bacillus subtilis* (12, 17). The method was validated with clinically available compounds, and the bioassay results were compared with and found to be similar to well-established pharmacokinetic data from the literature. Data from the literature show for INH at 25 mg/kg a peak serum level of 28.2 µg/ml obtained at 0.25 h after administration and for RIF at 10 mg/kg a peak level of 10.58 µg/ml obtained at 1.33 h after administration (5). In earlier studies, streptomycin when injected intravenously at 50 mg/kg reached a peak level of 9.6 µg/ml after 1 h (22). As for moxifloxacin, peak serum levels after an oral dose of 100 mg/kg are described as 7.8 µg/ml at 0.25 h and are expected to be 30 µg/ml after a 400-mg/kg dose (14, 26). In the bioavailability assay, ciprofloxacin shows the poorest result, with drug serum levels below the detection limit of the bioassay at all time points past 1 h after dosing (6). As these data show, the basic pharmacokinetic data, such as C_{max} and time to maximum concentration of drug in serum, of clinically available compounds obtained with the bioavailability assay are in the same range as those described in the literature after more-extensive pharmacokinetic studies using high-performance liquid chromatography or liquid chromatography-mass spectrometry analysis.

Since the bioavailability assay measures bioactivity, the determined drug levels in the bioassay are in fact free, unbound bioactive product (which can be the parent drug and/or drug metabolites). It is well established that only unbound compound shows antimicrobial activity. The detection limit of the assay is 10× the MIC_{90} of the tested compound, due to the restriction of using a maximum of 10% serum in the *M. tuberculosis* bioassay. Therefore, the bioavailability assay is designed for assessing absorption of the compound in the bloodstream and is not intended for advanced pharmacokinetic work, due to this lower sensitivity. By using more time points, however, the assay can provide preliminary information on basic pharmacokinetic parameters, such as C_{max} time to maximum concentration of drug in serum, and half-life of a compound series, which is important information for the medicinal chemist to refine the compounds further. The assay has also been proven useful to establish optimal pharmacokinetic conditions for a compound before in vivo efficacy testing (different routes of administration, formulations, and dosing frequencies).

On the same assay plate, the MIC of the compound is tested in the presence of and without 10% mouse serum. The obtained MIC of the compound in 10% mouse serum gives an indication of the protein binding capacity of a compound to plasma proteins. This valuable information can be used as one of many criteria for prioritization within a compound series, as well as during lead optimization. In our assay, RIF showed a twofold increase in the MIC in the presence of 10% serum, which indicates the drug has significant protein binding. This is consistent with data from the literature in which equilibrium dialysis showed that RIF has 83.8% protein binding (8). Since a maximum of 10% of serum can be used in the assay without inhibiting the growth of *M. tuberculosis*, the obtained results will give only an indication of protein binding.

This approach of integrating certain aspects of drug metabolism and pharmacokinetics into drug discovery is a recent development seen in the pharmaceutical industry in general by changing from a traditional linear pharmaceutical development approach to a parallel approach of drug development (1). In a traditional linear approach, discovery and development are two independent functions. By integrating the early preclinical program into drug discovery in the alternative, parallel approach, immediate feedback after every screen will be provided to the medicinal chemists to assist in further compound refinement (1). To our knowledge, this report is the first attempt to implement this parallel screening approach in the tuberculosis field. By implementing the described assays, we propose a screening sequence of in vitro assays and in vivo models for testing compounds against *M. tuberculosis* that can provide information faster, can be adapted to semi-high-throughput screening, and can add relevant physicochemical, basic killing kinetics, and basic pharmacological criteria to the decision process of drug discovery.

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