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Fast Standardized Therapeutic-Efficacy Assay for Drug Discovery against Tuberculosis[▽]

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Murine models of *Mycobacterium tuberculosis* infection are essential tools in drug discovery. Here we describe a fast standardized 9-day acute assay intended to measure the efficacy of drugs against *M. tuberculosis* growing in the lungs of immunocompetent mice. This assay is highly reproducible, allows good throughput, and was validated for drug lead optimization using isoniazid, rifampin, ethambutol, pyrazinamide, linezolid, and moxifloxacin.

The appearance of clinical resistance to first-line drugs (8) and the long treatments have prompted a renewed effort in tuberculosis drug discovery. Efficacy in animal models is a key criterion for selection of compounds. In order to address its complexity, tuberculosis can be deconstructed by modeling specific pathological characteristics of the human infection separately (15). Assays designed to measure the ability of a drug to kill *Mycobacterium tuberculosis* actively replicating in the lungs can be easily modeled in mice (12) and are relevant because most fatalities are caused by lung infections. The model using C57BL/6 (B6) gamma interferon (IFN-γ)-disrupted mice (GKO) and low-dose aerosol infection (9) is particularly well suited for the objective above since (i) the lungs are the primary infected organs, (ii) antitubercular efficacy is almost exclusively due to drug action, since GKO mice do not mount an effective adaptive immune response (6), and (iii) the model is reproducible and sensitive (9). However, this model requires 30 days from infection to sacrifice, and GKO mice are relatively expensive animals. In order to overcome these drawbacks, a fast therapeutic-efficacy assay against replicating *M. tuberculosis* H37Rv in the lungs of wild-type B6 mice was developed and standardized.

The assay should (i) allow significant bacterial growth after lung infection, (ii) enable detection of drug exposures that kill bacteria in lungs, (iii) minimize the duration of the assay, and (iv) maximize the duration of treatment. Bacterial growth of about 2 logs over the initial inoculum was determined to be a level that would provide enough dynamic range to detect statistically significant growth inhibition. Infection was initiated by nonsurgical intratracheal instillation of *M. tuberculosis* H37Rv. In brief, mice were anesthetized with 3% isoflurane and intubated with a metal probe (catalog number 27134; Unimed SA, Lausanne, Switzerland) as described previously (2). The inoculum (10^5 CFU/mouse suspended in 50 μl of phosphate-buffered saline) was put into the probe and delivered through forced inhalation with a syringe (14). To measure infection burden in lungs, all lobes were aseptically removed and homogenized. The homogenates were supplemented with 5% glycerol and stored frozen (−80°C) until plating. No significant differences were observed between fresh and frozen homogenates obtained from mice not treated or treated with antituberculars and having bacterial loads in the dynamic range of the assay (data not shown). After 14 days of culture, colonies were counted using an automatic colony counter (aCOLyte-Supercount; Synoptics Ltd., Cambridge, United Kingdom) and confirmed by visual inspection to correct potential misreadings. Consistent with an *in vivo* duplication time of approximately 24 h, the 2 logs of growth was achieved 9 days after infecting 8- to 10-week-old B6 female mice (Harlan, Barcelona, Spain) (Fig. 1). The initial inoculum selected for the assay (10^5 CFU/mouse) led to 6.94 ± 0.38 $\log_{10}$ CFU/lung (mean ± standard deviation [SD]) at day 9 (normally distributed; Shapiro-Wilk normality test, $P = 0.46$; $n = 154$ mice, pooled from 31 different experiments). Thus, this enabled us to detect a 0.7-log reduction in CFU/lung for the 5% confidence level and 90% power using $n = 5$ mice/group. Under these conditions it is possible to detect drug exposures insufficient to achieve net inhibition of bacterial growth (dynamic range, ~2 logs) as well as drug exposures able to kill *M. tuberculosis* (dynamic range, ~3 logs). Finally, the duration of treatment with test drugs was maximized by allowing a period of 24 h for phagocytosis of instilled bacteria and another additional 24 h for clearance of compounds before organ harvesting.

Under our experimental conditions the adaptive immune response likely did not impair growth of *M. tuberculosis* in B6 mice during the assay period (4, 5), since infection of wild-type B6 or GKO female mice (The Jackson Laboratory, Bar Harbor, ME) with 10^5 CFU/mouse showed the same rate of bacterial growth (Fig. 1) and it was lethal to both murine strains (median survival times, defined as a weight loss of >20% of

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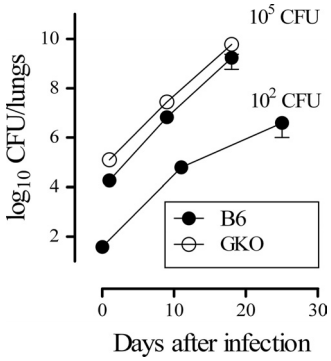

FIG. 1. Kinetics of growth of H37Rv *in vivo*. B6 mice were infected by intratracheal instillation with 10^2 or 10^5 CFU H37Rv per mouse. CFU counts were measured from lungs obtained at different time points. GKO (B6.129S7-Ifngtm1Ts/J) mice were infected by intratracheal instillation with 10^5 CFU H37Rv per mouse. Data are the means ± SDs of the $\log_{10}$ CFU/lung of five mice per time point.

initial weight, were 15.1, 15.7, and 18 days for B6 and 17.4 and 20.7 days for GKO mice in five independent experiments). In addition, lungs of infected B6 and GKO mice at day 9 after infection displayed similar uniformly scattered lymphoid or multicellular aggregates (lymphocytes, neutrophils, macrophages, and foamy macrophages) of less than 0.5 mm containing bacilli inside macrophages.

In order to validate the assay, different doses of known antituberculars were tested (Fig. 2 and Table 1). The individual $\log_{10}$ CFU/lung versus $\log_{10}$ dose administered fit a logistic equation in which compounds showed characteristic Hill's slopes and maximum inhibition (Fig. 2). The therapeutic efficacy of each drug was assessed at day 9 and expressed as the dose that inhibited 99% of CFU/lung with respect to untreated controls (ED_{99}) and the lowest dose at which maximum inhibition of CFU/lung (ED_{max}) was achieved. The ED_{99} measures a compound's potency and is an accurate estimate of the limit between doses with no net inhibitory or net killing effect *in vivo* (1), while the ED_{max} is the optimal dose for treatment (Table 1). Although not directly comparable to other assays, the ranking of potencies found was consistent with previously published data (13). Interestingly, compounds more active against replicating bacteria (isoniazid and ethambutol) appeared more potent in this assay than in chronic models, while rifampin and pyrazinamide lost potency in our assay (10). These data are consistent with the known activity profiles of the compounds and our target assay design.

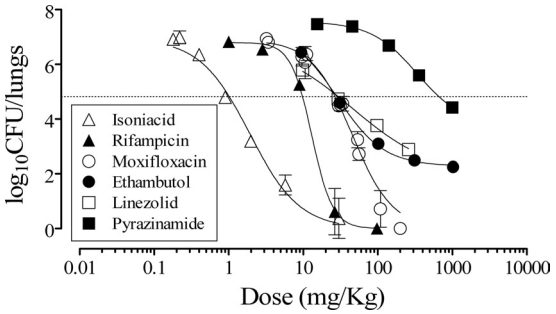

FIG. 2. Therapeutic efficacy of antituberculars against H37Rv *in vivo*. B6 mice were infected by intratracheal instillation with 10^5 CFU H37Rv per mouse. The mice were treated with antituberculars orally once a day from day 1 to day 8. Data are the means ± SDs of the $\log_{10}$ CFU/lung of five mice per time point. The dashed line indicates the $\log_{10}$ CFU/lung at the ED_{99}.

TABLE 1. Therapeutic efficacies against *M. tuberculosis* H37Rv replicating in the lungs of B6 mice[a]

Compound	Vehicle	ED_{99} (mg/kg)[b]	ED_{max} (mg/kg)[c]
Isoniazid	Water	0.95 (0.86–1.07)	25
Rifampin	Water–20% Encapsine	9.8 (9.0–10.7)	30
Pyrazinamide	Water–1% methylcellulose	362.0 (319.0–409.0)	≥1,000[d]
Ethambutol	Water–1% methylcellulose	21.6 (19.9–23.5)	300
Moxifloxacin	Water–20% Captisol	27.7 (26.2–29.3)	100
Linezolid	Water–1% methylcellulose	28.0 (23.7–32.9)	≥300[d]

[a] Data are from five mice/group.
[b] The dose that results in a 2-log reduction in bacterial burden in the lungs of mice. Data were calculated from individual $\log_{10}$ CFU/lung values fit to a logistic equation. Data are expressed as the ED_{99}, with the 95% confidence interval of the ED_{99} shown in parentheses.
[c] Estimated empirically as the lowest dose administered to mice that reduced bacterial burden in the lungs to a level statistically indistinguishable from reductions achieved with higher doses.
[d] Highest dose used in the assay.

The assay was found to be very reproducible using moxifloxacin, which is stable and potent and shows a low frequency of spontaneous resistant mutants compared to controls (ED_{99} for moxifloxacin, 28.1, 26.7, and 26.9 mg/kg of body weight in three independent experiments). Thereafter, a group of mice treated with moxifloxacin at 30 mg/kg, a dose close to its ED_{99}, was always included in each assay in order to obtain an assay quality parameter [Δ_{MOX} = ($\log_{10}$ CFU/lung in untreated controls) − ($\log_{10}$ CFU/lung at the ED_{99} for moxifloxacin)]. An *in vivo* assay is deemed valid if the $\log_{10}$ CFU/lung in untreated controls is in the range of 6.18 to 7.7 and Δ_{MOX} is in the range of 2.21 to 2.92 $\log_{10}$ CFU/lung.

In this paper we have proposed a standardized assay intended to evaluate new drugs against *M. tuberculosis* replicating in the lungs. Its design enables ranking of antituberculars according to their potency (ED_{99}), a parameter currently being used in lead optimization of *M. tuberculosis* gyrase B and InhA inhibitors. In addition, the ED_{max} is proposed as another parameter of efficacy that is not intended to directly compare compounds but to estimate the lowest dose of a drug candidate to be tested in chronic models of infection. As actively replicating mycobacteria may be a subpopulation present in the lungs of chronically infected mice (3, 11), the starting drug exposures chosen should be capable of killing at least that subpopulation. Of note, rifampin's ED_{max} is ≥30 mg/kg (Table 1), which is higher than doses usually employed in murine chronic models and is already suspected to be suboptimal (7). Overall, the standardized assay described in this paper may be a suitable tool for drug discovery that could guide subsequent *in vivo* studies, for which the validity of the approach awaits confirmation.

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All the experiments were approved by the GlaxoSmithKline Diseases of the Developing World (DDW) Ethical Committee on Animal Research, performed at the DDW Laboratory Animal Science facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and conducted according to European Union legislation and GlaxoSmithKline policy on the care and use of animals.

REFERENCES

1. **Andes, D., and W. A. Craig.** 2006. Pharmacodynamics of a new streptogramin, XRP 2868, in murine thigh and lung infection models. Antimicrob. Agents Chemother. **50:**243–249.
2. **Brown, R. H., D. M. Walters, R. S. Greenberg, and W. Mitzner.** 1999. A method of endotracheal intubation and pulmonary functional assessment for repeated studies in mice. J. Appl. Physiol. **87:**2362–2365.
3. **Cardona, P. J.** 2009. A dynamic reinfection hypothesis of latent tuberculosis infection. Infection **37:**80–86.
4. **Chackerian, A. A., J. M. Alt, T. V. Perera, C. C. Dascher, and S. M. Behar.** 2002. Dissemination of *Mycobacterium tuberculosis* is influenced by host factors and precedes the initiation of T-cell immunity. Infect. Immun. **70:** 4501–4509.
5. **Cooper, A. M.** 2009. Cell-mediated immune responses in tuberculosis. Annu. Rev. Immunol. **27:**393–422.
6. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
7. **Davies, G. R., and E. L. Nuermberger.** 2008. Pharmacokinetics and pharmacodynamics in the development of anti-tuberculosis drugs. Tuberculosis (Edinburgh) **88**(Suppl. 1)**:**S65–S74.
8. **Jassal, M., and W. R. Bishai.** 2009. Extensively drug-resistant tuberculosis. Lancet Infect. Dis. **9:**19–30.
9. **Lenaerts, A. J., V. Gruppo, J. V. Brooks, and I. M. Orme.** 2003. Rapid in vivo screening of experimental drugs for tuberculosis using gamma interferon gene-disrupted mice. Antimicrob. Agents Chemother. **47:**783–785.
10. **Matsumoto, M., H. Hashizume, T. Tomishige, M. Kawasaki, H. Tsubouchi, H. Sasaki, Y. Shimokawa, and M. Komatsu.** 2006. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. PLoS Med. **3:**e466.
11. **Muñoz-Elías, E. J., J. Timm, T. Botha, W. T. Chan, J. E. Gómez, and J. D. McKinney.** 2005. Replication dynamics of *Mycobacterium tuberculosis* in chronically infected mice. Infect. Immun. **73:**546–551.
12. **Nikonenko, B. V., K. A. Sacksteder, S. Hundert, L. Einck, and C. A. Nacy.** 2008. Preclinical study of new TB drugs and drug combinations in mouse models. Recent Pat. Antiinfect. Drug Discov. **3:**102–116.
13. **Nikonenko, B. V., R. Samala, L. Einck, and C. A. Nacy.** 2004. Rapid, simple in vivo screen for new drugs active against *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **48:**4550–4555.
14. **Oka, Y., M. Mitsui, T. Kitahashi, A. Sakamoto, O. Kusuoka, T. Tsunoda, T. T. Mori, and M. Tsutsumi.** 2006. A reliable method for intratracheal instillation of materials to the entire lung in rats. J. Toxicol. Pathol. **19:**107–109.
15. **Young, D.** 2009. Animal models of tuberculosis. Eur. J. Immunol. **39:**2011–2014.