# LETTERS

## The association between sterilizing activity and drug distribution into tuberculosis lesions

Brendan Prideaux<sup>1</sup>, Laura E Via<sup>2</sup>, Matthew D Zimmerman<sup>1</sup>, Seokyong Eum<sup>3</sup>, Jansy Sarathy<sup>1</sup>, Paul O'Brien<sup>1</sup>, Chao Chen<sup>1</sup>, Firat Kaya<sup>1</sup>, Danielle M Weiner<sup>2</sup>, Pei-Yu Chen<sup>1</sup>, Taeksun Song<sup>3</sup>, Myungsun Lee<sup>3</sup>, Tae Sun Shim<sup>4</sup>, Jeong Su Cho<sup>5</sup>, Wooshik Kim<sup>6</sup>, Sang Nae Cho<sup>7</sup>, Kenneth N Olivier<sup>8</sup>, Clifton E Barry III<sup>2,9</sup> & Véronique Dartois<sup>1</sup>

Finding new treatment-shortening antibiotics to improve cure rates and curb the alarming emergence of drug resistance is the major objective of tuberculosis (TB) drug development. Using a matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging suite in a biosafety containment facility, we show that the key sterilizing drugs rifampicin and pyrazinamide efficiently penetrate the sites of TB infection in lung lesions. Rifampicin even accumulates in necrotic caseum, a critical lesion site where persisting tubercle bacilli reside<sup>1</sup>. In contrast, moxifloxacin, which is active in vitro against a subpopulation of Mycobacterium tuberculosis that persists in specific niches under drug pressure and has achieved treatment shortening in mice<sup>2</sup>, does not diffuse well in caseum, concordant with its failure to shorten therapy in recent clinical trials. We suggest that such differential spatial distribution and kinetics of accumulation in lesions may create temporal and spatial windows of monotherapy in specific niches, allowing the gradual development of multidrugresistant TB. We propose an alternative working model to prioritize new antibiotic regimens based on quantitative and spatial distribution of TB drugs in the major lesion types found in human lungs. The finding that lesion penetration may contribute to treatment outcome has wide implications for TB.

Standard treatment of drug-susceptible tuberculosis requires six months of combination therapy, including three key drugs: isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA). This regimen was named 'short-course' therapy because it provided, at the time of its introduction, a dramatic shortening of the previous two-year treatment<sup>3</sup>. Reduced chemotherapy duration was achieved by the introduction of RIF and PZA, called 'sterilizing' drugs because they clear difficult-to-eradicate forms of the pathogen<sup>4</sup>. The main objective of contemporary TB drug development is still to shorten curative

treatment duration, in hopes of reducing nonadherence and the emergence of genetically drug resistant Mycobacterium tuberculosis, which present major challenges in global TB control. Here we use matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to show that the patterns of drug penetration provide insight into the ability of individual drugs to sterilize lesion compartments, where persisting bacteria have been shown to reside<sup>1,5</sup>. Such distinct patterns of drug partitioning may generate temporal and spatial windows of monotherapy, with the potential for emergence of genetic drug resistance. This approach may help guide the rational selection of new drug regimens for clinical development to improve the odds of success in future trials.

Historically, the contribution of each drug in the standard regimen to durable cure has been explained by the unique metabolic characteristics of M. tuberculosis subpopulations associated with lesion-specific microenvironments<sup>4</sup>. It is believed that, in aerobic microenvironments with neutral pH, the bacilli are metabolically active and susceptible to INH and RIF. In contrast, anaerobic microenvironments slow the growth of the obligate aerobic bacillus, which then become tolerant to INH, a cell wall synthesis inhibitor, while remaining susceptible to RIF, an RNA polymerase inhibitor. Other specialized microenvironments such as the phagolysosome of infected macrophages are thought to be acidic, resulting in slow growing bacilli that are specifically susceptible to PZA, which is only active at acidic pH. In this situation, RIF and PZA would be sterilizing because they are uniquely capable of killing metabolically adapted, drug-tolerant 'persister' populations. The gyrase inhibitor moxifloxacin (MXF), which was recently tested in clinical trials for shortening front-line treatment, kills growing as well as anaerobic nonreplicating bacteria in vitro<sup>6</sup>, similarly to RIF. MXF was therefore expected to have 'sterilizing' activity in vivo, and mouse efficacy trials further supported this expectation<sup>7</sup>. However, two large phase III trials failed to confirm the preclinical results and showed no shortening of duration of treatment<sup>8,9</sup>. In the wake of

<sup>1</sup>Public Health Research Institute, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, New Jersey, USA. <sup>2</sup>Tuberculosis Research Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. <sup>3</sup>International Tuberculosis Research Center, Changwon, Republic of Korea. <sup>4</sup>Asan Medical Center, Seoul, Republic of Korea. <sup>5</sup>Pusan National University Hospital, Pusan, Republic of Korea. <sup>6</sup>National Medical Center, Seoul, Republic of Korea. <sup>7</sup>Department of Microbiology and Institute of Immunology and Immunological Disease, Yonsei University College of Medicine, Seoul, Republic of Korea. <sup>8</sup>Pulmonary Clinical Medicine, Cardiovascular Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA. <sup>9</sup>Institute of Infectious Disease and Molecular Medicine, Department of Clinical Laboratory Sciences, University of Cape Town, Cape Town, South Africa. Correspondence should be addressed to C.E.B. (cbarry@niaid.nih.gov) or V.D. (veronique.dartois@rutgers.edu).

Received 1 May; accepted 6 August; published online 7 September 2015; doi:10.1038/nm.3937

**Figure 1** Quantitative drug distribution in human pulmonary lesions. (**a**–**d**) Concentrations of INH (**a**), RIF (**b**), PZA (**c**) and MXF (**d**) and their respective metabolites (**a**–**c**) in homogenized closed nodules, cavity wall and cavity caseum samples from 11-12 lesions from each subject (n = 3 subjects per group). INH is a prodrug activated by *M. tuberculosis*' catalase, the product of which forms transient NAD adducts that may display a distinct distribution pattern but cannot be captured by liquid chromatography–mass spectrometry or MALDI imaging. The dotted line indicates the lower limit of quantification. Clear boxes, clinical MIC range<sup>32</sup>; gray shaded boxes, clinical MAC<sup>6</sup> range; orange shaded box (**c**), range of PZA activity in acidic conditions<sup>13</sup>. Each drug and metabolite was quantified in 173 lesions obtained from 15 subjects.

these outcomes, we have considered an alternative working model to predict sterilizing activity on the basis of quantitative and spatial distribution of TB drugs in the major lesion types found in human lungs<sup>10</sup>.

Here we recruited a cohort of 15 TB-infected participants who were scheduled to undergo lung resection surgery for drug-refractory disease (Supplementary Table 1). All 15 volunteers received a single dose of a cocktail containing INH, RIF, PZA and MXF at prespecified times before surgery. The times of drug administration were randomly assigned to five groups of three subjects and ranged from 2 to 24 h before surgery (Supplementary Fig. 1). Upon lung resection, an average of 12 discrete lesions per subject was dissected and processed for histopathology, determination of drug concentration and mass spectrometry imaging. Dissected tissues were classified as uninvolved lung, closed nodule (cellular, necrotic or fibrotic), cavity wall, cavity caseum or nodule caseum. Lesion weights and dimensions were recorded. We measured the absolute drug concentrations over the 24-h dosing interval of INH, RIF, PZA and MXF in plasma (Supplementary Fig. 2), lung (Supplementary Fig. 3) and lesions (Fig. 1), as well as the major drug metabolites inactive acetyl-INH; desacetyl-RIF, which is one-third as potent as RIF; and active POA, which is produced by both the host and the pathogen<sup>11</sup>. Concentrations of drugs and metabolites were measured in homogenized lesions relative to the aerobic minimum inhibitory concentration (MIC) and the minimum anaerobic cidal concentration (MAC), a measure of drug activity against bacilli persisting in necrotic lesions where anaerobic conditions prevail<sup>12</sup> (Fig. 1). The MAC values are averages obtained using laboratory strains and may not capture population-specific variability. INH concentrations were above the MIC in most lesions (65%) but never reached the MAC, as the drug lacks activity against nonreplicating anaerobic bacilli (Fig. 1a). RIF concentrations were above the MIC range in almost all lesions, and at or above the MAC in 64% of lesions (Fig. 1b). The concentrations of PZA, which inhibits growth only at acidic pH in vitro<sup>13</sup>, were within its 'acidic' MIC range (Fig. 1c). Pyrazinoic acid, an active metabolite of PZA, distributed into all lesion types to the same extent as PZA (Supplementary Fig. 4). MXF, unlike INH, RIF and PZA, was consistently present at higher concentrations in lesions than in plasma (Supplementary Fig. 5a-e). MXF concentrations were above the MIC in all lesion homogenates and were at or above the MAC in 38% of lesions (Fig. 1d), indicating that MXF might reach nongrowing persisters at therapeutic concentrations.

In lesion homogenates, absolute drug concentrations are a useful measure of exposure at the site of infection, but important information on the spatial distribution of drugs in distinct compartments is lost. The spectrum of TB lesions is diverse: it includes cellular granulomas, in which a rim of lymphocytes surrounds neutrophils and macrophages; caseous granulomas, in the center of which necrosis or



apoptosis of infected cells generates a mass of cellular debris devoid of blood supply; and cavities, which result from the destructive erosion of lesions into major airways<sup>14</sup>. Mature lesions are encapsulated in layers of fibroblasts, which further serve to wall off the infection. The presence and extent of cavitary disease are correlates of poor clinical outcome and relapse<sup>15,16</sup>. To study the spatial distribution of TB drugs in intact lesions, we used a MALDI mass spectrometry suite17,18 inside a biosafety level 3 high-containment facility, allowing imaging of unlabeled drug molecules in human lesions (Fig. 2a). We obtained high-resolution (30-100 µm) images of RIF, PZA, MXF and acetyl-INH (used as a surrogate of INH on the basis of its similar distribution into lesions (Figs. 2 and 3 and Supplementary Figs. 6 and 7)) in necrotic granulomas from biopsies collected 3-25 h after drug administration and superimposed these images onto histological staining of adjacent sections, revealing lesion structure. The resulting maps showed differences in penetration of each drug or metabolite in caseous foci and cellular layers. PZA (Fig. 2b) and acetyl-INH (Supplementary Fig. 6) diffused favorably and rapidly into the necrotic cores and subtending cellular layers, where persister bacilli are found in the acidic phagolysosome of macrophages<sup>19</sup>. Thus, PZA's sterilizing activity could result from rapid and effective distribution in the caseum and the cellular region of granulomas, where it reaches and kills both extracellular and intraphagosomal bacterial

Image

reconstruction

100 %

0%

Figure 2 Two-dimensional imaging of MXF and PZA by MALDI mass spectrometry. (a) MALDI mass spectrometry imaging of small molecules in TB-infected lung tissue. The relative ion abundance of specific analytes in regions of interest delineated on the basis of histology staining can be measured to provide semiquantitative data. (b) Ion maps of PZA and MXF in representative (selected from more than 200 lesions) lung lesions sampled throughout the dosing interval; signal intensity is fixed for each drug. Hematoxylin and eosin (H&E) staining of adjacent sections is also shown (bottom). Outlines highlight the necrotic center of each lesion. Scale bars, 5 mm. (c) Left, diffusion of MXF in caseum as a function of caseum cellularity. \*\*P < 0.05, two-tailed unpaired t-test. Error bars, mean ± s.d. (n = 3). Right, a typical H&E example representative of each cellularity score.

а

abundance

b

С

MXF

PZA [M+H]<sup>+</sup> *m/z* 124

H&E stain

MXF caseum/cellular concentration ratio

1.0

0.8 0.6

0.4

0.2

2 3 4

Cellularity score

1

[M+H]<sup>+</sup> m/z 402

Dissection

Sectioning

Matrix

application

m/z

lon

quantification in regions of

interest

Acquisition

populations. Overall, INH, PZA and its active metabolite POA, which are small and polar molecules, showed homogeneous distribution across tissue types, with similar concentrations in the cavity wall and caseum of subjects with cavitary disease (**Supplementary Figs. 4** and **8a–c**). MXF exhibited a very different distribution pattern. Whereas the drug accumulated in cellular regions, it did not diffuse well into acellular caseum (**Fig. 2b** and **Supplementary Fig. 8d**). Relative drug abundance measured in regions of interest of

selected ion maps confirmed the visual trend of distribution in cellular rim and caseous foci (**Supplementary Fig. 8e**). Upon closer examination of MALDI images and absolute concentrations in lesions, we found that MXF penetration into the caseum of cavities and closed nodules was variable. Histological staining of lesions indicated that caseum had a spectrum of 'cellularity', from fully acellular and made exclusively of cellular debris, to >50% intact cells. Because MXF is known to accumulate in immune cells, particularly activated macrophages<sup>20</sup>, *in vitro*, we hypothesized that this variability in caseum partitioning could be associated with the degree of caseum cellularity. Indeed, we found a correlation between caseum cellularity and the extent of MXF partitioning into caseum<sup>20</sup> (**Fig. 2c**). Thus, failure of MXF to shorten TB treatment in clinical trials<sup>8,9</sup> might be explained by the drug's inability to achieve adequate concentrations in critical niches where the bacilli reside. The MXF distribution patterns observed here agree well with previous quantitative analysis<sup>21</sup> and imaging<sup>22</sup> of this drug in necrotic rabbit granulomas. Overall, the two-dimensional maps of RIF, PZA, POA and MXF in rabbit lesions (**Supplementary Fig. 9**) were similar to the corresponding drug maps of human lesions, confirming that the rabbit model is an adequate tool to study lesion pharmacokinetics.

To investigate drug accumulation in tissues at steady state, we focused on a subset of subjects who had received INH, PZA or MXF daily as standard of care for several months before surgery (**Supplementary Table 1**). None of the drugs accumulated to higher concentrations in the lesions of these subjects than in those who received a single dose (**Fig. 2** and **Supplementary Figs. 5** and **8e**), indicating rapid clearance of INH, PZA and MXF from the tissues,



**Figure 3** Penetration of RIF in TB lesions. (a) Ion maps of RIF in lung lesions collected after a single dose. Scale bars, 5 mm. (b) RIF accumulates in necrotic foci at steady state. Hematoxylin and eosin (H&E) staining of adjacent sections are shown below each set of ion maps. Outlines highlight the necrotic center of each lesion. Scale bar, 5 mm. (c) Average RIF concentration in plasma, uninvolved lung tissue and lesions in one subject at steady state. The samples were collected 26 h after the last of 180 RIF doses was administered. Error bars, mean ± s.d. of 3–5 lung pieces or distinct lesions.

## LETTERS

**Figure 4** Factors affecting drug diffusion into caseum. Relationship between caseum binding (light orange), accumulation into macrophages (dark orange), lipophilicity (cLogP) and diffusion into caseum *in vivo* (ion maps) for the study drugs. Each assay was done in biological triplicate (i.e., with different batches of samples on different days). Error bars, mean  $\pm$  s.d. Scale bars, 5 mm. Ac-INH, acetyl-INH; I/E, intracellular/extracellular concentration ratio.

as observed in plasma (Supplementary Fig. 2). The three drugs distributed rapidly into lesions, as demonstrated by plotting average lesion/plasma concentration ratios over the course of one dosing interval (Supplementary Fig. 5a-e). In contrast to the cumulative dose-independent distribution of INH, PZA and MXF, RIF (and desacetyl-RIF) accumulated in caseum after multiple doses (Fig. 3a,b, Supplementary Figs. 7 and 8e) and remained readily detectable in necrotic lesions after falling below the limit of detection in uninvolved lung and plasma (Fig. 3b,c). Thus, whereas the caseumto-cellular concentration ratio of MXF is <1 and independent of the number of doses received, RIF accumulates in necrotic foci to reach caseum/cellular concentration ratios >10, more than 20-fold higher at steady state than after a single dose (the former being more clinically relevant). This sustained accumulation in the necrotic core of nodules and cavities, combined with the unique potency of RIF against nonreplicating bacilli<sup>4,6</sup>, may explain RIF's excellent sterilizing activity in patients.

We next applied our lesion penetration approach to clofazimine (CFZ), a leprosy drug currently in clinical trials for use in TB. CFZ is very efficacious in BALB/c mice, where granulomas are exclusively cellular, but has minimal activity in C3HeB/FeJ mice, which develop large necrotic lesions with high bacillary burden<sup>23,24</sup>. We quantified and imaged the distribution of CFZ in three subjects (two were part of the cohort of 15; the other was not) who had received the drug for several months as part of their prescribed regimen. CFZ showed a contrasting partitioning between the cellular rim and necrotic core of lesions, with strong accumulation in cellular layers relative to caseum (Supplementary Fig. 10a). CFZ quantification in plasma, cavity wall and cavity caseum confirmed the imaging results (Supplementary Fig. 10b). Thus, the lack of CFZ diffusion into necrotic foci may contribute to its limited activity in C3HeB/FeJ mice, a finding that should be considered when selecting drug regimens for future clinical trials.

What mechanisms drive the distribution of drugs into avascular caseum? We reasoned that drug molecules present in the interstitial fluid at the interface between the cellular rim and the necrotic core can be subject to (i) active transport into macrophages and other immune cells<sup>25</sup>, (ii) binding to extracellular macromolecules or proteins and (iii) free diffusion through nonvascularized caseum. To understand drug partitioning at the cellular-necrotic interface, we measured the intramacrophage uptake and ex vivo caseum binding of RIF, acetyl-INH, PZA, MXF and CFZ. We observed a wide range of intracellular uptake in macrophages, in agreement with published data for CFZ and MXF<sup>20,26</sup>. As octanol-water partitioning and thus hydrophobicity increases, nonspecific protein binding increases, with two important implications: the free fraction available to passively diffuse through caseum decreases, and intracellular accumulation in macrophages increases owing to uptake of protein-drug complexes by endocytosis and lysosomal trapping (among other mechanisms). Less lipophilic drugs may also dissolve in interstitial fluid and from there gradually accumulate inside the caseum. Under this model, favorable diffusion through caseum is the result of a delicate balance



between active transport, protein binding and physicochemical properties driving solubility and diffusion ability. RIF may lie in the 'sweet spot' by combining relatively low uptake into macrophages and ideal caseum binding to lead to sustained accumulation into necrotic cores (**Fig. 4** and **Supplementary Fig. 11**).

We have demonstrated that different drugs exhibit different spatial and temporal patterns of distribution across TB lesion types and compartments. The results provide a spatial pharmacokinetic and pharmacodynamic explanation for the treatment-shortening properties (or lack thereof) of current TB drugs. Neither favorable penetration nor activity alone is sufficient, as exemplified by INH and MXF, respectively. The key sterilizing drugs RIF and PZA achieve adequate concentrations in critical lesion compartments. PZA is distributed equally well in caseous and cellular compartments, thus reaching two critical persister populations: extracellular anaerobic bacilli in caseum and intracellular bacteria in acidic phagolysosomes<sup>27</sup>. RIF showed sustained accumulation over time in necrotic caseum where extracellular anaerobic bacteria reside. MXF did not reach concentrations required to kill nonreplicating bacteria in the caseum of cavities and nodules, a potential explanation for its performance in recent trials. Such differences in drug distribution and kinetics of accumulation in lesions may create temporal and spatial windows of monotherapy in specific niches, allowing the multiplication of single-drug-resistant mutants and the gradual development of multidrug-resistant TB. The stepwise development of drug resistance has been demonstrated in individual patients through conventional typing methods<sup>28</sup> and whole-genome sequencing<sup>29</sup>. Deep lesion sequencing has revealed complex intrapatient microevolution of M. tuberculosis, leading to diverging clonal populations with distinct resistance patterns<sup>30,31</sup>. The selection of new drug combinations should be supported by lesion-penetration data in animal models of caseous lesions to ensure multidrug coverage in a given lesion compartment and limit the occurrence of de facto monotherapy. Our results suggest that persistent pathology may be as important as persistent organisms for determining sterilizing activity and pave the way for a more rational approach in which agents are combined to reach all target bacterial populations at therapeutic concentrations. This approach would be a significant departure from the current-and mostly empirical-paradigm. Such a strategy is expected to reduce costly attrition in late-phase clinical development, an important consideration for a disease that largely affects resourcelimited populations.

#### **METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

#### ACKNOWLEDGMENTS

We thank D. Young and the funders of the Grand Challenge for Global Health (GCGH 11) consortium for their faith and support. We are grateful to the subjects who enrolled in the study; Y. Kim, M.S. Kong, Y. Cai, J. Gonzales and L. Goldfeder; and the clinical teams at the three Korean centers (Asan Medical Centre, Pusan National University Hospital, and the National Medical Center) for their efforts in making the study a success. We thank S. Dorman, D. Alland and H. Boshoff for sharing clinical MIC and in vitro MAC data sets, M. Stoeckli and D. Staab for providing access to the MALDI imaging platform at the Novartis Institutes for Biomedical Research, E. Eugenin, J. Flynn and J. Mattila for sharing images of M. tuberculosis in nonhuman primate lesions, and T. Dick for discussions and advice. This work was carried out with funding from US National Institutes of Health (NIH) grant 1R01AI106398-01 (V.D.), grant OPP1066499 (V.D.) from the Bill and Melinda Gates Foundation, the Intramural Research Program of the NIH National Institute of Allergy and Infectious Diseases (C.E.B.) and the National Heart, Lung and Blood Institute (K.N.O.), as well as funding from the Korean Centers for Disease Control of the Korean Ministry of Health and Welfare to the International Tuberculosis Research Center.

#### AUTHOR CONTRIBUTIONS

B.P. and P.-Y.C. developed all MALDI MSI methods and imaged the study drugs in lung biopsies; L.E.V., C.E.B., S.N.C. and V.D. conceived the study and wrote the clinical protocol; L.E.V. and S.E. developed the dissection protocol and conducted lung dissections; M.D.Z. and F.K. quantified all study drugs in plasma, lung and lesions; J.S., P.O'B., D.M.W. and F.K. developed methods to sterilize TB-infected samples and extract the study drugs; J.S. and C.C. developed the caseum binding and macrophage uptake assay and quantified the study drugs; T.S. collated clinical and radiological data and facilitated sample collection and transport; M.L. coordinated patient enrollment, confirmed study eligibility and facilitated case reporting; T.S.S., J.S.C., W.K., S.N.C. and K.N.O. coordinated lung resection surgeries; C.E.B. and V.D. wrote the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/ reprints/index.html

- 1. Hoff, D.R. et al. Location of intra- and extracellular M. tuberculosis populations in lungs of mice and guinea pigs during disease progression and after drug treatment. PLoS ONE 6, e17550 (2011).
- Li, S.Y. et al. Evaluation of moxifloxacin-containing regimens in pathologically distinct murine tuberculosis models. Antimicrob. Agents Chemother. 59, 4026-4030 (2015).
- Fox, W., Ellard, G.A. & Mitchison, D.A. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. Int. J. Tuberc. Lung Dis. 3, S231-S279 (1999)
- Mitchison, D.A. The action of antituberculosis drugs in short-course chemotherapy. Tubercle 66, 219-225 (1985).
- Lenaerts, A.J. et al. Location of persisting mycobacteria in a Guinea pig model of tuberculosis revealed by r207910. Antimicrob. Agents Chemother. 51, 3338-3345 (2007)
- 6. Lakshminarayana, S.B. et al. Comprehensive physicochemical, pharmacokinetic and activity profiling of anti-TB agents. J. Antimicrob. Chemother. 70, 857-867 (2015)
- 7. Nuermberger, E.L. et al. Moxifloxacin-containing regimen greatly reduces time to culture conversion in murine tuberculosis. Am. J. Respir. Crit. Care Med. 169, 421-426 (2004).

- 8. Gillespie, S.H. et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. N. Engl. J. Med. 371, 1577-1587 (2014).
- Jindani, A. et al. High-dose rifapentine with moxifloxacin for pulmonary tuberculosis. N. Engl. J. Med. 371, 1599–1608 (2014).
- 10. Dartois, V. The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. Nat. Rev. Microbiol. 12, 159-167 (2014).
- 11. Via, L.E. et al. Host-mediated bioactivation of pyrazinamide: implications for efficacy, resistance, and therapeutic alternatives. ACS Infect. Dis. 1, 203-214 (2015).
- 12. Via, L.E. et al. Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and non-human primates. Infect. Immun. 76, 2333-2340 (2008).
- 13. Zhang, Y., Scorpio, A., Nikaido, H. & Sun, Z. Role of acid pH and deficient efflux of pyrazinoic acid in unique susceptibility of Mycobacterium tuberculosis to pyrazinamide. J. Bacteriol. 181, 2044-2049 (1999).
- 14. Canetti, G. The Tubercle Bacillus in the Pulmonary Lesion of Man: Histobacteriology and Its Bearing on the Therapy of Pulmonary Tuberculosis (Springer, 1955).
- 15. Aber, V.R. & Nunn, A.J. Short term chemotherapy of tuberculosis. Factors affecting relapse following short term chemotherapy [in French]. Bull. Int. Union Tuberc. **53**, 276–280 (1978).
- 16. Chang, K.C., Leung, C.C., Yew, W.W., Ho, S.C. & Tam, C.M. A nested case-control study on treatment-related risk factors for early relapse of tuberculosis. Am. J. Respir. Crit. Care Med. 170, 1124-1130 (2004).
- 17. Prideaux, B. & Stoeckli, M. Mass spectrometry imaging for drug distribution studies. J. Proteomics 75, 4999-5013 (2012).
- 18. Reyzer, M.L. & Caprioli, R.M. MALDI-MS-based imaging of small molecules and proteins in tissues. Curr. Opin. Chem. Biol. 11, 29-35 (2007).
- 19. Tischler, A.D. & McKinney, J.D. Contrasting persistence strategies in Salmonella and Mycobacterium. Curr. Opin. Microbiol. 13, 93-99 (2010).
- 20. Michot, J.M., Seral, C., Van Bambeke, F., Mingeot-Leclercq, M.P. & Tulkens, P.M. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. Antimicrob. Agents Chemother. 49, 2429-2437 (2005).
- Kjellsson, M.C. *et al.* Pharmacokinetic evaluation of the penetration of antituberculosis agents in rabbit pulmonary lesions. *Antimicrob. Agents Chemother.* 56, 446-457 (2012).
- 22. Prideaux, B. et al. High-sensitivity MALDI-MRM-MS imaging of moxifloxacin distribution in tuberculosis-infected rabbit lungs and granulomatous lesions. Anal. Chem. 83, 2112-2118 (2011).
- 23. Irwin, S.M. et al. Limited activity of clofazimine as a single drug in a mouse model of tuberculosis exhibiting caseous necrotic granulomas. Antimicrob. Agents Chemother. 58, 4026-4034 (2014).
- 24. Pichugin, A.V., Yan, B.S., Sloutsky, A., Kobzik, L. & Kramnik, I. Dominant role of the sst1 locus in pathogenesis of necrotizing lung granulomas during chronic tuberculosis infection and reactivation in genetically resistant hosts. Am. J. Pathol. 174, 2190-2201 (2009).
- 25. Kell, D.B. & Oliver, S.G. How drugs get into cells: tested and testable predictions to help discriminate between transporter-mediated uptake and lipoidal bilayer diffusion. Front. Pharmacol. 5, 231 (2014).
- 26. Baik, J. & Rosania, G.R. Macrophages sequester clofazimine in an intracellular liquid crystal-like supramolecular organization. PLoS ONE 7, e47494 (2012).
- 27. Nathan, C. & Barry, C.E. III. TB drug development: immunology at the table. Immunol. Rev. 264, 308-318 (2015).
- 28. Rastogi, N. et al. Emergence during unsuccessful chemotherapy of multiple drug resistance in a strain of Mycobacterium tuberculosis. Eur. J. Clin. Microbiol. Infect. Dis. 11, 901-907 (1992).
- 29. Calver, A.D. et al. Emergence of increased resistance and extensively drug-resistant tuberculosis despite treatment adherence, South Africa. Emerg. Infect. Dis. 16, 264-271 (2010).
- 30. Merker, M. et al. Whole genome sequencing reveals complex evolution patterns of multidrug-resistant Mycobacterium tuberculosis Beijing strains in patients. PLoS ONE 8, e82551 (2013).
- 31. Sun, G. et al. Dynamic population changes in Mycobacterium tuberculosis during acquisition and fixation of drug resistance in patients. J. Infect. Dis. 206, 1724-1733 (2012)
- 32. Lee, J. et al. Sensititre MYCOTB MIC plate for testing Mycobacterium tuberculosis susceptibility to first- and second-line drugs. Antimicrob. Agents Chemother. 58, 11-18 (2014).

#### **ONLINE METHODS**

Clinical research study design and institutional approvals. Adults with pulmonary MDR-TB scheduled for elective lung resection surgery were asked to participate in the study "Pharmacokinetics of Standard First and Second Line anti-TB Drugs in the Lung and Lesions of Subjects Elected for Resection Surgery" (ClinicalTrials.gov NCT00816426). The institutional review boards of the National Institute of Allergy and Infection Disease, National Institutes of Health and the Pusan National University Hospital, Asan Medical Center and National Medical Center all approved the study. The procedures followed were in accordance with the ethical standards of the Helsinki Declaration. Of the 19 subjects who gave consent for the study, three were found to be ineligible owing to underlying conditions or the investigator's decision, and one withdrew consent. Fifteen consenting subjects were randomized to receive three first-line and one second-line anti-TB agents as follows: 600 mg RIF (450 mg for patients less than 50 kg in body weight), 300 mg INH, 1,500 mg PZA and 400 mg MXF administered orally (all 15 subjects received the four study drugs). All 5 anti-TB drugs were administered concomitantly at 2, 4, 8, 12 or 24 h before scheduled surgery, with 3 subjects randomized to each of the 5 target time points. Subjects were assigned to one of the 5 drug administration times by simple randomization. A single balanced list of identification numbers with the 5 drug dose times permuted randomly across the identification numbers was provided by the statistician to the centralized monitoring organization who managed the randomization assignments for the three clinical sites. Each time a subject consented and enrolled, a designee at that site contacted the monitoring organization who assigned the next study ID and drug dosing time on the list.

All subjects had been treated for TB before the study and had thus received a specific drug regimen tailored to their drug-susceptibility profile. Thus, a subset of the subjects had received INH, PZA or MXF as part of their background drug regimen for several weeks or months before surgery (see **Supplementary Table 1** for a complete list of background drugs) and were at steady state for these drugs. Two subjects had received CFZ as part of their standard of care. During the surgery, the exact time of pulmonary artery ligation was recorded and used to back-calculate the time of drug administration relative to surgery. Actual times of drug administration varied from 3.3 to 25 h before pulmonary vessel ligation. Three surgeries took longer than anticipated, with the target time point after drug administration being longer than the target window (by 1 h to 4 h 30 min). This did not affect the data set, as the actual time elapsed from drug administration to termination of tissue blood flow was used in each participant's case.

One adult with chronic *Mycobacterium abscessus* infection scheduled for elective surgery at the NIH Clinical Centre gave written consent to participate under NIAID "Study of Mycobacterial Infections" (ClinicalTrials. gov NCT00018044). This participant had pulmonary nontuberculous mycobacterial disease presenting with cavitary lesions, well-organized necrotizing granulomas and non-necrotizing granulomas similar to those described previously in lung disease caused by non-TB mycobacteria and *M. tuberculosis*<sup>33–35</sup>. This 73-year-old female participant was administered 600 mg RIF and 100 mg CFZ daily among other antimycobacterial medications as part of her standard drug regimen (24 h target group, 26 h (for RIF) and 27 h (for CFZ) actual times of vessel ligation after drug administration). This individual was at steady state for both RIF and CFZ.

All participants had at least one prior episode of anti-TB or antimycobacterial treatment (median, 1 episode; range, 1–8). The median age was 45 (range, 23–59); nine subjects were male, and all were Asian. The weight range was 49.8–84.0 kg, with a median of 57.8 kg. Mean weights ( $\pm$ s.d.) were 65  $\pm$  12 kg and 53  $\pm$  3 kg for males and females, respectively. **Supplementary Table 2** summarizes subjects' demographics. Subjects were followed for one month after surgery for drug-related adverse effects. Investigators were not blinded to group allocation according to drug administration time.

**Sample-size justification.** The sample size for this study was determined on the basis of the number of subjects necessary to detect a 50% difference in the permeability coefficient into caseous necrotic nodules between two different drugs with 80% power. Comparison of the permeability coefficient was chosen as the primary endpoint rather than drug exposure in lesions relative to plasma. This type of endpoint is often adopted in case of sparse pharmacokinetics (PK) sampling design, where each subject contributes

plasma and tissue PK measurements at one or few time points. In such case, Area Under the Curve (AUC) or drug exposure over time cannot be estimated accurately but is rather fit to a model. However, since each time point and subject was expected to contribute serial plasma, lung tissue and at least 2-5 lesion measurements (largely exceeded in this study), an estimation of the permeability coefficient (P) for each drug, using pooled PK data across subjects, can be made with much better precision than an estimation of AUC. The variability of P across subjects or between similar lesions within one subject was expected to be around 50% on the basis of permeability coefficients in abscesses<sup>36</sup>. With these assumptions, and normally distributed differences in the coefficient P between two drugs, a paired t-test would have required 12 subjects. A 20% inflation factor was applied to the sample size to allow for loss of data due to some administrative or analytical issues. The required sample size for this study is thus 15 subjects. The total number of lesions sampled was 173 across 15 subjects, thus 11.6 lesions per subject. All lesion samples contained the 4 study drugs.

Excluded from the study were: (i) subjects <20 years of age; (ii) women of childbearing potential, who were pregnant, breastfeeding or unwilling to avoid pregnancy (i.e., through use of appropriate contraception, including oral and subcutaneous implantable hormonal contraceptives, condoms, diaphragm, intrauterine device (IUD) or abstinence from sexual intercourse; prospective female participants of childbearing potential were required to have negative pregnancy test (urine) within 48 h before study entry); (iii) people having an allergy or hypersensitivity to any of the 5 study drugs or any fluoroquinolone, aminoglycoside, or rifamycin; (iv) people with severe gout; (v) people with severe claustrophobia or gadolinium hypersensitivity (to be confirmed); (vi) renal, hepatic, auditory and/or vestibular impairment, determined as follows: serum creatinine >2.0 mg/dl (renal), aspartate aminotransferase (AST or SGOT) >100 IU/L (LFTs), alanine aminotransferase (ALT or SGPT) >100 IU/L (LFTs), total bilirubin >2.0 mg/dl (LFTs); (vii) documented QT interval (time between the start of the Q wave and the end of the T wave in the heart's electrical cycle) prolongation; (viii) use of any of RIF, rifapentine or rifabutin within 30 d before the study; (ix) HIV infection, determined by a positive HIV test performed with the past 6 months; (x) the use of any of systemic cancer chemotherapy or chronic systemic corticosteroids (oral or IV only) within 30 d before study (with the following exceptions: intranasal, topical, and inhaled corticosteroids; a short course (10 d or less) of corticosteroids for a nonchronic condition completed at least 2 weeks before enrollment in this study; systemic investigational new drug other than linezolid; antiretroviral medications, or growth factors); (xi) the need for ongoing therapy with warfarin, phenytoin, lithium, cholestyramine, levodopa, cimetidine, disulfiram, ergot derivatives, fosphenytoin, carbamazepine, cyclosporine, tacrolimus, sirolimus, amiodarone or phenobarbital. (Subjects stopping any of these drugs (except amiodarone) per standard of care were required to have stopped at least 1 d before receiving the study drug; because of amiodarone's long half-life and potential for QT prolongation, it was to be stopped at least 60 d before receiving study drugs.)

**Sample collection and processing.** Plasma was collected from each subject before dose and at 2 h and 6 h following administration of the study drugs, at the time of pulmonary artery ligation and of lung resection. Blood was not collected at 6 h for the subjects who were randomized to receive the study drugs 2 or 4 h before surgery.

For each subject, resected lung tissue was collected in a sterile container by the study nurse from the surgical suite and delivered immediately to the dissection team. The tissue was photographed and palpated to identify the location of the lesions observed in the pre-surgery computed tomographic scan. Apparently nondiseased tissue specimens were removed and frozen in liquid nitrogen. The lung tissue was then section in approximately 0.5-cm slices to reveal the cavities and nodules and other lesions. Each lesion was measured, classified, mapped, and divided into parts for analytical drug quantification by HPLC coupled to tandem mass spectrometry (LC/MS-MS) MALDI mass spectrometry imaging (MSI), and histology as described previously<sup>21,22</sup>. For analytical drug measurement, lesions were carefully dissected away from the surrounding tissue, and the portion of the lesion used for MALDI imaging was left embedded in the surrounding tissue. When lesions greater than 4 mm (in at least one dimension) had separable necrotic contents, separate samples of the lesion wall and caseous

material were stored for determination of drug levels. Lesion samples for histological analysis were placed in neutral buffered formalin overnight, dehydrated and embedded in paraffin by standard methods.

Tissue samples were weighed and homogenized in approximately—but accurately recorded—5–7 volumes PBS. Homogenization was achieved using a FastPrep-24 instrument (MP Biomedicals) and 1.4 mm zirconium oxide beads (Precellys). Proteins were precipitated by adding 9 volumes of 1:1 acetonitrile and methanol containing 0.5  $\mu$ g/ml of internal standards to 1 volume of plasma or homogenized tissue sample. The mixtures were vortexed for 5 min and centrifuged at 4,000 r.p.m. for 5 min. The supernatant was then transferred for LC/MS-MS analysis. All tissue samples were homogenized and diluted fivefold in PBS. Homogenization was achieved using a FastPrep-24 instrument (MP Biomedicals) and 1.4-mm zirconium oxide beads (Precellys). Proteins were precipitated by adding 180  $\mu$ l of 1:1 acetonitrile and methanol containing relevant internal standards to 20  $\mu$ l of plasma or homogenized tissue sample. The mixtures were vortexed for 5 min and centrifuged at 4,000 r.p.m. for 5 min. The supernatant was then transferred for LC/MS-MS analysis.

LC/MS-MS analytical methods. LC/MS-MS analysis was performed on a Sciex Applied Biosystems Qtrap 4000 triple-quadrupole mass spectrometer coupled to an Agilent 1260 HPLC system to quantify each drug in the clinical samples. PZA and pyrazinoate chromatography was performed with an Agilent Zorbax SB-C8 column ( $4.6 \times 75$  mm; particle size,  $3.5 \mu$ m) using a reverse phase gradient elution. Cinnamaldehyde-INH derivative, MXF, RIF, desacetyl-RIF, and CFZ chromatography was performed on an Agilent Zorbax SB-C8 column (2.1  $\times$  30 mm; particle size, 3.5  $\mu$ m) using a reverse phase gradient elution. Acetyl-INH chromatography was performed on a Cogent Diamond Hydride column (2.1  $\times$  50 mm; particle size, 4  $\mu$ m) using a normal phase gradient. All gradients used 0.1% formic acid in Milli-Q deionized water for the aqueous mobile phase and 0.1% formic acid in acetonitrile for the organic mobile phase. Multiple-reaction monitoring of parent-daughter transitions in electrospray positive-ionization mode was used to quantify the analytes. Sample analysis was accepted if the concentrations of the quality-control samples were within 20% of the nominal concentration. Data processing was performed using Analyst software (version 1.6.2; Applied Biosystems Sciex).

Neat 1 mg/ml DMSO stocks for all compounds were serial diluted in 50:50 acetonitrile and water to create standard curves and quality-control spiking solutions. 20  $\mu$ l neat spiking solution was added to 20  $\mu$ l drug-free plasma or control tissue homogenate, and extraction was performed by adding 180  $\mu$ l of acetonitrile-methanol (50:50) protein precipitation solvent containing the internal standards for all drugs except INH. Extracts were vortexed for 5 min and centrifuged at 4,000 r.p.m. for 5 min. The supernatant was transferred for LC-MS/MS analysis. INH extraction was performed by adding 30  $\mu$ l of 35% trichloroacetic acid (TCA) in water to study samples to precipitate proteins. Extracts were vortexed for 5 min, and 80  $\mu$ l of internal standard solution (INH-D4 in 1% formic acid in water) was added to the extract, vortexed again for 5 min and centrifuged at 4,000 r.p.m. for 5 min. The supernatant was transferred to a 96-well plate and trans-cinnamaldehyde (50× diluted in methanol) was added to the extracts 1:1 (vol:vol) to derivatize INH and INH-D4.

Human control plasma (K<sub>2</sub>EDTA, Bioreclamation IVT, NY) was used to build standard curves. Gamma-irradiated lung, lesion, and caseum from tuberculosis-infected New Zealand White rabbits was used as a surrogate matrix for human tissues to build standard curves. Surrogate matrices were homogenized by adding four parts PBS buffer and 1 part surrogate tissue. The tissues were homogenized using a SPEX Sample Prep Geno/Grinder 2010 for 5 min at 1,500 r.p.m. 5  $\mu$ l 75 mg/ml ascorbic acid was added to the RIF standards and study samples before extraction to improve the autosampler stability of the extracts. The drugs and metabolites, corresponding labeled internal standards, MRM transitions and lower and upper limits of quantification (LLOQ and ULOQ) are listed in **Supplementary Table 2**. A list of drug and standard manufacturers is provided in **Supplementary Table 3**. The complete raw data set of study drugs and metabolites in plasma, uninvolved lung and lesions can be found in **Figure 1** Source Data and **Supplementary Data 1** and **2**.

Tissue sectioning and matrix application. Tissue sections (12  $\mu$ m thick) were prepared using a Leica CM1850 cryostat (Buffalo Grove, IL) and thaw

mounted onto stainless steel slides (for MALDI MSI analysis) or frosted glass microscope slides (for H&E staining). After sectioning, tissue sections were immediately transferred to a -80C freezer for storage.

Prior to MALDI-MSI analysis, tissue sections were removed from the -80 °C freezer and allowed to reach room temperature for 15 min. For PZA, MXF and CFZ analysis, 3 ml of 50% methanol containing 2 pmol/µl MXF-D3 (C/D/N Isotopes, Quebec, Canada) was applied to the surface by airspray deposition at 40 p.s.i., followed by 25 mg/ml 2,5-dihydroxybenzoic acid (Sigma-Aldrich, St. Louis, MO) (50% methanol, 0.1% TFA). The airbrush (Paasche Model VL, Chicago, IL) was positioned at a distance of 30 cm from the tissue, and 30 passes over the tissue were performed, with the tissue being allowed to dry for 30 s between coatings. For RIF analysis, 3 ml 50% methanol containing 3 pmol/µl RIF-D3 (TRC, Toronto) was applied to the surface by airspray deposition at 40 p.s.i., followed by 15 mg/ml 2',4',6'-trihydroxyacetophenone monohydrate (Acros Organics, Morris Plains, NJ) (50% methanol). The airbrush was positioned at a distance of 30 cm from the tissue, and 20 passes over the tissue were performed, with the tissue being allowed to dry for 30 s between coatings. For acetyl-INH, 5 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO) (50% ACN 0.1% TFA containing 2 pmol/µl INH-D4) was applied to the surface by airspray deposition at 40 p.s.i. The airbrush was positioned at a distance of 30 cm from the tissue, and 30 passes over the tissue were performed, with the tissue being allowed to dry for 30 s between coatings.

**MALDI mass spectrometry imaging analysis.** MALDI-MSI analysis was performed using a MALDI LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a resolution of 60,000 at m/z 400, full width at half maximum. The resolution was sufficient to resolve the drug and respective labeled standards peaks from background without the requirement for MS/MS and subsequent loss of signal. However, drug peak identities were confirmed by acquiring several MS/MS spectra directly from the dosed tissues.

Standards of PZA, MXF, acetyl-INH, RIF and desacetyl-RIF were analyzed both direct from the stainless steel target plate and spiked into control rat lung tissue to optimize instrument parameters. The limits of detection were determined as follows. Standards were spotted on drug-naïve tissue (3-mm diameter circles) and the weight of the underlying tissue (7.07 mm<sup>2</sup>) was estimated at 0.042 mg, using the measured average weight of a  $1 \times 1.5$  cm  $\times 12 \,\mu$ m section piece as 0.9 mg. The limit of detection (LOD) was assessed from signals detected on spotted standards on granuloma and uninvolved lung sections following matrix application by airspray. The LOD values were 1 pmol or 3  $\mu$ g/g for PZA, 50 fmol or 200 ng/g for MXF, 100 fmol or 430 ng/g for acetyl-INH, 50 fmol or 410 ng/g for RIF and desacetyl-RIF, and 50 fmol or 240 ng/g for CFZ.

For PZA, MXF, CFZ and acetyl-INH analysis, spectra were acquired in positive mode and with a mass window of m/z 100–500. This range covered the three small molecules, expected metabolites and any potential salt adduct peaks. A laser energy of 7.5  $\mu$ J was applied, and 50 laser shots were fired at each position (total of 1 microscan per position). The laser step size was set at 75–100  $\mu$ m (depending upon the size of the tissue section), at which small necrotic areas within lesions could easily be resolved and no overlapping of the laser spot on adjacent acquisitions was observed. Images of the dehydrated cholesterol ion ([M–H<sub>2</sub>O+H]<sup>+</sup> at m/z 369.351 derived from in-source fragmentation of cholesterol and cholesterol esters were generated from the same acquisitions. For RIF analysis, spectra were acquired in negative ion mode with a mass window of m/z 500–900. This range covered both RIF and its major metabolite desacetyl-RIF. A laser energy of 25  $\mu$ J was applied, and 35 laser shots were fired at each position (total of 1 microscan per position). The laser step size was set to 75  $\mu$ m.

Data visualization was performed using Thermo ImageQuest software. Normalized ion images of MXF were generated by dividing MXF [M+H]<sup>+</sup> signal ( $m/z 402.182 \pm 0.003$ ) by MXF-D3 [M+H]<sup>+</sup> signal ( $m/z 405.201 \pm 0.003$ ). Normalized ion images of PZA [M+2H]<sup>+</sup> ( $m/z 125.058 \pm 0.003$ ) were generated by normalizing to PZA[<sup>15</sup>ND<sub>3</sub>] [M+2H]<sup>+</sup> ( $m/z 129.075 \pm 0.003$ ). Normalized ion images of RIF were generated by dividing RIF [M-H]<sup>-</sup> signal ( $m/z 821.397 \pm 0.003$ ) by RIF-D3 [M–H]<sup>-</sup> signal ( $m/z 824.416 \pm 0.003$ ). Normalized ion images of acetyl-INH and CFZ were generated by dividing acetyl-INH [M+H]<sup>+</sup> signal ( $m/z 180.077 \pm 0.003$ ) and CFZ [M+H]<sup>+</sup> signal ( $m/z 473.129 \pm 0.003$ ) by the

total ion count (TIC). TIC normalization of acetyl-INH and CFZ images was validated by airspray deposition of 3 ml of 5 pmol solution of acetyl-INH or CFZ onto the tissue surface followed by 30 passes of 25 mg/ml DHB matrix. When either acetyl-INH [M+H]<sup>+</sup> or CFZ [M+H]<sup>+</sup> signal was normalized to the TIC, all tissue-derived ion suppression was compensated for, resulting in a homogenous signal across the tissue uninvolved lung, cellular and caseous tissue areas. Comparative TIC normalized images of acetyl-INH and CFZ versus raw images are shown in **Supplementary Figure 12**.

For all drugs imaged, the signal responds linearly up to 1 nmol/ml of internal standard, several orders of magnitude higher than levels achieved in tissues at therapeutic doses.

**Ion quantification in regions of interest.** Relative quantification of drug levels within caseum and cellular lesion regions was performed using MSiReader<sup>37</sup>. The Thermo RAW data format was converted to imzML using Raw to imzML converter to enable importation into MSiReader<sup>38</sup>. Regions of interest (ROIs) were drawn in each image covering either cellular lesion or caseum, and the mean drug signal intensity within that region (normalized to the corresponding drug internal standard) was calculated following exportation of the ROI peak list into Excel.

Lesion histology and cellularity of caseum. Sections (5  $\mu$ m thick) of each tissue block were cut, mounted on glass slides, and stained with hematoxylin and eosin (H&E)<sup>39</sup>. Lesions were classified into non-necrotizing, cellular granulomas, necrotizing granulomas, and cavities. The cellularity of the necrotic material (or caseum) in large nodules and cavities was assessed by a reader masked to all data other than histology. Slides were read, and caseum cellularity was scored from 1 to 4 using an adaption of the method of MacCarrick *et al.*<sup>40</sup> according to the estimated percentage of the lesion necrotic region with infiltrating leukocytes as follows: 1, 0–5%; 2, 6–20%; 3, 21–50%; 4, >50% (representative H&E stain images of each quartile are shown in **Fig. 2b**).

Drug accumulation assay in human THP-1 cells. Human macrophage-like THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 medium (1× without L-glutamine, Corning Cellgro 15-040-CV) supplemented with 10% FBS and 2 mM L-glutamine (Sigma G7513) at 37 °C in a 5% CO<sub>2</sub> incubator. THP-1 cells were initiated at a density of  $2 \times 10^5$  to  $4 \times 10^5$  cells/ml in 75 cm<sup>2</sup> flasks. After 3 d of incubation, the number of viable cells was counted using the trypan blue exclusion protocol (http://www.lifetechnologies.com/us/en/ home/references/gibco-cell-culture-basics/cell-culture-protocols/trypan-blueexclusion.html) and diluted to 1 × 10<sup>6</sup> cells/ml. Phorbol 12-myristate 13-acetate (PMA) was added to a final concentration of 100 nM, and  $1\times 10^5$  cells were seeded into each well of 96-well tissue culture-treated plates (Greiner Bio One, 50-823-592). Plates were incubated overnight at 37 °C in a 5% CO<sub>2</sub> incubator to allow cells to attach to the bottom of the wells. After overnight incubation, cells were gently washed twice with equal volume of PBS to remove unattached and dead cells. To count and estimate the number of cells per well, cells were detached by incubation with 5 mM EDTA in PBS for 20 min from a subset of wells. Viable cells were counted using the trypan blue exclusion protocol.

To incubate cells with drugs, old medium was removed carefully, and medium supplemented with drugs was added. Cells were incubated at 37 °C for 30 min under normal atmospheric conditions. After 30-min incubation, media were removed carefully, and cells were gently washed twice with equal volume of ice-cold PBS to remove any extracellular drug residuals. Cells were then lysed with equal volume of deionized water for 1 h at 37 °C. Lysates were transferred to 1.5-ml centrifuge tubes and stored at -20 °C or analyzed right away.

Five anti-TB drugs were tested for accumulation in human THP-1 cells: INH (30  $\mu$ M, 40 × MIC), PZA (4 mM, 40 × MIC), MXF (5  $\mu$ M, 4 × MIC), RIF (4  $\mu$ M, 4 × MIC), CFZ (1  $\mu$ M, 4 × MIC). When INH and PZA were first tested at 4 × MIC concentrations, LC/MS-MS signals were below the lowest limit of detection. Therefore, higher concentrations (40 × MIC) were used in subsequent assays.

**Statistical analysis.** A two-tailed *z*-test for proportions was used to compare the proportions of lesions that were above the minimum anaerobic bactericidal concentration for the study drugs. Two-tailed unpaired *t*-tests were used to analyze the correlation between moxifloxacin

caseum/cellular concentration ratios and caseum cellularity scores. Statistical significance was defined as P < 0.05.

**Caseum-binding assay.** Specific pathogen–free, individually housed female New Zealand White (NZW) rabbits were used for aerosol infection by *M. tuberculosis* HN878 or *M. bovis* AF2122, as previously described<sup>12,41</sup>. Briefly, rabbits were exposed to *M. tuberculosis*– or *M. bovis*–containing aerosol using a nose-only delivery system. Three hours after infection, three rabbits were euthanized, and serial dilutions of the lung homogenates were cultured on Middlebrook 7H11 agar plates to enumerate the number of bacterial colony forming units (CFUs) implanted in the lungs. The infection was allowed to progress for 4 weeks (*M. bovis*) or 12–16 weeks (*M. tuberculosis*) before necropsy and collection of cavity caseum. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, with approval from the Institutional Animal Care and Use Committee of the New Jersey Medical School, Newark, NJ, and the National Institute of Allergy and Infectious Diseases (National Institutes of Health), Bethesda, MD.

The rapid equilibrium dialysis (RED) device comprises two side-by-side chambers separated by a vertical cylinder of dialysis membrane (molecular weight cut-off ~8,000)<sup>42</sup>. Caseum samples were diluted tenfold in PBS and homogenized. Drug stock solutions were added to the homogenized samples to give the final concentration of 5  $\mu$ M (<1% DMSO). The inserts were placed in the open wells of the Teflon base plate. 200  $\mu$ l spiked plasma was placed in the sample chambers and the buffer chambers were filled with 350  $\mu$ l PBS. The plates were sealed and incubated at 37 °C for 4 h on an orbital shaker set at 200 r.p.m. Following incubation, 50  $\mu$ l plasma from the sample chamber was transferred to tube containing 50  $\mu$ l PBS. Similarly, 50  $\mu$ l PBS was transferred from the buffer chamber to a tube containing 50  $\mu$ l neat plasma. 400  $\mu$ l organic solvent mixture (30/70 methanol/ACN with internal standard) was added to each sample. Samples were vigorously vortexed and centrifuged. The supernatant was transferred to 96-well plates and stored at -20 °C.

Fraction unbound  $(f_u)$  in undiluted caseum was calculated as

Undiluted 
$$f_u = \frac{1/D}{\left(\left(\frac{1}{f_u}\right) - 1\right) + 1/D}$$

where the dilution factor D = 10. Recovery (mass balance) for each assay was calculated as

$$Recovery = \frac{\text{mass in sample chamber + mass in buffer chamber}}{\text{mass in sample chamber at } t = 0} \times 100\%$$

- O'Connell, M.L. et al. Lung manifestations in an autopsy-based series of pulmonary or disseminated nontuberculous mycobacterial disease. Chest 141, 1203–1209 (2012).
- Okumura, M. *et al.* Clinical factors on cavitary and nodular bronchiectatic types in pulmonary *Mycobacterium avium* complex disease. *Intern. Med.* 47, 1465–1472 (2008).
- 35. Yuan, M.K. et al. Comparative chest computed tomography findings of nontuberculous mycobacterial lung diseases and pulmonary tuberculosis in patients with acid fast bacilli smear-positive sputum. BMC Pulm. Med. 14, 65 (2014).
- Wagner, C., Sauermann, R. & Joukhadar, C. Principles of antibiotic penetration into abscess fluid. *Pharmacology* 78, 1–10 (2006).
- Robichaud, G., Garrard, K.P., Barry, J.A. & Muddiman, D.C. MSiReader: an opensource interface to view and analyze high resolving power MS imaging files on Matlab platform. *J. Am. Soc. Mass Spectrom.* 24, 718–721 (2013).
- Schramm, T. et al. imzML—a common data format for the flexible exchange and processing of mass spectrometry imaging data. J. Proteomics 75, 5106–5110 (2012).
- Via, L.E. *et al.* Infection dynamics and response to chemotherapy in a rabbit model of tuberculosis using [18F]2-fluoro-deoxy-d-glucose positron emission tomography and computed tomography. *Antimicrob. Agents Chemother.* 56, 4391–4402 (2012).
- MacCarrick, M.J. et al. Does hypercapnia ameliorate hyperoxia-induced lung injury in neonatal rats? Lung 188, 235–240 (2010).
- Subbian, S. *et al.* Chronic pulmonary cavitary tuberculosis in rabbits: a failed host immune response. *Open Biol.* 1, 110016 (2011).
- Waters, N.J., Jones, R., Williams, G. & Sohal, B. Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding. *J. Pharm. Sci.* 97, 4586–4595 (2008).