Matrix Metalloproteinase Inhibition in a Murine Model of Cavitary Tuberculosis Paradoxically Worsens Pathology

### Abstract
Matrix metalloproteinases (MMPs) degrade extracellular matrix and are implicated in tuberculosis (TB) pathogenesis and cavitation. In particular, MMP-7 is induced by hypoxia and highly expressed around pulmonary cavities of Mycobacterium tuberculosis-infected C3HeB/FeJ mice. In this study, we evaluated whether administration of cipemastat, an orally available potent inhibitor of MMP-7, could reduce pulmonary cavitation in M. tuberculosis-infected C3HeB/FeJ mice. We demonstrate that compared to untreated controls, cipemastat treatment paradoxically increases the frequency of cavitation (32% versus 7%; P = 0.029), immunopathology and mortality. Further studies are needed to understand the role of MMP inhibitors as adjunctive treatments for pulmonary TB.

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ABSTRACT WORD COUNT: 99
SUMMARY OF ARTICLE’S MAIN POINT

Treatment with cipemastat, a selective MMP inhibitor, paradoxically worsens disease in a mouse model of TB. Our investigation demonstrates that host-directed therapies in TB could have unpredicted deleterious effects. Consequently, careful pre-clinical evaluation is required before progression to clinical trials.
CONFLICT OF INTEREST STATEMENTS:

Alvaro A. Ordonez: No conflicts of interest
Supriya Pokkali: No conflicts of interest
Julian Sanchez-Bautista: No conflicts of interest
Mariah H. Klunk: No conflicts of interest
Michael E. Urbanowski: No conflicts of interest
André Kübler: No conflicts of interest
William R. Bishai: No conflicts of interest
Paul T. Elkington: No conflicts of interest
Sanjay K. Jain: No conflicts of interest

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ABSTRACT

Matrix metalloproteinases (MMPs) degrade extracellular matrix and are implicated in tuberculosis (TB) pathogenesis and cavitation. In particular, MMP-7 is induced by hypoxia and highly expressed around pulmonary cavities of *Mycobacterium tuberculosis*-infected C3HeB/FeJ mice. In this study, we evaluated whether administration of cipemastat, an orally available potent inhibitor of MMP-7, could reduce pulmonary cavitation in *M. tuberculosis*-infected C3HeB/FeJ mice. We demonstrate that compared to untreated controls, cipemastat treatment paradoxically increases the frequency of cavitation (32% versus 7%; *P* = 0.029), immunopathology and mortality. Further studies are needed to understand the role of MMP inhibitors as adjunctive treatments for pulmonary TB.

Keywords: tuberculosis, matrix metalloproteinases, cavities, cipemastat, mouse
BACKGROUND

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that degrade collagen and remodel the extracellular matrix. Multiple MMPs have been associated with tuberculosis (TB) pathogenesis [1, 2], and MMP-1 (interstitial collagenase), MMP-9 (gelatinase B) and MMP-7 (matrilysin) in particular have been associated with active TB and cavitation [3]. MMP-7 is induced by hypoxia [4], and highly expressed in cavitary and hypoxic pulmonary lesions of *Mycobacterium tuberculosis*-infected C3HeB/FeJ mice [5].

Cipemastat (Trocade, Ro 32-3555) is an orally available drug that inhibits several MMPs, including MMP-7 with high potency [6]. In this study, we evaluated whether administration of cipemastat could alter the development of pulmonary cavities in *M. tuberculosis*-infected C3HeB/FeJ mice.

METHODS

All protocols were approved by the Johns Hopkins Biosafety, Radiation Safety, and Animal Care and Use Committees.

**In vivo aerosol infection.** Four to six-week-old female C3HeB/FeJ (Jackson Laboratory) mice were aerosol infected with frozen stocks of *M. tuberculosis* H37Rv, using the Middlebrook Inhalation Exposure System (Glas-Col). Three mice were sacrificed using isoflurane (Henry Schein) overdose one day after infection to determine the number of bacilli implanted in the lungs. Mice were randomized into the cipemastat treatment group and the control untreated group. At each time point, a subset of four infected mice from each group was sacrificed to determine the bacillary burden as log$_{10}$ colony forming units (CFU). The entire lungs were
harvested, homogenized in PBS, and then plated by serial dilution in triplicate onto Middlebrook 7H11 selective plates (Becton Dickinson). All plates were incubated at 37°C for 4 weeks before colonies were counted.

Chemotherapy. Cipemastat (F. Hoffmann-La Roche) was administered via oral gavage at 100 mg/kg daily (divided over two equal doses) for 10 weeks. The control group received sham treatment with phosphate buffered saline (PBS) twice daily via oral gavage.

Bio-containment and CT imaging. At 4, 8 and 10 weeks post infection, M. tuberculosis-infected animals were serially imaged within a sealed bio-containment bed (Minerve) modified in-house to be compliant with biosafety level 3 (BSL3) containment, as described previously [5]. Computed tomography (CT) was performed using the NanoSPECT/CT (Bioscan) in vivo animal imager; images were reconstructed and visualized using VivoQuant 2.5 (Invicro). A cavity was defined as a macroscopic region of air (density <−900 Hounsfield units) within the diseased lung parenchyma.

Histopathology. The lungs were harvested after systemic perfusion with PBS under deep anesthesia, fixed in 4% paraformaldehyde and sectioned to 5 μm thickness. Hematoxylin-eosin staining was performed following standard procedures. The slides were scanned using the Apeiro digital scanner (Leica). The total area involved with disease (including pneumonia, granulomas, and cavities) was measured and compared to total lung area using ImageJ (NIH). Briefly, we utilized the free-hand tool in Image J and manually demarcated the diseased areas. The ROI manager tool was used to quantify diseased areas and divided by the total area for that section.
**Immunohistochemistry.** The lungs from *M. tuberculosis*-infected mice (untreated control) were processed as described above and paraffin-embedded sections were rehydrated in graded alcohols, steamed in citrate buffer at pH 6, probed at room temperature for 2 hours for MMP-7 (rabbit polyclonal; 1:250; Abcam) and processed with a polymer-HRP kit (BioGenex) with diaminobenzidine development and Mayer hematoxylin counterstaining.

**Statistical analysis.** Statistical analysis using the two-tailed Fisher’s exact test, two-tailed Student’s t test or Log-rank test was performed as indicated using Prism 6 version 6.07 (GraphPad). Data are presented as mean ± standard deviation on a logarithmic (CFU) or linear scale.

**RESULTS**

The pulmonary bacterial burden one day after infection was $2.6 \pm 0.4 \log_{10}$ CFU. MMP-7 was highly expressed in *M. tuberculosis*-infected pulmonary lesions (Supplementary Figure 1).

There were no differences in the pulmonary bacterial burden in animals with or without cipemastat treatment over the course of the study (Figure 1A). However, animals receiving cipemastat had a significant increase in the proportion of cavitation at 10 weeks post-infection, 32% versus 7% ($P = 0.029$; two-tailed Fisher’s exact test) (Figure 1B and Table 1). Details on location and size of each cavitary lesion are provided in Supplementary table 1, demonstrating a trend towards an increased size of cavities in the cipemastat treated versus untreated mice (3.65 versus 1.48 mm$^3$). Post-mortem gross pathological lung samples at 10 weeks post-infection are shown in Supplementary Figure 2, showing extensive pathology in all infected mice. Post-
mortality histopathological also demonstrated significantly increased disease severity in cipemastat-treated mice (Figure 1C; \( P = 0.003; \) two-tailed Student’s t test). Representative hematoxylin-eosin stained sections are shown in Supplementary Figure 3. Both treated and untreated groups had a similar percentage area of granulomas, but cipemastat-treated mice had a significantly larger area of pneumonia-like disease compared to controls (Supplementary Figure 4; \( P = 0.004, \) two-tailed Student’s t test). Finally, there was also a trend demonstrating increased mortality amongst the cipemastat-treated mice versus the control group (Figure 1D; \( P = 0.17; \) Log-rank test).

**DISCUSSION**

Destruction of lung extracellular matrix is a prerequisite for cavity formation in TB disease and is associated with collagenase activity [6-8]. The presence of cavitary lesions in TB patients correlates with worse outcomes, infectiousness and higher rates of drug-resistance [9]. These observations have led to an interest in developing adjuvant therapies that could modulate collagen remodeling and reduce cavitary disease. Cipemastat is an orally available selective MMP inhibitor, originally developed by Roche Pharmaceuticals as an anti-arthritis agent. Cipemastat inhibits several collagenases (MMP-1, MMP-8, and MMP-13) as well as matrilysin (MMP-7), all of which are upregulated in *M. tuberculosis* infection [6-8]. Although mice do not express an ortholog of MMP-1 [10], MMP-1 does not seem to be essential for cavity formation in this model. Mice do express MMP-7 as well as other collagenases (MMP-8, MMP-13), and both MMP-7 and MMP-13 are potently (IC\(_{50} < 9 \) nm) inhibited by cipemastat [6, 7].

We therefore utilized a murine model of pulmonary TB that develops well-organized, hypoxic TB granulomas, as well as cavitary lesions after aerosol infection [5, 11], and evaluated
whether cipemastat treatment could reduce cavitary formation and immunopathology.

Unexpectedly, we found that cipemastat-treated animals developed more cavities, worse histopathology and a trend towards increased mortality. These data are consistent with those reported by Urbanowski et al. where cipemastat monotherapy in a rabbit model of cavitary TB increased both the frequency and volume of cavitary disease compared to control animals [12]. Rabbits do express MMP-1 as well as other key MMPs. Furthermore, prior studies have demonstrated that daily administration of 10-25 mg/kg of cipemastat provide high plasma levels in rodents (Supplementary table 2) and a dose ranging study in mice demonstrated that treatment with 10, 25 or 50 mg/kg cipemastat were effective in preventing tissue damage and reduce disease progression in experimentally induced arthritis [7]. However, while we used an adequately high dose of 100 mg/kg, we did not measure intra-lesional concentrations of cipemastat. In addition, it is also possible that other MMPs or processes are involved with cavitation which were not affected by cipemastat treatment.

Interestingly, Xu et al have recently demonstrated that administration of marimastat (a selective MMP-2 and MMP-9 inhibitor) alone was not protective in M. tuberculosis-infected C57BL/6J mice. However, when administered as adjunctive treatment with either rifampin or isoniazid, marimastat increased drug exposures in infected lung tissues and led to a reduction (0.5-1 log_{10}) in the pulmonary bacterial burden compared to animals treated with rifampin or isoniazid alone [13]. Similarly, we have also recently demonstrated improved pulmonary bacterial burden or stable (relapse-free) cure in C3HeB/FeJ mice receiving adjunctive anti-MMP-9 antibody in combination with multi-drug first-line TB treatment, compared to standard therapy alone, although these results were no different compared to control animals receiving adjunctive isotype control antibody [14]. Consequently, MMP inhibition in TB seems to have
divergent effects when administered alone or in combination with effective TB treatment. These findings have parallels in the cancer field, where MMP inhibitors were proposed as adjunctive therapy but subsequent clinical trials were disappointing [15], an outcome ascribed to the complexity of protease pathways and diversity of MMP function in normal physiology.

In summary, we demonstrate that treatment with cipemastat, a selective MMP inhibitor, paradoxically worsens disease and increases cavitation in a mouse model of TB. Our investigation of monotherapy with an MMP inhibitor demonstrates that host-directed therapy in TB, which would be co-administered with antibiotics, may have unpredicted deleterious effects. Consequently, careful pre-clinical evaluation is required before progression to clinical trials.

**AUTHOR CONTRIBUTIONS:** A.A.O., S.P., P.T.E., and S.K.J. designed the study. A.A.O., S.P., J.S-B., and M.H.K. performed the studies. M.E.U., A.K., and W.R.B. obtained cipemastat and helped plan animal dosing. A.A.O., S.P., P.T.E., and S.K.J. analyzed the data. A.A.O. and S.K.J. wrote the initial draft and all authors edited the manuscript.
REFERENCES


Table. Proportion of cavitation in *M. tuberculosis*-infected mice. Number of mice with cavitary lesions / total number of scanned mice at each time point.

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 4</th>
<th>Week 8</th>
<th>Week 10*</th>
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<tr>
<td>Cipemastat-treated</td>
<td>0/45 (0%)</td>
<td>2/29 (7%)</td>
<td>7/22 (32%)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0/45 (0%)</td>
<td>1/34 (3%)</td>
<td>2/29 (7%)</td>
</tr>
</tbody>
</table>

*P = 0.029; two-tailed Fisher’s exact test
FIGURE LEGEND

Figure. Effect of Cipemastat in *M. tuberculosis*-infected mice. (A) Pulmonary bacterial burden represented as colony forming units (CFU) in treated and untreated animals after infection. (B) Transverse CT images of representative mice that developed cavitary lesions (yellow arrow) scanned at 4, 8 and 10 weeks post-infection. (C) The disease severity at 10 weeks post-infection, quantified as percentage of lung involved with disease seen on hematoxylin-eosin stained sections, was significantly higher in treated mice (n=13) compared to untreated controls (n=19). *P = 0.003. (D) Survival of the infected mice treated with cipemastat was lower compared to controls. Data represented as mean ± standard deviation.
June 4, 2018

David Hooper
Deputy Editor
The Journal of Infectious Diseases

Re: Revised manuscript JID-64600

Dear Dr. Hooper,

We are submitting a revised manuscript entitled, “Matrix Metalloproteinase Inhibition in a Murine Model of Cavitary Tuberculosis Paradoxically Worsens Pathology” to be considered as a Brief Report for publication in The Journal of Infectious Diseases. We thank you and the reviewers for evaluating our original manuscript and making suggestions that further strengthen it. Each comment has been addressed and a point-by-point response to all of the reviewers' comments are listed.

REVIEWER 1: This Brief Report describes treatment of a cavitary TB murine model with an MMP inhibitor. Authors unexpectedly found cipemastat worsened disease. The results are accurate, original & provide new insight, and the manuscript is largely acceptable. However, a few minor changes and clarifications would strengthen it.

1. **Reviewer comment:** Using mice with a different genetic background, others (Xu PLoS Path 2018) have recently reported that MMP inhibitors attenuate TB infection when used in conjunction with antimicrobials. Would like to see integration of those data with these data, in the Discussion.

   **Author’s response:** This is an excellent point and we have added this to the discussion, on lines 154-164.

2. **Reviewer comment:** Most of the focus in this paper is on cavities, but MMPs contribute to granuloma formation as well. In fact Figure S1 shows MMP7 staining associated with many lesions that are not cavitory lesions. What's the evidence that worsening of disease was caused by increased cavities and not increased granulomas? Are changes in the cellular content? Figure S2 appears to show more or larger granulomas in the treated animals. Would like to see quantification of granulomas & pneumonia in treated vs untreated mice as well as cavities, if authors want to keep the claim that disease worsening is due to more cavities, rather than other manifestations of TB.

   **Author’s response:** We thank the reviewer for pointing this out. We agree that worse pathology is not only due to cavitation but also due to granulomas and pneumonia. Based on the histological analysis, cipemastat-treated animals had more lung involvement compared to untreated mice. This included granulomas as well as pneumonic regions in the lung. We have now clarified this better in the revised version on lines 121-123. Analysis for granulomas and pneumonia alone was also performed and is shown in Supplementary Figure 4. These data demonstrate that cipemastat-treated animals had more pneumonic involvement than controls, as the reviewer postulated.
3. **Reviewer comment:** The Authors say treated mice "had a significant increase in the proportion of cavitation". It is unclear to me what this means - does the number of mice with cavitary lesions increases? Or does the area of the lung with cavities increase (as in 1C)? Figure 1B points out a single cavitation that increases in volume, but cavity volume changes are not discussed. Authors say their results are consistent with Urbanowski et al., but this claim would be strengthened by quantifying frequency and volume of cavities. Would like to see clarified wording in Results, quantification of changes in number and size of cavities on a per-mouse basis, and clarification (or new quantification) of whether the number of mice with cavities per group changes.

**Author’s response:** We agree with the reviewer that the additional information regarding cavitation frequency and volume would be beneficial. While we had initially quantified the number of animals with cavitary disease for each group and time point (Table), we now provide additional information on the number of cavities per mouse and the volume of each cavity (Supplementary table 1), both of which were increased in cipemastat-treated animals.

**REVIEWER 2:** This brief report provides evidence that cipemastat, a potent inhibitor of metalloproteinase MMP-7, exacerbated progression of pulmonary tuberculosis in an experimental mouse model. The authors used a mouse model of human-like pulmonary TB, where necrosis and MMP expression has been well documented, yet the MMP inhibitor was inefficient. This result has translational importance, since MMP inhibitors are suggested as adjunct therapies for human TB. Although the study produced negative result, it provided definitive and important information on an important topic debated within the TB community. Of note, the authors used state of the art imaging technology in combination with traditional methods to support their conclusion. To improve the quality of the manuscript, however, more detailed description of the methods is required.

1. **Reviewer comment:** Comparing lung images in Suppl. Fig.2 - there is no clear evidence of necrotic granulomas in the non-treated control mice. Is it a representative image? Please provide description on the number of sections that were evaluated from each animal. Were necrotic granulomas observed in non-treated mice as well?

**Author’s response:** We agree with the reviewer and thank them for pointing this out. The histological analysis was performed in the widest area of the lungs for each animal (13 cipemastat-treated lung tissues and 19 untreated controls). Although necrotic granulomas are not seen in the data presented in Supplementary Figure 3A (Supp. Fig. 2A in the initial submission), multiple necrotic granulomas were indeed seen in the untreated group. The reason for including the said panel (Supplementary Figure 3A) was to correspond with the MMP staining shown in Supplementary Figure 1 (they are from the same animal). However, to better represent these data, we have now included a panel from another untreated animal in Supplementary Figure 3A which shows necrotic granulomas.

2. **Reviewer comment:** Please describe tools and/or addons utilized within Image J software to annotate the number of cavities, necrotic areas and total area involved.

**Author’s response:** We have now updated the methods with this information and apologize for the omission in the original submission. Briefly, we utilized the free-hand tool in Image J and manually demarcated the diseased areas. The ROI manager tool was used to quantify diseased areas and divided by the total area for that section.

3. **Reviewer comment:** Describe statistical tools in Prizm used for the analyses. Was the survival difference statistically significant?
Author’s response: We have now included the information on statistical analysis for each comparison in the methods and results sections. The two-tailed Fisher’s exact test was used for comparison between cavitation rates in treated and untreated groups and a two-tailed Student’s t test for comparison of diseased area in histology. Although the survival analysis showed a trend towards increased mortality in the cipemastat-treated group, the statistical difference was not significant using a Log-rank test ($P = 0.17$). Details have been added to the methods.

4. **Reviewer comment:** Are gross lung images of the different treatment groups available? If possible they should be included as well.

**Author’s response:** As requested, the gross pathology of whole lungs for both the treated and untreated groups at 10 weeks post-infection has been added as Supplementary Figure 2.

**REVIEWER 3:** The very focused study by Ordonez very convincingly demonstrates that an MMP inhibitor worsens outcomes of Mtb infections when given as a monotherapy. The strength of the study are the use of the C3HeB/FeJ mouse model which better reflects human lung pathology and a very elegant CT-based imaging approach to quantify cavitary lesions.

1. **Reviewer comment:** The major weakness of the study is that it does not investigates how cipemastat works in adjunctive therapy. It could be possible that the worsened pathology makes Mtb bacterial actually more accessible to the conventional drugs and so that paradoxically in that setting cipemastat could actually be beneficial.

**Author’s response:** We agree with the reviewer and have added information regarding this in the discussion (see also Reviewer 1, comment 1). In the current study, we wanted to understand the effects of cipemastat in a mouse strain that develops necrotic pulmonary TB lesions and mirror some of the work performed in the rabbit model (Urbanowski et al. JID 2018). We now discuss the combination with antibacterial therapy in the revised submission on lines 154-164.

Each co-author has read and approved the manuscript. This information is completely new and is not under review for publication elsewhere including on the internet. All authors do not have any other commercial or association that might pose a conflict of interest. All protocols and safety procedures were reviewed and approved by the relevant Johns Hopkins University committees.

Thank you for considering this manuscript.

Sincerely,

Sanjay K. Jain, MD
Professor of Pediatrics, Radiology and Radiological Sciences
Director, Center for Infection and Inflammation Imaging Research
Supplementary Data

Matrix Metalloproteinase Inhibition in a Murine Model of Cavitary Tuberculosis Paradoxically Worsens Pathology

Alvaro A. Ordonez\textsuperscript{1,2,3}, Supriya Pokkali\textsuperscript{1,2,3}, Julian Sanchez-Bautista\textsuperscript{1,2,3}, Mariah H. Klunk\textsuperscript{1,2,3}, Michael E. Urbanowski\textsuperscript{1,4}, André Kübler\textsuperscript{5}, William R. Bishai\textsuperscript{1,4}, Paul T. Elkington\textsuperscript{6,7}, Sanjay K. Jain\textsuperscript{1,2,3}.

\textsuperscript{1}Center for Tuberculosis Research, \textsuperscript{2}Center for Infection and Inflammation Imaging Research \textsuperscript{3}Department of Pediatrics, \textsuperscript{4}Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. \textsuperscript{5}Queen’s Hospital, Barking, Havering and Redbridge University Hospital National Health Service Trust, Romford, Essex, UK. \textsuperscript{6}NIHR Biomedical Research Centre, Clinical and Experimental Sciences Academic Unit, Faculty of Medicine, \textsuperscript{7}Institute of Life Sciences, University of Southampton, Southampton, UK.
Supplementary Figure 1. MMP-7 expression in *M. tuberculosis*-infected pulmonary lesions.

MMP-7 immunostaining from a mouse infected with *M. tuberculosis*. Paraffin-embedded sections were rehydrated in graded alcohols, steamed in citrate buffer at pH 6, probed at room temperature for 2 hours for MMP-7 (rabbit polyclonal; 1:250; Abcam) and processed with a polymer-HRP kit (BioGenex) with diaminobenzidine development and Mayer hematoxylin counterstaining. Representative sections at low and high (inset) power are shown.
Supplementary Figure 2. Gross pathology images of whole lungs at week 10 post-infection.

Ten weeks after aerosol infection with *M. tuberculosis*, mice were sacrificed in both the untreated control group (A) and the cipemastat treatment group (B). Subsequently, the lungs were harvested, fixed in 4% paraformaldehyde and gross images were acquired.
Supplementary Figure 3. Hematoxylin-eosin staining of lungs at week 10 post-infection.

Animals infected with *M. tuberculosis* from both the cipemastat treated group and untreated controls were sacrificed 10 weeks post-infection. The lungs were fixed with 4% paraformaldehyde, sectioned to 5-μm thickness and stained with hematoxylin-eosin. Representative sections for untreated control mice (A) and cipemastat treated animals (B) are shown with dotted lines surrounded the lung involved with disease. These areas were quantified using ImageJ and divided by the total lung area to calculate the percentage of the area involved with disease.
Supplementary Figure 4. The disease severity at 10 weeks post-infection was quantified using ImageJ in hematoxylin-eosin stained sections of cipemastat-treated mice (n=13) compared to untreated controls (n=19). (A) The percentage area involving only granulomas was not different between groups ($P = 0.25$, two-tailed Student’s t test). (B) However, the area involving only areas with pneumonia was significantly higher in the cipemastat-treated group compared to controls ($P = 0.004$, two-tailed Student’s t test).
Supplementary Table 1. Location and size of cavitary lesions.

<table>
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<tr>
<th>Mouse #</th>
<th>Cavity location</th>
<th>Time after infection</th>
<th>Cavity size (mm$^3$)</th>
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<tr>
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<td>Week 8</td>
<td>Week 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cavity size</td>
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<tr>
<td><strong>Cipemastat-treated</strong></td>
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<tr>
<td>M63</td>
<td>RML</td>
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<tr>
<td></td>
<td>LL</td>
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<td>M68</td>
<td>RSL</td>
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<tr>
<td></td>
<td>LL</td>
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<td>0.22</td>
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<tr>
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<td>RSL</td>
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<tr>
<td>M81</td>
<td>RML</td>
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<td></td>
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<td>M91</td>
<td>RIL</td>
<td>0.98</td>
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<tr>
<td>M100</td>
<td>RSL</td>
<td>8.12</td>
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<td>M114</td>
<td>LL</td>
<td>0.72</td>
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<tr>
<td><strong>Average size ± SD</strong></td>
<td></td>
<td>0.28 ± 0.26</td>
<td>3.65 ± 3.96</td>
</tr>
</tbody>
</table>

| **Untreated controls** | | | |
| M2       | RML             | 0.06                 | 2.09                 |
| M25      | LL              |                      | 0.87                 |
| **Average size ± SD** | | 1.48 ± 0.87         |

RSL=right superior lobe; RML=right middle lobe; RIL=right inferior lobe; LL=left lung.
<table>
<thead>
<tr>
<th>Dose (mg/Kg)</th>
<th>Route</th>
<th>Animal model</th>
<th>Results</th>
<th>Reference</th>
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<td>10</td>
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<td>Rats</td>
<td>½ life = 2.95 hours, ( T_{\text{max}} ) = 0.05 hours, ( C_{\text{max}} ) = 7728 ng/ml</td>
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<td>25</td>
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<td>Rats</td>
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<td>10</td>
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<tr>
<td>50</td>
<td>Oral (once daily for 14 days)</td>
<td>Rats (\text{Propionibacterium acnes} -induced arthritis model)</td>
<td>Less collagen degradation</td>
<td>[1]</td>
</tr>
<tr>
<td>50, 25, 10</td>
<td>Oral (twice daily for 14 days)</td>
<td>Rats (injected in the paw with killed \text{M. tuberculosis})</td>
<td>No inhibition of acute inflammatory changes (first 14 days)</td>
<td>[1]</td>
</tr>
<tr>
<td>50</td>
<td>Oral once daily x 12 weeks</td>
<td>Mice (SRT/ORT model for osteoarthritis)</td>
<td>Reduced joint space narrowing and knee damage. Less cartilage degradation.</td>
<td>[2]</td>
</tr>
<tr>
<td>75</td>
<td>Intraperitoneal (twice daily for 42 hours)</td>
<td>Newborn rats with \text{Streptococcus pneumoniae} meningitis</td>
<td>Reduced mortality. Neuroprotection by reducing hippocampal apoptosis and cortical necrosis.</td>
<td>[3]</td>
</tr>
<tr>
<td>100</td>
<td>Oral (once daily for 5 weeks)</td>
<td>Rabbits</td>
<td></td>
<td>[4]</td>
</tr>
<tr>
<td>50 mg</td>
<td>Oral (single dose)</td>
<td>Human (healthy volunteers)</td>
<td>½ life = 24 hours, ( T_{\text{max}} ) = 0.6 hours, ( C_{\text{max}} ) = 1272 ng/ml</td>
<td>[5]</td>
</tr>
<tr>
<td>25-150 mg</td>
<td>Oral (once daily for 28 days)</td>
<td>Human (rheumatoid arthritis patients)</td>
<td></td>
<td>[6]</td>
</tr>
</tbody>
</table>
Supplementary References


TYPE OF ARTICLE: Brief Report

MANUSCRIPT TITLE: Matrix Metalloproteinase Inhibition in a Murine Model of Cavitary Tuberculosis Paradoxically Worsens Pathology

RUNNING TITLE: Inhibition of MMPs and Cavitary TB

AUTHORS AND AFFILIATIONS:
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WORD COUNT: 1833

ABSTRACT WORD COUNT: 99
Treatment with cipemastat, a selective MMP inhibitor, paradoxically worsens disease in a mouse model of TB. Our investigation demonstrates that host-directed therapies in TB could have unpredicted deleterious effects. Consequently, careful pre-clinical evaluation is required before progression to clinical trials.
CONFLICT OF INTEREST STATEMENTS:

Alvaro A. Ordonez: No conflicts of interest
Supriya Pokkali: No conflicts of interest
Julian Sanchez-Bautista: No conflicts of interest
Mariah H. Klunk: No conflicts of interest
Michael E. Urbanowski: No conflicts of interest
André Kübler: No conflicts of interest
William R. Bishai: No conflicts of interest
Paul T. Elkington: No conflicts of interest
Sanjay K. Jain: No conflicts of interest

SOURCES OF FINANCIAL SUPPORT:

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ABSTRACT

Matrix metalloproteinases (MMPs) degrade extracellular matrix and are implicated in tuberculosis (TB) pathogenesis and cavitation. In particular, MMP-7 is induced by hypoxia and highly expressed around pulmonary cavities of Mycobacterium tuberculosis-infected C3HeB/FeJ mice. In this study, we evaluated whether administration of cipemastat, an orally available potent inhibitor of MMP-7, could reduce pulmonary cavitation in M. tuberculosis-infected C3HeB/FeJ mice. We demonstrate that compared to untreated controls, cipemastat treatment paradoxically increases the frequency of cavitation (32% versus 7%; $P = 0.029$), immunopathology and mortality. Further studies are needed to understand the role of MMP inhibitors as adjunctive treatments for pulmonary TB.

Keywords: tuberculosis, matrix metalloproteinases, cavities, cipemastat, mouse
Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that degrade collagen and remodel the extracellular matrix. Multiple MMPs have been associated with tuberculosis (TB) pathogenesis [1, 2], and MMP-1 (interstitial collagenase), MMP-9 (gelatinase B) and MMP-7 (matrilysin) in particular have been associated with active TB and cavitation [3]. MMP-7 is induced by hypoxia [4], and highly expressed in cavitary and hypoxic pulmonary lesions of *Mycobacterium tuberculosis*-infected C3HeB/FeJ mice [5].

Cipemastat (Trocade, Ro 32-3555) is an orally available drug that inhibits several MMPs, including MMP-7 with high potency [6]. In this study, we evaluated whether administration of cipemastat could alter the development of pulmonary cavities in *M. tuberculosis*-infected C3HeB/FeJ mice.

**METHODS**

All protocols were approved by the Johns Hopkins Biosafety, Radiation Safety, and Animal Care and Use Committees.

*In vivo aerosol infection.* Four to six-week-old female C3HeB/FeJ (Jackson Laboratory) mice were aerosol infected with frozen stocks of *M. tuberculosis* H37Rv, using the Middlebrook Inhalation Exposure System (Glas-Col). Three mice were sacrificed using isoflurane (Henry Schein) overdose one day after infection to determine the number of bacilli implanted in the lungs. Mice were randomized into the cipemastat treatment group and the control untreated group. At each time point, a subset of four infected mice from each group was sacrificed to determine the bacillary burden as $\log_{10}$ colony forming units (CFU). The entire lungs were
harvested, homogenized in PBS, and then plated by serial dilution in triplicate onto Middlebrook 7H11 selective plates (Becton Dickinson). All plates were incubated at 37°C for 4 weeks before colonies were counted.

Chemotherapy. Cipemastat (F. Hoffmann-La Roche) was administered via oral gavage at 100 mg/kg daily (divided over two equal doses) for 10 weeks. The control group received sham treatment with phosphate buffered saline (PBS) twice daily via oral gavage.

Bio-containment and CT imaging. At 4, 8 and 10 weeks post infection, *M. tuberculosis*-infected animals were serially imaged within a sealed bio-containment bed (Minerve) modified in-house to be compliant with biosafety level 3 (BSL3) containment, as described previously [5]. Computed tomography (CT) was performed using the NanoSPECT/CT (Bioscan) in vivo animal imager; images were reconstructed and visualized using VivoQuant 2.5 (Invicro). A cavity was defined as a macroscopic region of air (density <−900 Hounsfield units) within the diseased lung parenchyma.

Histopathology. The lungs were harvested after systemic perfusion with PBS under deep anesthesia, fixed in 4% paraformaldehyde and sectioned to 5 μm thickness. Hematoxylin-eosin staining was performed following standard procedures. The slides were scanned using the Apeiro digital scanner (Leica). The total area involved with disease (including pneumonia, granulomas, and cavities) was measured and compared to total lung area using ImageJ (NIH). Briefly, we utilized the free-hand tool in Image J and manually demarcated the diseased areas. The ROI manager tool was used to quantify diseased areas and divided by the total area for that section.
101 Immunohistochemistry. The lungs from *M. tuberculosis*-infected mice (untreated control) were processed as described above and paraffin-embedded sections were rehydrated in graded alcohols, steamed in citrate buffer at pH 6, probed at room temperature for 2 hours for MMP-7 (rabbit polyclonal; 1:250; Abcam) and processed with a polymer-HRP kit (BioGenex) with diaminobenzidine development and Mayer hematoxylin counterstaining.

108 Statistical analysis. Statistical analysis using the two-tailed Fisher’s exact test, two-tailed Student’s t test or Log-rank test was performed as indicated using Prism 6 version 6.07 (GraphPad). Data are presented as mean ± standard deviation on a logarithmic (CFU) or linear scale.

113 RESULTS

The pulmonary bacterial burden one day after infection was $2.6 \pm 0.4 \log_{10}$ CFU. MMP-7 was highly expressed in *M. tuberculosis*-infected pulmonary lesions (Supplementary Figure 1).

There were no differences in the pulmonary bacterial burden in animals with or without cipemastat treatment over the course of the study (Figure 1A). However, animals receiving cipemastat had a significant increase in the proportion of cavitation at 10 weeks post-infection, 32% versus 7% ($P = 0.029$; two-tailed Fisher’s exact test) (Figure 1B and Table 1). Details on location and size of each cavitary lesion are provided in Supplementary table 1, demonstrating a trend towards an increased size of cavities in the cipemastat treated versus untreated mice (3.65 versus 1.48 mm$^3$). Post-mortem gross pathological lung samples at 10 weeks post-infection are shown in Supplementary Figure 2, showing extensive pathology in all infected mice. Post-
mortem histopathological also demonstrated significantly increased disease severity in cipemastat-treated mice (Figure 1C; $P = 0.003$; two-tailed Student’s t test). Representative hematoxylin-eosin stained sections are shown in Supplementary Figure 3. Both treated and untreated groups had a similar percentage area of granulomas, but cipemastat-treated mice had a significantly larger area of pneumonia-like disease compared to controls (Supplementary Figure 4; $P = 0.004$, two-tailed Student’s t test). Finally, there was also a trend demonstrating increased mortality amongst the cipemastat-treated mice versus the control group (Figure 1D; $P = 0.17$; Log-rank test).

**DISCUSSION**

Destruction of lung extracellular matrix is a prerequisite for cavity formation in TB disease and is associated with collagenase activity [6-8]. The presence of cavitary lesions in TB patients correlates with worse outcomes, infectiousness and higher rates of drug-resistance [9]. These observations have led to an interest in developing adjuvant therapies that could modulate collagen remodeling and reduce cavitary disease. Cipemastat is an orally available selective MMP inhibitor, originally developed by Roche Pharmaceuticals as an anti-arthritis agent.

Cipemastat inhibits several collagenases (MMP-1, MMP-8, and MMP-13) as well as matrilysin (MMP-7), all of which are upregulated in *M. tuberculosis* infection [6-8]. Although mice do not express an ortholog of MMP-1 [10], MMP-1 does not seem to be essential for cavity formation in this model. Mice do express MMP-7 as well as other collagenases (MMP-8, MMP-13), and both MMP-7 and MMP-13 are potently (IC$_{50}$ < 9 nm) inhibited by cipemastat [6, 7].

We therefore utilized a murine model of pulmonary TB that develops well-organized, hypoxic TB granulomas, as well as cavitary lesions after aerosol infection [5, 11], and evaluated
whether cipemastat treatment could reduce cavitary formation and immunopathology.

Unexpectedly, we found that cipemastat-treated animals developed more cavities, worse histopathology and a trend towards increased mortality. These data are consistent with those reported by Urbanowski et al. where cipemastat monotherapy in a rabbit model of cavitary TB increased both the frequency and volume of cavitary disease compared to control animals [12]. Rabbits do express MMP-1 as well as other key MMPs. Furthermore, prior studies have demonstrated that daily administration of 10-25 mg/kg of cipemastat provide high plasma levels in rodents (Supplementary table 2) and a dose ranging study in mice demonstrated that treatment with 10, 25 or 50 mg/kg cipemastat were effective in preventing tissue damage and reduce disease progression in experimentally induced arthritis [7]. However, while we used an adequately high dose of 100 mg/kg, we did not measure intra-lesional concentrations of cipemastat. In addition, it is also possible that other MMPs or processes are involved with cavitation which were not affected by cipemastat treatment.

Interestingly, Xu et al have recently demonstrated that administration of marimastat (a selective MMP-2 and MMP-9 inhibitor) alone was not protective in M. tuberculosis-infected C57BL/6J mice. However, when administered as adjunctive treatment with either rifampin or isoniazid, marimastat increased drug exposures in infected lung tissues and led to a reduction (0.5-1 log$_{10}$) in the pulmonary bacterial burden compared to animals treated with rifampin or isoniazid alone [13]. Similarly, we have also recently demonstrated improved pulmonary bacterial burden or stable (relapse-free) cure in C3HeB/FeJ mice receiving adjunctive anti-MMP-9 antibody in combination with multi-drug first-line TB treatment, compared to standard therapy alone, although these results were no different compared to control animals receiving adjunctive isotype control antibody [14]. Consequently, MMP inhibition in TB seems to have
divergent effects when administered alone or in combination with effective TB treatment. These findings have parallels in the cancer field, where MMP inhibitors were proposed as adjunctive therapy but subsequent clinical trials were disappointing [15], an outcome ascribed to the complexity of protease pathways and diversity of MMP function in normal physiology.

In summary, we demonstrate that treatment with cipemastat, a selective MMP inhibitor, paradoxically worsens disease and increases cavitation in a mouse model of TB. Our investigation of monotherapy with an MMP inhibitor demonstrates that host-directed therapy in TB, which would be co-administered with antibiotics, may have unpredicted deleterious effects. Consequently, careful pre-clinical evaluation is required before progression to clinical trials.

**AUTHOR CONTRIBUTIONS:** A.A.O., S.P., P.T.E., and S.K.J. designed the study. A.A.O., S.P., J.S-B., and M.H.K. performed the studies. M.E.U., A.K., and W.R.B. obtained cipemastat and helped plan animal dosing. A.A.O., S.P., P.T.E., and S.K.J. analyzed the data. A.A.O. and S.K.J. wrote the initial draft and all authors edited the manuscript.
REFERENCES


Table. Proportion of cavitation in *M. tuberculosis*-infected mice. Number of mice with cavitary lesions / total number of scanned mice at each time point.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after <em>M. tuberculosis</em> infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 4</td>
</tr>
<tr>
<td>Cipemastat-treated</td>
<td>0/45 (0%)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0/45 (0%)</td>
</tr>
</tbody>
</table>

*P = 0.029; two-tailed Fisher’s exact test
Figure. Effect of Cipemastat in *M. tuberculosis*-infected mice. (A) Pulmonary bacterial burden represented as colony forming units (CFU) in treated and untreated animals after infection. (B) Transverse CT images of representative mice that developed cavitary lesions (yellow arrow) scanned at 4, 8 and 10 weeks post-infection. (C) The disease severity at 10 weeks post-infection, quantified as percentage of lung involved with disease seen on hematoxylin-eosin stained sections, was significantly higher in treated mice (n=13) compared to untreated controls (n=19). *P = 0.003. (D) Survival of the infected mice treated with cipemastat was lower compared to controls. Data represented as mean ± standard deviation.
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\(^1\)Center for Tuberculosis Research, \(^2\)Center for Infection and Inflammation Imaging Research, \(^3\)Department of Pediatrics, \(^4\)Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. \(^5\)Queen’s Hospital, Barking, Havering and Redbridge University Hospital National Health Service Trust, Romford, Essex, UK. \(^6\)NIHR Biomedical Research Centre, Clinical and Experimental Sciences Academic Unit, Faculty of Medicine, \(^7\)Institute of Life Sciences, University of Southampton, Southampton, UK.

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Sources of Financial Support:

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**METHODS**

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**Statistical analysis.** Statistical analysis was performed using Prism 6 version 6.07 (GraphPad). Data are presented as mean ± standard deviation on a logarithmic (CFU) or linear scale.

**RESULTS**

The pulmonary bacterial burden one day after infection was $2.6 \pm 0.4 \log_{10} \text{CFU}$. MMP-7 was highly expressed in *M. tuberculosis*-infected pulmonary lesions (Supplementary Figure 1). There were no differences in the pulmonary bacterial burden in animals with or without cipemastat treatment over the course of the study (Figure 1A). However, animals receiving cipemastat had a significant increase in the proportion of cavitation, 32% versus 7% ($P = 0.029$; two-tailed Fisher’s exact test) (Figure 1B and Table 1). Post-mortem histopathological also demonstrated significantly increased disease severity in cipemastat-treated mice (Figure 1C; $P = 0.003$; two-tailed Student’s t test). Representative hematoxylin-eosin stained sections are shown in Supplementary Figure 2. Finally, there was also a trend demonstrating increased mortality amongst the cipemastat-treated mice versus the control group (Figure 1D; $P = 0.17$; Log-rank test).

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REFERENCES


**Table. Proportion of cavitation in *M. tuberculosis*-infected mice.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 4</th>
<th>Week 8</th>
<th>Week 10*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cipemastat-treated</td>
<td>0/45 (0%)</td>
<td>1/29 (3%)</td>
<td>7/22 (32%)</td>
</tr>
<tr>
<td>Control</td>
<td>0/45 (0%)</td>
<td>0/34 (0%)</td>
<td>2/29 (7%)</td>
</tr>
</tbody>
</table>

*P = 0.029; two-tailed Fisher’s exact test*
Figure. Effect of Cipemastat in *M. tuberculosis*-infected mice. (A) Pulmonary bacterial burden represented as colony forming units (CFU) in treated and untreated animals after infection. (B) Transverse CT images of representative mice that developed cavitary lesions (yellow arrow) scanned at 4, 8 and 10 weeks post-infection. (C) The disease severity at 10 weeks post-infection, quantified as percentage of lung involved with disease seen on hematoxylin-eosin stained sections, was significantly higher in treated mice (n=13) compared to untreated controls (n=19). *P = 0.003. (D) Survival of the infected mice treated with cipemastat was lower compared to controls. Data represented as mean ± standard deviation.