In vivo activity of thiophene-containing trisubstituted methanes against acute and persistent infection of non-tubercular Mycobacterium fortuitum in a murine infection model

Vivek Kr. Kashyap1†, Ravi Kr. Gupta1†, Rahul Shrivastava1†, Brahm S. Srivastava1, Ranjana Srivastava1*, Maloy Kumar Parai2, Priyanka Singh2, Saurav Bera2 and Gautam Panda2

1Microbiology Division, Central Drug Research Institute, CSIR, Lucknow 226001, India; 2Medicinal and Process Chemistry Division, Central Drug Research Institute, CSIR, Lucknow 226001, India

*Corresponding author. Tel: +91-522-4007058; Fax: +91-522-2623405; E-mail: ranjanasrivastava5@gmail.com
†Authors contributed equally.

Received 28 October 2011; returned 28 November 2011; revised 18 December 2011; accepted 22 December 2011

Objectives: Mycobacterium fortuitum causes opportunistic non-tubercular infection in humans. Chronic infection of M. fortuitum has been clinically documented and requires prolonged chemotherapy. The objectives of this study were to characterize acute and persistent infection of M. fortuitum in a murine infection model and to screen thiophene-containing trisubstituted methanes active against both acute and persistent infection.

Methods: A murine infection model of M. fortuitum was used. Bacillary count, bioluminescence, disease symptoms, host immune response, drug susceptibility and mortality were measured. Reactivation of persistent bacilli was induced by dexamethasone. Trisubstituted methanes containing thiophene rings were synthesized and screened in vitro by agar dilution and BACTEC assay and in mice. Cytotoxicity was tested with Vero monkey kidney cells using a resazurin assay.

Results: The acute infection in mice was marked by a 3 log rise in viable counts, the appearance of disease symptoms and a rise in the Th1 immune response. Bacilli were susceptible to fluoroquinolones. This was followed by persistent infection, in which disappearance of disease symptoms, a decline in Th1 response and non-susceptibility to fluoroquinolones was observed. When the mice were immunocompromised on day 40 post-infection (persistent state) by dexamethasone, a rise in viable counts, symptoms and susceptibility to fluoroquinolones and a prominent Th1 response reappeared. Two lead compounds were found that cleared the mice of bacilli in acute infection and caused a 2.29–2.99 log reduction in cfu of persistent bacilli.

Conclusions: The study established acute and persistent infection in mice and identified two promising anti-M. fortuitum compounds with a selectivity index >10.

Keywords: in vivo drug screening, NTM, antimycobacterials, replicating and latent M. fortuitum

Introduction

Non-tubercular mycobacteria (NTM) are considered important opportunistic pathogens as they have become a major cause of mortality and morbidity in immunocompromised individuals.1 The rise in incidence of NTM infections worldwide has caused serious concern because NTM are generally resistant to standard antimycobacterials and antibiotics. Mycobacterium fortuitum, a rapidly growing NTM, accounts for approximately 67% of NTM isolated from respiratory specimens, skin and soft tissues, joint, bursae and wound infections. Mortality due to localized M. fortuitum infection is rare, but may result from extensive pulmonary or disseminated disease like sepsicaemia, meningitis and endocarditis, exclusively in the setting of severe immunosuppression, especially AIDS, or in those who use corticosteroids.1–4 M. fortuitum infection requires long-term chemotherapy; first-line antituberculous drugs (e.g. isoniazid, rifampicin and pyrazinamide) have no role in the treatment of M. fortuitum infection.1,5 The clinical manifestation of NTM is like tuberculosis as they cause both symptomatic and asymptomatic infection and require long-term chemotherapy—resembling latent infection by tubercular mycobacteria.5–7 However, very little is known about the pathogenesis and state of persistence in M. fortuitum, nor is a suitable animal model of persistent infection available—essential for the development of new therapeutics against M. fortuitum infection.
Characteristic features of M. fortuitum infection have been reproduced in goldfish (Carassius auratus) and mice, which have been used as animal models. In goldfish, the systemic, chronic disease is characterized by the presence of granulomatous reaction in visceral organs accompanied by continuing mortalities in the infected stock,8 while in mouse it causes the neurological disorder ‘spinning disease.’9–11 In both mice and fish, granulomas were seen in the infected organs.8

In the present study, we were able to partition acute and asymptomatic persistent infection in mice on the basis of tissue bacillary load, bioluminescence, disease symptoms, immune profiling and drug susceptibility. This model was successfully used to screen compounds from a chemical library of trisubstituted methanes containing thiophene rings. Two compounds completely cleared the mice of acute infection and reduced persistent bacillary infection by more than 2 log. These compounds are interesting because they are bactericidal and effective against both acute and persistent infection, with a selectivity index (SI) >10. SI was evaluated as the concentration for 50% cellular cytotoxicity (CC50) divided by the MIC.

Materials and methods

Bacterial strains, plasmids and culture conditions

M. fortuitum ATCC 6841 and isogenic M. fortuitum[pCDlux] expressing firefly luciferase were used. Plasmid pCDlux is an integrative mycobacterial expression vector derived from pMV306 expressing the firefly luciferase gene under control of the BCG Pshp60 promoter.12 Mycobacteria were grown at 37°C with shaking in Middlebrook 7H9 broth (Difco) supplemented with 0.5% glycerol and 0.05% Tween 80 (Sigma), then plated in nutrient agar containing 0.05% Tween 80 (NAT) for cfu count determination. Kanamycin at 25 mg/L was used during proliferation of M. fortuitum[pCDlux]. Moxifloxacin was dissolved in 0.1 M NaOH, then diluted in sterile distilled water. Ofloxacin was dissolved in sterile distilled water.

Murine infection model

Female BALB/c mice (aged 4–6 weeks, 18–20 g) were bred in the animal house facility of the institute and infected intravenously with M. fortuitum[pCDlux]. The cultures were grown to mid-logarithmic phase in Middlebrook 7H9 broth containing kanamycin (25 mg/L) and Tween 80 (0.05%). An aliquot (150 μL) of culture containing 5 × 10⁷ cfu was injected into each mouse through the tail vein. Mice were observed for mortality and disease symptoms (lethargy, spinning and restlessness). At different timepoints, six mice were sacrificed per group, and kidneys were removed aseptically and homogenized in 2 mL of Tween 80/normal saline. After appropriate dilution, tissue bacillary load and bioluminescence were measured. The protocol standardized for infection with wild-type M. fortuitum was used.10 All the animal protocols were approved by the Institutional Animal Ethics Committee.

For in vivo drug susceptibility experiments, BALB/c mice were infected intravenously with M. fortuitum at a dose of 5 × 10⁷ cfu. The test compounds were finely emulsified in 2.5% gum arabic/0.2% Tween 80 solution and administered orally by gavage (once a day, 6 times per week, for 30 days). Mice were observed for frequency of spinning disease during the course of infection. At regular intervals, six mice were sacrificed from the treated and untreated (control) groups, and kidney homogenates were plated for colony counts on NAT/kanamycin plates. Ofloxacin and moxifloxacin were used as positive controls.

Bioluminescence

Bioluminescence was measured by mixing 100 μL of culture or fresh kidney homogenate with 250 μL of sodium citrate buffer (0.1 M, pH 5.0) and 100 μL of 1 mM luciferin (Promega, USA). Luminescence was measured in a luminometer (Lumat LB 9507, EG&G/Berthold) for 10 s and expressed as relative light units (RLU).12,13

Reactivation

Dexamethasone (0.08 mg/day/mouse) was injected intraperitoneally after 40 days of infection.14,15 Dexamethasone was purchased as a sterile injection (4 mg/mL; Zydus Cadila Healthcare Ltd, India) and diluted in autoclaved water before use.

Immunological profiling of mice infected with M. fortuitum

Th1/Th2 cytokine response was measured by a Becton Dickinson Cytokine Bead Array (CBA) Kit. Mice from different groups were sacrificed to collect blood by cardiac puncture. Collected blood was kept at 37°C for 3 min, to facilitate clotting, and then centrifuged at 9000 g for 10 min at 4°C. The upper layer of serum was aspirated and immediately frozen at −80°C for later analysis of interferon-γ, tumour necrosis factor-α, interleukin (IL)-2, IL-4 and IL-5. Aliquots (50 μL) of sera were used for each assay after dilution in the assay diluent buffer provided in the kit.

Chemical synthesis

Synthesis and characterization information for all thiophene-containing trisubstituted methane derivatives are provided [Figure 1 and Figure S1 (available as Supplementary data at JAC Online); see also additional information in the Supplementary data].

Drug susceptibility

The in vitro drug susceptibility of test compounds was determined by a standard microdilution method.16,17 Aliquots (2 mL) of MB7H9 broth containing serial dilutions of candidate drugs (100–0.78 mg/L) were prepared in 15 mL screw cap tubes. Tubes were inoculated with M. fortuitum test strain (10⁶–10⁷ cells) and incubated at 37°C with shaking. After 48 h of incubation, growth was monitored by measuring absorbance at 600 nm, and serial dilutions were plated on NAT plates for determination of cfu. A tube containing bacteria without drug was used as a control. The lowest concentration of the drug that caused no visible turbidity was considered the MIC of the drug, and the lowest concentration of the drug that caused a 99% reduction in cfu relative to that on day 0

<Figure 1. Prototype of trisubstituted methanes with basic amino alkyl chains with antitubercular activity.>
(99% kill) was considered the MBC of the drug. Moxifloxacin (0.0625–0.5 mg/L) and ofloxacin (1.0–4.0 mg/L) were used as positive controls.

For in vivo activity evaluation, two regimens of drug administration were carried out: (i) day 1 post-infection, and (ii) day 40 post-infection; treatment was continued for 30 days. Infected untreated mice were used as a control. Treated uninfected mice were used to study drug toxicity. Ofloxacin (50 mg/kg body weight), moxifloxacin (100 mg/kg body weight) and test compounds (100 mg/kg body weight) were administered orally, 6 days a week for 30 days.

Cytotoxicity

The compounds for cytotoxicity were tested in an in vitro model for toxicity with Vero monkey kidney cells using a resazurin assay. Briefly, the Vero cells were seeded overnight at 1×10⁴ cells per well in a 96-well plate at 37°C in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and 5% CO₂. Cells were exposed to dilutions of experimental and control drugs in triplicate for 24 h, with each compound in a range of concentrations from 100 to 1.56 mg/L. Moxifloxacin and ofloxacin were used as controls at the same concentrations. Each well had 100 μL of the test material in serially descending concentrations. After 72 h of incubation, 10 μL of resazurin indicator solution (0.1%) was added and incubation was continued for 4–5 h. The colour change was assessed visually. Any colour change from purple to pink or to colourless was recorded as positive. The fluorescence of each sample was measured (excitation 530 nm, emission 590 nm) using a Polar Star Galaxy (BMG). CC₅₀ values were calculated by plotting fluorescence values using a Microsoft Excel spreadsheet.

Statistical analysis

Statistical significance of the data was analysed by two-way ANOVA followed by a Newman-Keuls test. P<0.05 and P<0.01 were considered significant and highly significant, respectively.

Results

Phase of acute infection

During the acute phase of infection, viable counts of infected M. fortuitum[pCDlux] increased 3 log to more than 10⁸ cfu/g of kidney at 10–12 days. Bioluminescence (RLU) measured in kidney homogenates correlated with viable counts. Along with an increase in cfu in kidney, RLU also peaked at 10–12 days and declined with decreasing cfu (Figure 2). With M. fortuitum[pCDlux] expressing firefly luciferase, bioluminescence was used as an index of viability and metabolic activity. During acute infection, infected mice produced the characteristic spinning disease, in which mice hold their heads to one side and show shaking and twitching movements. These mice, when suspended by their tails, rotated vigorously. After 2 weeks, viable counts began to decline in tissue, and on day 25 post-infection, ~10⁵ cfu/g tissue were found. The disease symptoms began to diminish, and within this period 25%–30% of infected mice died. Survival of the mice at various timepoints is shown in Figure S2 (available as Supplementary data at JAC Online).

Phase of chronic persistent infection

From 25 days post-infection to the end of the observation period (60 days), the tissue bacillary load in kidney remained at ~10⁵ cfu/g of tissue. There was no increase in either cfu or RLU, suggesting persistent presence of M. fortuitum in the kidney (Figure 2). The disease symptoms began to disappear after 25 days of infection, and mice were asymptomatic by 40 days post-infection.

Differential susceptibility to fluoroquinolones during acute and persistent infection

Differential drug susceptibility of acute and persistent bacilli was determined to moxifloxacin and ofloxacin, the two commonly used fluoroquinolones against M. fortuitum infection. Mice were cleared of infection to below the limit of detection if drugs were administered 1 day and 25 days post-infection (Table 1 and Table S1 (available as Supplementary data at JAC Online)). Since the mice were cleared of infection, these mice were treated with dexamethasone to see if reactivation of disease occurred. Interestingly, these mice remained free of symptoms of disease, suggesting definite clearance of infection by fluoroquinolone treatment, whereas symptoms appeared in untreated infected control mice treated with dexamethasone (data not shown).

However, if drugs were administered on day 40 post-infection, bacilli were comparatively non-susceptible to fluoroquinolone treatment, because kidney homogenates had viable bacilli that formed colonies on NAT plates. The log reduction after 30 days...
**Compounds against replicating and persistent *M. fortuitum***

**Table 1. In vivo activity of compounds against *M. fortuitum* in mice**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; cfu on day 30</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; reduction compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.40 ± 0.55</td>
<td>0.0</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>3.72 ± 0.57</td>
<td>1.33</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>3.91 ± 0.61</td>
<td>1.14</td>
</tr>
<tr>
<td>Compound 10</td>
<td>4.62 ± 0.23</td>
<td>0.43</td>
</tr>
<tr>
<td>Compound 11</td>
<td>2.06 ± 0.34</td>
<td>2.99</td>
</tr>
<tr>
<td>Compound 18</td>
<td>2.76 ± 0.64</td>
<td>2.29</td>
</tr>
</tbody>
</table>

Mice were infected with 5 × 10<sup>7</sup> cfu. Drug/test compounds were administered 1 day post-infection for 30 days, and cfu in whole kidney homogenate were determined. Whole kidney homogenate was plated for cfu determination. The results are means of three independent experiments (±SD); P < 0.01.

**Table 2. In vivo activity evaluation in persistent state of infection**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; cfu on day 30</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; reduction compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.05 ± 0.61</td>
<td>0.0</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>3.72 ± 0.57</td>
<td>1.33</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>3.91 ± 0.61</td>
<td>1.14</td>
</tr>
<tr>
<td>Compound 10</td>
<td>4.62 ± 0.23</td>
<td>0.43</td>
</tr>
<tr>
<td>Compound 11</td>
<td>2.06 ± 0.34</td>
<td>2.99</td>
</tr>
<tr>
<td>Compound 18</td>
<td>2.76 ± 0.64</td>
<td>2.29</td>
</tr>
</tbody>
</table>

The mice were infected with 5 × 10<sup>7</sup> cfu of *M. fortuitum*. Drug/test compounds were administered 40 days post-infection for 30 days, and cfu in kidney homogenate were determined. The results are means of three independent experiments (±SD); P < 0.01.

Dexamethasone-induced reactivation of persistent infection

Infected mice were subjected to dexamethasone treatment 40 days post-infection according to the protocol. Dexamethasone, a broad-spectrum steroid, has been used in various studies to suppress the immune system. The treatment resulted in reactivation of persistent infection. Appearance of disease symptoms commenced and peaked within 10 ± 2 days of dexamethasone treatment. Bacterial counts in kidney increased up to 10<sup>8</sup> cfu/g of tissue. Increase in cfu correlated with increase in bioluminescence in kidney homogenate of reactivated mice (Figure 2). In contrast, the control mice (infected, but not treated with dexamethasone) remained asymptomatic (no increase in cell count or bioluminescence). When continued, the treatment resulted in 100% mortality at 18 ± 3 days. Withdrawal of dexamethasone on day 10 resulted in reduction in bacillary load, mortality and disease symptoms (Figure 2). This demonstrated that during 40–60 days post-infection, bacilli were in a persistent state, but reactivation occurred by dexamethasone treatment suggesting that the mice were harbouring persistent bacilli and that reactivation was a consequence of immunosuppression.

**Immune profiling**

During the entire period of observation, Th1/Th2 cytokines were measured. Following infection, increases in Th1 cytokines (interferon-γ, IL-2 and tumour necrosis factor-α) were observed, and these attained peak levels on day 10 post-infection, matching peak bacillary load. As the bacillary load declined, Th1 response was found to reduce. During the persistent infection phase, the Th1 response was minimal, and there appeared to be a balanced Th1/Th2 response (Figure S3, available as Supplementary data at JAC Online). Reactivation of *M. fortuitum* following immunosuppression by dexamethasone treatment resulted in a shift from Th1 to Th2 cytokines (IL-4 and IL-5). This change in the pattern of cytokine production was temporary, and the cytokine profile shifted back to a Th1-type response with an increase in cfu (Figure S3).

**Screening of chemical library**

An in-house library of thiophene-containing trisubstituted methanes was screened against *M. fortuitum* ATCC 6841 by the microdilution method. The prototype trisubstituted methanes with basic amino alkyl chains are shown in Figure 1. Thirteen compounds were found to be active against *M. fortuitum*, with MICs between 1.56 and 50 mg/L. The structures of the compounds and their properties are described in Table 3. Compounds with MICs in the range 1.56–3.125 mg/L were selected and tested for cytotoxicity and bactericidal activity as described in the Materials and methods section. Four compounds (10, 11, 17 and 18; Table 3) had bactericidal activity with SI >10, a criterion for advancement of a molecule to in vivo evaluation. These compounds (Table 3) were administered to infected mice on day 1 or day 40 post-infection and thereafter for 30 days (6 days/week), and monitored for mortality and visible disease symptoms.

**Drug administration 1 day post-infection**

Infected untreated mice (control) produced disease symptoms within 8–10 days of infection, which included twitching, tilting, lethargy and spinning. Three categories of compounds were identified: treatment with compounds 11 and 18 completely inhibited disease symptoms, with no mortality; treatment with compound 10 resulted in disease symptoms being observed in 20% of mice with 10% mortality; and mice treated with compound 17 died rapidly, accompanied by hair loss, which presumably was due to toxicity of the compound. With moxifloxacin and ofloxacin, neither mortality nor disease symptoms were observed (Figure S2). The symptoms correlated with tissue bacillary load in the kidney (Table 1). Treatment with compounds 11 and 18 for 30 days cleared the infection in mice, as no cfu were detected in kidney homogenate on NAT plates (>5 log reduction in cfu; Table 1).

**Drug administration 40 days post-infection**

When compounds were administered on day 40 post-infection for 30 days, mice were not cleared of bacilli. In the case of the control, a 0.1–0.2 log increase in cfu was found.
Table 3. *In vitro* activity of synthesized compounds against *M. fortuitum*

<table>
<thead>
<tr>
<th>Name and structure of compound</th>
<th>MIC (mg/L)</th>
<th>CS LogP ± SD[^a]</th>
<th>CS BBB ± SD[^b]</th>
<th>CC50 (mg/L)</th>
<th>SI</th>
<th>MBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>3.91 ± 1.34</td>
<td>0.05 ± 0.29</td>
<td>ND</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>3.125</td>
<td>4.67 ± 1.01</td>
<td>0.23 ± 0.24</td>
<td>20</td>
<td>6.4</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>3.29 ± 1.12</td>
<td>0.09 ± 0.31</td>
<td>ND</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>1.56</td>
<td>2.79 ± 0.84</td>
<td>0.21 ± 0.29</td>
<td>15.12</td>
<td>9.6</td>
<td>2</td>
</tr>
</tbody>
</table>

Continued
Table 3. Continued

<table>
<thead>
<tr>
<th>Name and structure of compound</th>
<th>MIC (mg/L)</th>
<th>CS LogP ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CS BBB ± SD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (mg/L)</th>
<th>SI</th>
<th>MBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3.125</td>
<td>4.27 ± 0.93</td>
<td>0.32 ± 0.2</td>
<td>37.13</td>
<td>11.8</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>3.125</td>
<td>4.35 ± 0.98</td>
<td>0.32 ± 0.25</td>
<td>44.27</td>
<td>14.11</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>6.25</td>
<td>3.66 ± 1.5</td>
<td>0.15 ± 0.27</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>6.25</td>
<td>4.25 ± 1.06</td>
<td>0.16 ± 0.26</td>
<td>ND</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Continued
Table 3. Continued

<table>
<thead>
<tr>
<th>Name and structure of compound</th>
<th>MIC (mg/L)</th>
<th>CS LogP ± SDa</th>
<th>CS BBB ± SDb</th>
<th>CC50 (mg/L)</th>
<th>SI</th>
<th>MBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 H₂CS</td>
<td>25</td>
<td>4.03 ± 1.42</td>
<td>-0.17 ± 0.34</td>
<td>ND</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10 H₂CS</td>
<td>50</td>
<td>3.85 ± 1.58</td>
<td>-0.22 ± 0.3</td>
<td>ND</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11 Cl₁₅₁₅₁₅₁₅₂</td>
<td>12.5</td>
<td>4.02 ± 1.54</td>
<td>0.15 ± 0.3</td>
<td>ND</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12 Cl₁₅₁₅₁₅₁₅₂</td>
<td>1.56</td>
<td>5.16 ± 0.96</td>
<td>0.3 ± 0.26</td>
<td>20</td>
<td>12.82</td>
<td>2</td>
</tr>
</tbody>
</table>

Continued
bacilli on day 40 were in a persistent state. In moxifloxacin- and ofloxacin-treated mice, a 1.14–1.33 log decrease was observed. Compound 10 showed a 0.43 log decrease, while in the case of compounds 11 and 18 a 2.29–2.99 log reduction relative to the control was found (Table 2). Therefore compounds 11 and 18 were found to be more effective than fluoroquinolones against persistent infection of M. fortuitum.

**Activity against other NTM and Mycobacterium tuberculosis**

In vitro drug susceptibility to selected compounds (10, 11 and 18) of other NTM (Mycobacterium abscessus, Mycobacterium avium, Mycobacterium chelonae, Mycobacterium smegmatis, Mycobacterium marinum and Mycobacterium kansasii) and M. tuberculosis H37Rv was determined (Table 4). Compounds...
11 and 18 were found to be effective against *M. fortuitum*, *M. avium*, *M. marinum* and *M. kansasii* (3.125 mg/L) and *M. abscessus* (6.25–12.5 mg/L), establishing them as active molecules against not only *M. fortuitum* but also other NTM. Compounds 10, 11 and 18 were also effective against *M. tuberculosis* H37Rv in the range 1.56–3.125 mg/L.

**Discussion**

In this study we have shown that acute and persistent infection of *M. fortuitum* can be simultaneously induced in mice and used to screen lead compounds effective against acute and persistent infection. Acute infection of *M. fortuitum* was defined as active multiplication of bacilli in kidney, the appearance of disease symptoms and 25% mortality. Persistent infection was marked by constant bacillary load and waning disease symptoms. A library of thiophene-containing trisubstituted methanes was screened with this model, and inhibitors against acute and persistent infections were selected. These compounds were also active against *M. tuberculosis*.

In the murine infection model described in this investigation, there was a rise in viable counts of infected bacteria between day 1 and day 10, followed by a decrease in viable count, which may have been due to a rise in the Th1 immune response. The appearance and waning of disease symptoms in infected mice corresponded with the number of bacilli and the immune status of the host. It has been reported that bacilli can persist for at least 40 days in granuloma-like formations in the kidneys of BALB/c mice. The bacilli were cleared from the kidney if ofloxacin or moxifloxacin was added at day 1 or day 25. However, when drugs were added at day 40, bacilli were not cleared from the kidney (Table 2). The two drugs have been shown to have appre-ciable antimycobacterial activities in vitro and in vivo against *M. fortuitum*, and against other NTM and *M. tuberculosis*. The differential susceptibility of bacilli to fluoroquinolones at day 40 suggests that the persistent bacilli had acquired physiological tolerance to fluoroquinolones, which were active against the replicating bacilli during the initial replication/acute phase. This phenotypic resistance is attributed to latency in *M. tuberculosis* infection. The key front-line antituberculosis drugs, which are effective in treating individuals with acute tuberculosis, are ineffect-ive in eliminating *M. tuberculosis* during the persistent stages of latent infection. It has been reported that latent tuberculosis bacilli become relatively resistant to antimycobacterial agents such as isoniazid. This latency might be the reason for the need for long-term chemotherapy required for treatment of NTM infection, and this has been examined in the murine infection model developed by us. The cytokine profile was consistent with a mycobacterial-induced cellular immune response.

The bacilli from day 40 onwards represented asymptomatic persistent infection because they were comparatively resistant to fluoroquinolones and responded to dexamethasone-induced immunosuppression. The results suggest that dexamethasone treatment caused reactivation of persistent bacilli with a rise in viable count, bioluminescence, appearance of disease symptoms, mortality and fluoroquinolone susceptibility (Figure 2 and Table S1). Reactivation of *M. fortuitum* following immunosuppres-sion by dexamethasone treatment resulted in an initial shift from Th1 to Th2 cytokines. Corticosterone has been shown to suppress cytokine production and to effect a shift in cytokine profiles from Th1 to Th2. Such corticosterone-induced suppression of Th1 cytokines is probably responsible for the reactivation of persistent mycobacteria. Our results are consistent with a previous clinical observation that infection of *M. fortuitum* responds to immunosuppressive conditions.

Thiophene-containing trisubstituted methanes have been shown to exhibit various biological activities; for example, they can act as BACE1 inhibitors, anti-inflammatory agents and anti-HIV protease inhibitors, and have anti-breast cancer, antimalarial and antitubercular activities. On the basis of MIC, cytotoxicity and bactericidal mode of action, four compounds were selected for in vivo activity determination. Bactericidal activity was considered an important parameter because it ensures a rapid reduction in infective load. This is important in the case of NTM infections as they often arise in patients with reduced cell-mediated immunity, where little help is expected in the eradication of the organism by the immune system. Compounds 11 and 18 appear to be very promising as they cleared the infection in mice when administered 1–25 days post-infection, and also showed in vivo efficacy against persistent bacilli strikingly better than moxifloxacin or ofloxacin. These compounds, representing new potential compounds for the treatment of mycobacterial infection, are based on trisubstituted methanes containing thiophene rings. Not only are these compounds effective against *M. fortuitum* in vivo, but they also showed comparable efficacy against other NTM (*M. avium, M. chelonae* and *M. abscessus*) and *M. tuberculosis* (Table 4). NTM have long been neglected in drug discovery efforts and the compounds described in this paper could become possible leads to develop therapeutic agents against *M. fortuitum* and other NTM. However, investigation of their absorption, distribution, metabolism and excretion (ADME) properties would be critical for further evaluation of their clinical potential.

In conclusion, two compounds have been identified that were found to be bactericidal against *M. fortuitum*, other NTM and *M. tuberculosis*. In addition, the results described in this paper present a comprehensive and robust animal model of acute and asymptomatic persistent infection of *M. fortuitum* that can be employed to screen compounds active against replicating and persistent mycobacteria.

**Acknowledgements**

We thank the Director of the Central Drug Research Institute for facilities and support. The mycobacterial strains were obtained from the National JALMA Institute of Leprosy & Other Mycobacterial Diseases. *M. abscessus* was provided by Dr A. Dasgupta. This is CDRI communication number 8177.

**Funding**

The study was supported by ICMR grant 58/16/2009-BMS to R. Srivastava, V. K. K., R. K. G., R. Shrivastava, M. K. P., P. S. and S. B. were recipients of Senior Research Fellowships from the Council of Scientific and Industrial Research (India).
Compounds against replicating and persistent M. fortuitum

Transparency declarations
None to declare.

Supplementary data
Figures S1, S2 and S3 and Table S1, as well as some additional information, are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References