An integrated surrogate model for screening of drugs against 
*Mycobacterium tuberculosis*

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**Objectives:** The intracellularly surviving and slow-growing pathogen, *Mycobacterium tuberculosis*, adapts the host cell environment for its active and dormant life cycle. It is evident that the lack of appropriate high-throughput screening of inhibitors within host cells is an impediment for the early stages of anti-tubercular drug discovery. We aimed to develop an integrated surrogate model that enhances the screening of large inhibitor libraries.

**Methods:** Different mycobacterial species were compared for their growth, drug susceptibility and intracellular uptake. A 6-well plate solid agar-based spot culture growth inhibition (SPOTi) assay was developed into a higher throughput format. The uptake and intracellular survival of *Mycobacterium aurum* within mouse macrophage cells (RAW 264.7) were optimized using 24/96-well plate formats.

**Results:** Fast-growing, non-pathogenic *M. aurum* was found to have an antibiotic-susceptibility profile similar to that of *M. tuberculosis*. The sensitivity to an acidic pH environment and the ability to multiply inside RAW 264.7 macrophages provided additional advantages for employing *M. aurum* in intracellular drug screening methods. A selection of anti-tubercular drugs inhibited the growth and viability of *M. aurum* inside the macrophages at different levels.

**Conclusions:** We present a rapid, convenient, high-throughput surrogate model, which provides a comprehensive evaluation platform for new chemical scaffolds against different physiological stages of mycobacteria within the primary cell environment of the host. The results using anti-tubercular drugs validate this model for screening libraries of existing and novel chemical entities.

**Keywords:** drug susceptibility testing, *Mycobacterium aurum*, RAW 264.7 macrophages, SPOTi assay, surrogate

**Introduction**

Tuberculosis (TB) is an ancient infectious disease that is still responsible for the highest human mortality worldwide from its single causal pathogen, *Mycobacterium tuberculosis*. In spite of intensive efforts towards eradicating TB, it remains a challenging problem, not only for developing countries but also for the developed world. According to the latest data, 1.6 million deaths from TB have been reported. Furthermore, the situation is becoming even more complicated due to issues such as (i) long, complex and ineffective chemotherapy against newly emerging, extensively drug-resistant (XDR)/totally drug-resistant (TDR) TB strains, and (ii) incompatibilities between anti-TB and anti-HIV drugs. Therefore, development of a new and more effective drug treatment against TB is urgently required.

A concerted effort has been recently made worldwide towards developing new anti-TB drugs by different consortia and organizations, such as the TB Alliance (http://www.tballiance.org/), TBIUK (http://www.tbd-uk.org.uk/), Open Source Drug Discovery (OSDD) (http://www.osdd.net/), New Medicines For Tuberculosis (NM4TB) (http://www.nm4tb.org/), the Working Group on New TB Drugs (WGNDB) (http://www.newtbdrugs.org/) and More Medicines for Tuberculosis (MM4TB) (http://www.mm4tb.org/), resulting in a large number of novel chemical libraries in the pipeline waiting for comprehensive evaluation at the preclinical stage of TB drug development.

During infection, *M. tuberculosis* is able to survive inside the host’s primary immune cells even under different stress conditions for an undefined period. Whilst the standard practice for screening inhibitors is to test them against actively dividing bacilli in vitro, most growth inhibitor hits fail at a later stage of the extremely expensive in vivo clinical trials. Furthermore, the slow growth, highly infectious nature of *M. tuberculosis* and the complex infrastructure pose major obstacles towards developing
a comprehensive high-throughput, intracellular, drug screening procedure.

To avoid the use of the highly pathogenic, slow-growing *M. tuberculosis* in the early drug screening process, different mycobacterial species, such as *Mycobacterium smegmatis*, *Mycobacterium fortuitum*, *Mycobacterium phlei*, *Mycobacterium aurum*, *Mycobacterium abscessus*, *Mycobacterium marinum* and *Mycobacterium bovis BCG* have been utilized as surrogates. Of these, the non-pathogenic fast-growing *M. aurum* possesses a similar cell wall profile to *M. tuberculosis*, as well as sharing intracellular therapeutic targets, gene organization and drug resistance patterns. Here, we compared *M. aurum* with *M. smegmatis* mc²155, *Mycobacterium neoaurum* (another species of the *M. aurum* clade) and *M. bovis BCG* for suitability in drug screening systems. *M. smegmatis* mc²155, which does not provide a consistent and reliable model for drug screening, was chosen as a negative control because it does not have the ability to survive inside macrophages.

Mouse macrophage cell line RAW 264.7 has been considered suitable for drug screening because of its high phагocytic activity, adherence capability and ease of culturing. The choice of mouse macrophages is also supported by the wide use of mice as an animal model for *in vivo* anti-TB drug testing. We have previously used the spot culture growth inhibition (SPOTi) assay, a solid agar-based method that has advantages in drug screening over over liquid-based colorimetric assays, and methods that employ cfu counting (the ‘gold standard’ quantification method).

In this study, we have integrated *M. aurum* (a surrogate for *M. tuberculosis*), RAW 264.7 macrophages (surrogates for primary immune cells) and SPOTi (a method for testing inhibitors) to develop a rapid and convenient intracellular-screening platform. This is the first report of such a comprehensive model for testing inhibitor libraries.

In developing the surrogate method for screening novel antimycobacterials against *M. tuberculosis* within a host cell environment, our objectives were: (i) to validate *M. aurum* as a good intracellular model for testing bacteriological inhibitors; (ii) to characterize the uptake and survival of *M. aurum* inside RAW 264.7 macrophage cells; and (iii) to examine the reliability of the screening system using known anti-TB drugs on *M. aurum* inside the RAW 264.7 macrophages.

**Materials and methods**

**Selected anti-TB drugs**

The known anti-TB front-line drugs [isonicotinic acid hydrazide (isoniazid), rifampicin, pyrazinamide and ethambutol] and second-line drugs (ethionamide, p-aminosalicylic acid, cycloserine, streptomycin, ofloxacin, phosphomycin, cephalosporin and metronidazole) were bought from Sigma. Isoniazid, streptomycin and cephalosporin were dissolved in water, and the others were dissolved in DMSO, to a concentration of 50 g/L. All antibiotics were sterilized using 0.2 μm filters (Sartorius). All chemicals were purchased from Sigma, unless otherwise stated.

**Growth and maintenance of mycobacterial species**

Biosafety level 2 mycobacterial species *M. neoaurum* (NC 10440) and *M. aurum* (NC 10437) were purchased from the UK National Collection of Type Cultures (NCTC). These mycobacterial cells, *M. smegmatis* mc²155 (ATCC 700084) and *M. bovis BCG* Pasteur (ATCC 35734) were grown in Middlebrook (MB) 7H9 medium (BD Biosciences) enriched with 10% (v/v) albumin/dextrose/catalase (ADC; BD Biosciences) for liquid growth, and in MB7H10 (BD Biosciences) with 10% (v/v) oleic acid/albumin/dextrose/catalase (OADC; BD Biosciences) for solid agar growth at 37°C. Stock cultures of log-phase cells were maintained in glycerol (25% final concentration of glycerol) at −80°C.

**Development of a higher throughput extracellular SPOTi assay**

The solid agar-based MIC-determining SPOTi screening method was extended from a 6-well to a 24-well format. Log-phase cultures of *M. smegmatis* mc²155, *M. neoaurum*, *M. aurum* and *M. bovis BCG* (Optical Density (OD)₆₀₀ ≈1) were first checked for quality control using cold Ziehl–Neelsen (ZN) staining (also called ‘acid fast staining’); TB-colour staining kit, BDH/Merck) according to the manufacturer’s protocol. Then 10³ cells (2 μL) were placed with 2 mL of MB7H10/OADC (containing antibiotics at 0, 0.39, 1.5, 6.25, 25 or 100 mg/L) in each well of a 24-well Costar plate (Appleton Woods). In order to obtain accurate MIC values, the MICs determined from the first set of experiments were used to select a narrower concentration range in a subsequent experiment. The cell growth at 37°C was recorded after 2 days for *M. smegmatis* mc²155, 3 days for *M. neoaurum*, 5 days for *M. aurum* and 14 days for *M. bovis BCG*. Wells with DMSO or H₂O were used as negative controls.

**Growth inhibition of mycobacteria at different pH**

*M. neoaurum* and *M. aurum* cultures (OD₆₀₀ ≈1.0) were harvested, and 2 μL of cells (undiluted, ×10 and ×100 diluted) were spotted on Petri dishes containing Sauton’s agar medium. The Sauton’s agar medium was prepared by adding 4.0 g L-asparagine, 2.0 g citric acid, 0.5 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.05 g ferric ammonium citrate, 15.0 g agar and 60 mL glycerol in deionized water. It was adjusted to different pH values (5.5, 5.8, 6.0, 7.0 and 7.2) using 5 M NaOH. The volume was adjusted to 1 L and then sterilized. To observe the antibiotic susceptibility under acidic stress, *M. neoaurum* cells (OD₆₀₀ ≈1.0) were washed with PBS (pH 7.0) twice before being stressed by placing in acidic Sauton’s liquid for 1 h at 37°C. Stressed cells (2 μL, 10³ cells/mL) were spotted onto 24-well plates containing Sauton’s agar medium at pH 5.5, 5.8, 6.0 and 7.0 with antibiotics at 0, 0.39, 1.5, 6.25, 25 and 100 mg/L.

**Growth and maintenance of mouse macrophage cell line (RAW 264.7)**

RAW 264.7 cells (NCTC #91062702) were maintained in 25 or 75 cm² tissue culture flasks (BD Biosciences) containing RPMI-1640 complete medium (RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (37°C, humidified 5% CO₂)), and passaged twice before the assay. Before passage, cells were washed twice with 1× PBS to remove unattached cells. Adhered cells were detached using a lidocaine/EDTA mixture (10 mM lidocaine HCl, 10 mM EDTA in 1× PBS) at room temperature for 10 min followed by hitting the side of the flask against the palm of the hand, and then diluting with an equal volume of fresh medium. Cells were then centrifuged at 1000 g for 5 min and resuspended in fresh medium. The number of viable cells was counted using a Trypan Blue assay. Stock cultures of the cells were maintained at −80°C by adding actively growing cells to an equal volume of Bambanker Cell Freezing Media (Anachem).

**Cell toxicity assay using RAW 264.7 macrophages**

The assay was performed in 96-well cell culture flat-bottom plates (Costar; Appleton Woods) in triplicate. First, 20 μL samples of the 50 g/L
stock solutions of antimicrobial compounds were placed in wells containing 200 μL of RPMI-1640 complete medium in the first row, and then 2-fold serial dilution was done six times (the last well did not contain any antibiotic and was used as the control). To each well, 100 μL of diluted macrophage cells (5×10^5 cells/mL) was added. After 48 h of incubation, the monocytes were washed twice with PBS, and fresh RPMI-1640 complete medium was added. Plates were then treated with 30 μL of a freshly prepared 0.01% resazurin solution and incubated overnight at 37°C. The following day the change in colour was observed and the fluorescence intensity was measured (λex=560 nm, λem=590 nm, FLUOstar OPTIMA microplate reader; BMG LABTECH GmbH). The 50% growth inhibitory concentration (GIC50) was determined based on a resazurin fluorescence assay, and the selectivity index (SI) was determined, as the ratio between the GIC50 against RAW 264.7 macrophages and the MIC against M. aurum, for all tested drugs.

**Optimizing the uptake of mycobacteria in RAW 264.7 macrophages**

M. smegmatis mc²155, M. neoaurum, M. aurum and M. bovis BCG were labelled with fluorescein 5(6)-isothiocyanate (FITC) by adding 10^7–10^9 cells to 1 mL of 0.01% FITC in 0.5 M carbonate/bicarbonate buffer pH 9.5 (0.5 M Na2CO3, 1 volume; 0.5 M NaHCO3 3 volumes) and incubating for 30 min at 25°C.20 The bacteria were then washed twice in RPMI-1640 complete medium to remove surplus dye and suspended in RPMI-1640 complete medium. Macrophages (100 μL, 1×10^6 cells/mL) in a 96-well clear-bottom black plate (Corning, Appleton Woods) were infected with an equal number of unlabelled and labelled bacilli at 10:1 multiplicity of infection (moi) ratio. At 2 h post-infection, the infected macrophages were washed with PBS containing 0.05% Tween 80, and this was followed by a wash with PBS alone. Cells were then suspended in 100 μL of PBS and the fluorescence was measured (λex=485 nm, λem=520 nm). For optimizing the uptake of M. aurum, macrophage cells were infected with unlabelled and labelled bacilli at different mois (5:1, 10:1 and 20:1) 2 h post-infection, and at different time-points (0, 30, 60 and 120 min) with a fixed moi (10:1). Only-macrophages and only-bacteria wells were used as controls, and only-PBS/RPMI-1640 was used as a blank. Fluorescence per μg of protein was calculated by equating the fluorescence intensity with protein concentration of the respective well. The protein concentration was measured using the Bradford colorimetric assay.

**Optimizing the infection of M. aurum in RAW 264.7 macrophages**

A macrophage cell suspension was adjusted to 1×10^6 cells/mL, and 2 mL was added onto circular coverslips (Appleton Wood) of 6-well cell culture flat-bottom plates. After 15 min of incubation, 10, 20 or 40 μL of mid-log phase M. aurum cells (1×10^5 cells/mL) harvested in RPMI-1640 complete medium were added to each well at different mois (5:1, 10:1 and 20:1) 2 h post-infection, and at different times (30, 60 and 120 min) with a fixed moi (10:1). Treated coverslips were washed thrice with RPMI-1640 medium containing 0.05% Tween 80 and assessed by ZN staining using a TB-colour staining kit. Co-infection was optimized by counting bacteria per macrophage cell under a bright field microscope (Zeiss) with coverslips mounted onto slides. Acid-fast-stained phagocytozed mycobacteria images were captured at ×1000 magnification.

**Optimizing the survival of M. aurum in RAW 264.7 macrophages**

For the survival assay, macrophages cells infected with M. aurum were lysed at different times (0, 24, 48 and 72 h) in distilled water at room temperature for 10 min followed by scrubbing using a syringe plunger. The lysed cells were spread onto plates containing MB7H10 OADC agar, and then incubated at 37°C to determine the cfu.

**Development of 24-well plate SPOTi assay with RAW 264.7 macrophages**

For the killing assay, macrophages (5×10^5 cells per well) were infected with M. aurum at 10:1 moi at 1 h at 37°C in a 24-well plate. The culture was washed with RPMI-1640 thrice and incubated with different concentration of inhibitors (0, 0.39, 1.5, 6.25, 25 and 100 μg/L) in RPMI-1640 complete medium. Inhibitors were incubated for different times (2 h, 24 h, 48 h and 72 h). Cells were then washed twice with RPMI-1640 and lysed in 500 μL of distilled water at room temperature for 10 min. The lysed cells were centrifuged and suspended in 50 μL of distilled water. Then, 5 μL was spotted onto wells of a 24-well plate containing MB7H10/OADC/agar and incubated at 37°C for 5 days to determine intracellular survival.

**Results**

**M. aurum as a bacteriological model for testing antimycobacterials**

**Comparative growth curves of mycobacterial species**

Based on cell morphology, generation time, antibiotic susceptibility and intracellular survival ability, we compared M. smegmatis mc²155, M. neoaurum, M. aurum and M. bovis BCG in order to establish a bacteriological model for this study. The mycobacterial species were first observed for their morphology using cold ZN staining. M. neoaurum and M. aurum showed a similar pattern to M. bovis BCG, while M. smegmatis mc²155 cells were more elongated (acid fast-stained cells at mid-log phase; Figure 1). The mycobacterial species were also studied for their physiological properties by plotting their growth curves (OD₆₀₀ against time). As both M. neoaurum and M. aurum are scotochromogenic species, a spectrum analysis was performed to exclude possible interference by chromogenic substrate at 600 nm. The growths of mycobacterial species were observed at three different growth rates: active, moderate, and slow. Although M. smegmatis mc²155 and M. neoaurum showed the fastest growth, comparative growth curves showed that M. aurum also take less time to enter the log phase compared with M. bovis BCG (Figure 1). For M. aurum, log-phase cells were collected after 36 h, SPOTi assays were performed after 5 days and colonies were obtained on plates after 8 days; for M. bovis BCG the corresponding values were 3–4 days, 14 days and 28 days (Table 1).

**Growth of mycobacterial species in the presence of anti-TB drugs (MIC)**

A solid agar-based SPOTi assay was performed in a 24-well plate format (Figure 2). The MICs of known antibiotics were determined for complete inhibition for M. smegmatis mc²155, M. neoaurum, M. aurum and M. bovis BCG. When we tested MICs of known anti-TB drugs at or below 100 μg/mL values (extracellular), 5 out of 12 antibiotics were not active against M. smegmatis mc²155, while 4 were not active against M. neoaurum and only 3 were not active against M. aurum, M. bovis BCG and M. tuberculosis H37Rv (Table 2). In comparison with M. smegmatis mc²155...
and *M. neoaurum*, the extracellular MICs of each of 9 of the 12 tested anti-TB drugs were within narrow ranges for *M. aurum*, *M. bovis* BCG and *M. tuberculosis* H37Rv: isoniazid (0.02–0.4 mg/L), rifampicin (0.1–0.5 mg/L), ethambutol (0.47–2.0 mg/L), ethionamide (0.6–5.0 mg/L), *p*-aminosalicylic acid (1.0 mg/L), *d*-cycloserine (5.0–25 mg/L), streptomycin (0.1–1.0 mg/L), ofloxacin (0.2–2.0 mg/L) and cephalosporin (25 mg/L), while the other three, phosphomycin, metronidazole and pyrazinamide, were not active (Table 2).

### Tolerance of mycobacterial species to pH
In order to select an intracellularly survivable bacteriological model, *M. neoaurum* and *M. aurum* were analysed at acidic pH.
for their survival ability, and the results were compared with earlier reports for M. smegmatis mc²155, M. bovis BCG and M. tuberculosis H37Rv.²⁴ We found that, similarly to M. bovis BCG and M. tuberculosis H37Rv, M. aurum was sensitive to acidic pH (Figure 3b): growth was undetected at ≤ pH 6.0, and full growth was seen only at pH 7.2 (Figure 3b). In contrast, similarly to M. smegmatis mc²155, M. neoaurum was not sensitive to pH 5.8, 6.0, 7.0 or 7.2, but we observed retarded growth at pH 5.5 (Figure 3a). We also performed the anti-TB drug susceptibility test against M. neoaurum in acidic stress conditions (pH 5.5–7.0) and found that the rifampicin, D-cycloserine and cephalosporin had increased effectivity in acidic conditions (Table 3), which indicates higher antibiotic susceptibility at low pH. We also observed modulation in MICs between an enriched medium (MB7H10) and minimal medium (Sauton’s) (Tables 2 and 3). Rifampicin, p-aminosalicylic acid, cephalosporin and ofloxacin were more active against M. neoaurum in enriched medium while isoniazid was more active against M. neoaurum in minimal medium.

RAW 264.7 for an ex vivo infection model

We observed that monolayers of RAW 264.7 spread uniformly, adhered well and started differentiating into active macrophages within 15 min. The 80% viability of macrophage cells was determined by using a Trypan Blue assay.

Toxicity of anti-TB drugs against RAW 264.7

Macrophages in logarithmic growth phase were used for testing the cytotoxicity of various antibiotics against RAW 264.7 macrophage cells before testing in intracellular inhibition. All anti-TB drugs were found to be non-toxic to RAW 264.7 macrophages, as determined by GI₅₀ values (cephalosporin 500 mg/L, rifampicin 700 mg/L, ethionamide 1000 mg/L, ofloxacin 1500 mg/L and others ≥3000 mg/L), resulting in SI values >20 for all drugs tested (Table 4).

Killing of M. aurum within RAW 264.7 macrophages

Intracellular survival was evaluated by cfu counting in the absence or presence of a subinhibitory concentration of isoniazid (Figure 4d). M. aurum was able to survive intracellularly and entered into logarithmic growth phase in 48 h, with a 10-fold increase in bacilli in the following 24 h (Figure 4d). Intracellular growth inhibition of M. aurum was observed at different time intervals in the presence and absence of front-line anti-TB drugs.

Uptake of mycobacterial species into RAW 264.7 macrophages

When phagocytosis of M. smegmatis mc²155, M. neoaurum, M. aurum and M. bovis BCG were compared by an FITC fluorescence uptake assay (2 h infection time), we found that M. aurum had similar uptake to M. bovis BCG, while M. smegmatis mc²155 and M. neoaurum had 3–5 times lower uptake (Figure 4c). This further supported the selection of M. aurum as a fast-growing mycobacterial model organism for drug screening.

Infection of M. aurum within RAW 264.7 macrophages

A small aliquot of M. aurum culture was used for screening against extracellular bacilli by performing a SPOTi assay, and the remainder was used further to optimize the uptake and killing assay. Uptake of M. aurum was assessed using ZN staining of infected and non-infected macrophages, and by a quantitative fluorescence assay. Staining results showed the presence of engulfed pink (carbol-fuchsin-stained) M. aurum inside blue (methylene-blue-stained) macrophage cells (Figure 5). Phagocytosis was observed between 30 min and 2 h (moi 10:1), while increasingly clumped bacilli were seen with increasing moi at fixed timepoints (Figure 5).

Quantitative analysis of unlabelled and FITC-labelled M. aurum-infected macrophages showed that maximum uptake was observed at 10:1 moi and 1 h infection time (Figure 4a and b).
## Table 2. Comparison of extracellular MICs of anti-tubercular drugs for *M. smegmatis mc²155, M. neoaurum, M. aurum* and *M. bovis* BCG using the SPOTi assay in MB7H10 medium and *M. tuberculosis* H37Rv (acquired from the literature)

<table>
<thead>
<tr>
<th>Anti-TB drugs</th>
<th>Structure</th>
<th>Mol. weight (g/M)</th>
<th>M. smegmatis mc²155</th>
<th>M. neoaurum</th>
<th>M. aurum</th>
<th>M. bovis BCG</th>
<th>M. tuberculosis H37Rv</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td><img src="https://example.com/INH.png" alt="INH structure" /></td>
<td>137.14</td>
<td>5 (36.46)</td>
<td>2 (14.58)</td>
<td>0.40 (3.65)</td>
<td>0.10 (0.73)</td>
<td>0.02–0.2 (0.15–1.46)</td>
</tr>
<tr>
<td>RIF</td>
<td><img src="https://example.com/RIF.png" alt="RIF structure" /></td>
<td>822.94</td>
<td>8 (9.72)</td>
<td>1 (1.22)</td>
<td>0.10 (0.12)</td>
<td>0.50 (0.61)</td>
<td>0.10 (0.12)</td>
</tr>
<tr>
<td>PYZ</td>
<td><img src="https://example.com/PYZ.png" alt="PYZ structure" /></td>
<td>123.11</td>
<td>&gt;100 (&gt;812.26)</td>
<td>&gt;100 (&gt;812.26)</td>
<td>&gt;100 (&gt;812.26)</td>
<td>&gt;100 (&gt;812.26)</td>
<td>&gt;100 (&gt;812.26)</td>
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<tr>
<td>ETM</td>
<td><img src="https://example.com/ETM.png" alt="ETM structure" /></td>
<td>204.31</td>
<td>2 (9.79)</td>
<td>10 (48.95)</td>
<td>0.5 (2.45)</td>
<td>2 (9.79)</td>
<td>0.47 (2.30)</td>
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<tr>
<td>ETH</td>
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<td>166.24</td>
<td>50 (300.76)</td>
<td>100 (601.53)</td>
<td>5 (30.08)</td>
<td>5 (30.08)</td>
<td>0.6–2.5 (3.60–15.04)</td>
</tr>
<tr>
<td>PAS</td>
<td><img src="https://example.com/PAS.png" alt="PAS structure" /></td>
<td>153.14</td>
<td>&gt;100 (&gt;653.02)</td>
<td>&gt;100 (&gt;653.02)</td>
<td>1 (6.53)</td>
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<td>1 (6.53)</td>
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<tr>
<td>DCS</td>
<td><img src="https://example.com/DCS.png" alt="DCS structure" /></td>
<td>102.09</td>
<td>75 (734.63)</td>
<td>25 (244.88)</td>
<td>25 (244.88)</td>
<td>5 (48.98)</td>
<td>5–25 (48.97–244.88)</td>
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<tr>
<td>STR</td>
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<td>581.57</td>
<td>1 (1.72)</td>
<td>0.4 (0.69)</td>
<td>0.2 (0.34)</td>
<td>1 (1.72)</td>
<td>0.1–0.5 (0.17–0.86)</td>
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<td>OFX</td>
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<td>361.37</td>
<td>1 (2.77)</td>
<td>0.2 (0.55)</td>
<td>0.2 (0.55)</td>
<td>1 (2.77)</td>
<td>1–2 (2.77–5.53)</td>
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<td>&gt;200 (&gt;1448.66)</td>
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<td>CEP</td>
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<td>478.79</td>
<td>&gt;100 (208.86)</td>
<td>50 (104.43)</td>
<td>25 (52.21)</td>
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<td>&gt;200 (&gt;1168.56)</td>
<td>&gt;200 (&gt;1168.56)</td>
</tr>
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</table>

INH, isoniazid; RIF, rifampicin; PYZ, pyrazinamide; ETM, ethambutol; ETH, ethionamide; PAS, *p*-aminosalicylic acid; DCS, d-cycloserine; STR, streptomycin; OFX, ofloxacin; PHO, phosphomycin; CEP, cephalosporin; MET, metronidazole. MIC was determined for complete inhibition.
Figure 3. Growth of (a) *M. neoaurum* and (b) *M. aurum* in plates containing Sauton's medium of different pH (5.5–7.2). The assay was performed with three biological replicates.

**Table 3.** MICs of anti-TB drugs for *M. neoaurum* in acidic environments using Sauton's medium

<table>
<thead>
<tr>
<th>Anti-TB drug</th>
<th>Drug targets</th>
<th>pH 5.5</th>
<th>pH 5.8</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>mycolic acid biosynthesis</td>
<td>0.39 (2.84)</td>
<td>0.39 (2.84)</td>
<td>0.39 (2.84)</td>
<td>0.39 (2.84)</td>
</tr>
<tr>
<td>RIF</td>
<td>RNA synthesis</td>
<td>0.39 (0.47)</td>
<td>0.39 (0.47)</td>
<td>1.56 (1.86)</td>
<td>1.56 (1.86)</td>
</tr>
<tr>
<td>PYZ</td>
<td>fatty acid synthesis pathway</td>
<td>&gt;100 (&gt;812.26)</td>
<td>&gt;100 (&gt;812.26)</td>
<td>&gt;100 (&gt;812.26)</td>
<td>&gt;100 (&gt;812.26)</td>
</tr>
<tr>
<td>DCS</td>
<td>peptidoglycan biosynthesis</td>
<td>6.25 (61.22)</td>
<td>6.25 (61.22)</td>
<td>25 (244.87)</td>
<td>25 (244.88)</td>
</tr>
<tr>
<td>PAS</td>
<td>tetrahydrofolate pathway and mycobactin biosynthesis</td>
<td>0.39 (2.55)</td>
<td>0.39 (2.55)</td>
<td>0.39 (2.55)</td>
<td>0.39 (2.55)</td>
</tr>
<tr>
<td>CEP</td>
<td>penicillin binding proteins</td>
<td>100 (208.86)</td>
<td>&gt;100 (&gt;208.86)</td>
<td>&gt;100 (&gt;208.86)</td>
<td>&gt;100 (&gt;208.86)</td>
</tr>
<tr>
<td>ETH</td>
<td>mycolic acid biosynthesis</td>
<td>100 (601.53)</td>
<td>100 (601.53)</td>
<td>100 (601.53)</td>
<td>100 (601.53)</td>
</tr>
<tr>
<td>OFX</td>
<td>DNA gyrase/topoisomerase</td>
<td>0.39 (1.08)</td>
<td>0.39 (1.08)</td>
<td>0.39 (1.08)</td>
<td>0.39 (1.08)</td>
</tr>
</tbody>
</table>

INH, isoniazid; RIF, rifampicin; PYZ, pyrazinamide; DCS, D-cycloserine; PAS, *p*-aminosalicylic acid; CEP, cephalosporin; ETH, ethionamide; OFX, ofloxacin.

**Table 4.** Comparison of extracellular and intracellular MICs for *M. aurum*, 50% eukaryotic cell growth inhibitory concentrations (GIC50s) and intracellular SIs (GIC50/ex vivo MIC) for different anti-TB drugs

<table>
<thead>
<tr>
<th>Anti-TB drug</th>
<th>extracellular MICs, mg/L (µM)</th>
<th>intracellular MICs, mg/L (µM)</th>
<th>GIC50s, mg/L (µM)</th>
<th>Intracellular SIs</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>0.39 (2.84)</td>
<td>0.39 (2.84)</td>
<td>3000 (21875.62)</td>
<td>7692.31</td>
</tr>
<tr>
<td>RIF</td>
<td>0.10 (0.12)</td>
<td>0.39 (0.47)</td>
<td>700 (850.61)</td>
<td>1794.87</td>
</tr>
<tr>
<td>PYZ</td>
<td>&gt;100 (100)</td>
<td>&gt;100 (100)</td>
<td>3000 (24367.86)</td>
<td>&lt;30</td>
</tr>
<tr>
<td>DCS</td>
<td>25 (244.87)</td>
<td>25 (244.87)</td>
<td>3500 (34282.80)</td>
<td>140</td>
</tr>
<tr>
<td>PAS</td>
<td>1 (6.53)</td>
<td>100 (65)</td>
<td>3000 (19590.56)</td>
<td>30</td>
</tr>
<tr>
<td>CEP</td>
<td>25 (52.21)</td>
<td>25 (52.21)</td>
<td>500 (1044.30)</td>
<td>20</td>
</tr>
<tr>
<td>ETH</td>
<td>5 (30.08)</td>
<td>6.25 (37.59)</td>
<td>1000 (6015.25)</td>
<td>160</td>
</tr>
<tr>
<td>ETM</td>
<td>0.5 (2.45)</td>
<td>0.39 (1.91)</td>
<td>3000 (14683.57)</td>
<td>7692.31</td>
</tr>
<tr>
<td>STR</td>
<td>0.2 (0.34)</td>
<td>1.56 (2.68)</td>
<td>3500 (6018.15)</td>
<td>2243.59</td>
</tr>
<tr>
<td>OFX</td>
<td>0.2 (0.55)</td>
<td>1.56 (4.32)</td>
<td>1500 (4150.89)</td>
<td>3846.15</td>
</tr>
</tbody>
</table>

INH, isoniazid; RIF, rifampicin; PYZ, pyrazinamide; DCS, D-cycloserine; PAS, *p*-aminosalicylic acid; CEP, cephalosporin; ETH, ethionamide; ETM, ethambutol; STR, streptomycin; OFX, ofloxacin.
Figure 4. Uptake fluorescence assay using FITC-labelled mycobacteria. (a) Uptake of M. aurum at different timepoints. (b) Uptake of M. aurum at different moi. (c) Different species uptake in 2 h and 10:1 moi. (d) Intracellular survival of M. aurum in the presence and absence of isoniazid (INH). Each bar represents the mean value of fluorescence from three independent experiments. The standard deviation of the mean for each is shown as an error bar.
drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) using the SPOTi assay (Figure 6). Significant inhibition by isoniazid and rifampicin was observed after 24 h, and complete inhibition was observed after 48 h. Therefore, a 48 h drug-incubation timepoint was chosen for our model (Figure 7). In conclusion, the \textit{M. aurum} with murine macrophage high-throughput drug-screening model was optimized with the following parameters: 10:1 moi uptake, 1 h infection, and 48 h drug incubation.

Intracellular MICs of all anti-TB drugs (isoniazid, rifampicin, pyrazinamide, \(\beta\)-cycloserine, \(\beta\)-aminosalicylic acid, cephalosporin, ethionamide, ethambutol, streptomycin and ofloxacin) for phagocytosed \textit{M. aurum} were calculated, except phosphomycin and metronidazole, which were not active against extracellular mycobacteria. We found that rifampicin, \(\beta\)-aminosalicylic acid, ethionamide, streptomycin and ofloxacin had slightly higher MIC values when inside the macrophages compared with extracellular values, while ethambutol was more active inside macrophages. It is noteworthy that pyrazinamide was not active and \(\beta\)-aminosalicylic acid was considerably less active against intracellular \textit{M. aurum}, although the extracellular MIC of the latter was 1 mg/L (Table 4).

**Figure 5.** Phagocytosis of \textit{M. aurum} at different moi and times of infection. (a) Control (without infection). (b) 5:1 moi, 2 h of infection. (c) 10:1 moi, 2 h of infection. (d) 20:1 moi, 2 h of infection. (e) 10:1 moi, 1 h of infection. (f) 10:1 moi, 30 min of infection. The assay was performed with three biological replicates.

**Discussion**

During pathogenesis, \textit{M. tuberculosis} cells enter the alveolar macrophage cells of the host, and are often able to survive in a nutrient-deficient, oxygen-deficient, redox-active and highly acidic environment, in various physiological states (replicating, not replicating or slowly replicating) for an undefined period of time. Therefore, it is important to have an inhibitor screening assay for these different physiological bacilli states within the host cell environment. In addition, the solubility, permeability, stability and eukaryotic cell toxicity of novel chemical entities are also relevant for antibacterial selectivity and subsequent progress through the drug development pipeline. Considering these, our study aimed to establish an integrated high-throughput method for testing libraries of compounds for in-cell growth inhibition of mycobacteria.

The use of the fast-growing, non-pathogenic, biosafety level 2 organism \textit{M. aurum} as surrogate model organism provides a rapid, less hazardous and more economic platform for the primary screening of inhibitors. Based on the similarity of its gene organization and enzymatic activity to those of \textit{M. tuberculosis}, recombinant \textit{M. aurum} has already been established as a model for screening inhibitors targeting known pathways. \(15,17\) RAW 264.7 cells were found to have better growth kinetics and ability to phagocyte mycobacteria than other cell lines. \(19\) Our results further support the identification of \textit{M. aurum} as an appropriate screening model for \textit{M. tuberculosis} over other tested fast-growing species, such as \textit{M. smegmatis} mc\(^2\)155 and \textit{M. neoaurum}.
In addition, the adaptation of the SPOTi method to a 24-well plate format provided further saving of resources, including consumption of inhibitors. The direct observation of growth inhibition in the SPOTi method affords reliability and reproducibility of results, superior to other available methods where growth can be compromised by contaminants (radioactivity, fluorescence or colorimetric counts in automated systems). Furthermore, the SPOTi method saves time compared with the authenticated but laborious gold standard method, cfu counting. The compatibility of the SPOTi assay with differing media components and pH in minimal media was shown, and it can be utilized for assaying actively dividing bacilli as well as mycobacteria in various physiological growth conditions, such as the Hu et al.25 models of dormant and drug-tolerant mycobacteria, the Wayne and Hayes26 dormancy model, the oxygen-depletion-induced model27 and the chemostatic model.28 Furthermore, this SPOTi-based model can be used to screen against drug-resistant as well as genetically modified mycobacteria.17,29

Figure 6. Growth inhibition of *M. aurum* inside RAW 264.7 cells at different time intervals for different drug concentrations of front-line anti-TB drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PYZ) and ethambutol (ETM). The assay was done in three biological replicates.

Figure 7. Schematic representation of the surrogate model.
Moreover, the natural pigmentation of \textit{M. aurum} helps to distinguish its spots from mycobacterial contaminants (if present). Extracellular antibiotic-susceptibility profiles of first- and second-line anti-tubercular drugs against \textit{M. aurum} showed close similarity to the MICs for \textit{M. tuberculosis} H37Rv (Table 2). We found that the sensitivity of \textit{M. aurum}, unlike \textit{M. neoaurum} and \textit{M. smegmatis}, to acidic pH is similar to that of \textit{M. tuberculosis} H37Rv (Figure 3). We further justified the use of \textit{M. aurum} for testing of inhibitors within infected macrophages (which are known to be acidic). Moreover, similar uptake of \textit{M. aurum} and \textit{M. bovis} BCG into macrophages (Figure 4c) provided additional validation of \textit{M. aurum} as an appropriate fast-growing, intracellularly surviving surrogate.

The developed model involves screening inhibitors against extracellular bacilli and host macrophages, and then screening against intracellular populations. Using an optimized SPOTi-based \textit{M. aurum} intracellular model, MIC patterns showed differences in drug susceptibility between actively dividing extracellular bacilli and bacilli at different physiological stages inside the host macrophage cells (Table 4). Interestingly, mycobacteria showed no such difference in susceptibility to pyrazinamide, and susceptibility to \textit{p}-aminosalicylic acid was significantly lower inside macrophages.

Pyrazinamide, a front-line anti-TB drug was found to be ineffective against actively dividing mycobacteria in vitro, yet active in a mouse model.\(^\text{30}\) In this study, pyrazinamide was found to be inactive both against \textit{M. neoaurum} grown in culture medium at acidic pH and against \textit{M. aurum} grown inside macrophages. Therefore, the effect of the intracellular environment and the requirement for the presence of other anti-TB drugs for the activation of the pro-drug are still debatable.\(^\text{31,32}\)

\textit{p}-Aminosalicylic acid is a second-line anti-TB drug reported to have more than one intracellular target. As a metabolic analogue, \textit{p}-aminosalicylic acid inhibits the tetrahydrofolate pathway, and as an antisyllate compound, it interferes with the conversion of salicylic acid to carboxymycobactin and mycobactin (iron-oxophores for iron transport). We observed that only \textit{p}-aminosalicylic acid showed a significant difference in activity against \textit{M. neoaurum} when tested in two different culture media (Sauton’s and MB7H10) (Tables 2 and 3). We infer, as MB7H10 medium has 8 times higher iron concentration than Sauton’s medium, that more \textit{p}-aminosalicylic acid is needed to inhibit iron uptake.\(^\text{33,34}\) \textit{p}-Aminosalicylic acid inhibits the uptake of iron by targeting mycobactin. We also found that \textit{p}-aminosalicylic acid was not active against \textit{M. smegmatis mc\(^\text{2}\)155} or \textit{M. neoaurum}, while it was active against \textit{M. aurum} and \textit{M. bovis} BCG (Table 2). In an earlier study it was found that \textit{M. smegmatis mc\(^\text{2}\)155}, unlike \textit{M. tuberculosis} H37Rv or \textit{M. bovis} BCG, has a second, salicylic acid-independent route of iron uptake; therefore, it is less susceptible to \textit{p}-aminosalicylic acid.\(^\text{35}\) Results in this study indicate that \textit{M. aurum} has an iron uptake mechanism similar to that of \textit{M. bovis} BCG and \textit{M. tuberculosis} H37Rv, while the mechanism in \textit{M. neoaurum} is similar to that in \textit{M. smegmatis mc\(^\text{2}\)155}. We have found that \textit{p}-aminosalicylic acid loses its activity against \textit{M. aurum} inside the macrophages; this supports a previous finding where no intracellular activity was detected in a mouse model.\(^\text{36}\)

The integration of \textit{M. aurum}-infected RAW 264.7 mouse macrophage cells with the SPOTi assay to yield a cost-effective and low-hazard microbiology containment system now provides an effective inhibitor screening method to address the large number of compound libraries, thus facilitating early stage anti-TB drug discovery.

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\section*{Transparency declarations}
None to declare.

\section*{References}
\begin{enumerate}
\item Guzman JD, Gupta A, Bucar F \textit{et al}. Antimycobacterials from natural sources: ancient times, antibiotic era and novel scaffolds. \textit{Front Biosci} 2012; 17: 1861–81.
\item Sun D, Scherman MS, Jones V \textit{et al}. Discovery, synthesis, and biological evaluation of piperidinol analogs with anti-tuberculosis activity. \textit{Biorg Med Chem} 2009; 17: 3588–94.
\end{enumerate}


