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A high content screening assay for identifying inhibitors against active and dormant state intracellular *Mycobacterium tuberculosis*

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ABSTRACT

The antitubercular drug development pipeline could start with an *in vitro* investigation of several compounds to examine their effect on active and dormant state *Mycobacterium tuberculosis* (Mtb). However, *in vitro* screening of dormant state bacilli cannot provide enough information on the simultaneous effect of a compound on the host. Therefore, we developed a live cell fluorescence based screening protocol by utilizing the high content system for determining the effect of inhibitors against active and dormant state intracellular mycobacteria. THP-1 macrophages infected with an actively growing and hypoxia derived dormant Mtb culture were standardized to develop the screening protocol. The signal to noise ratio and the Z′ factor of this assay were found to be 7.5–29 and 0.6–0.8, respectively, which confirm the robustness of the protocol. The protocol was then validated with standard inhibitors. This newly developed drug screening assay offers an easy, safe, image based high content screening tool to search for novel antitubercular inhibitors against both active and dormant state intracellular mycobacteria. Therefore, this assay could fill in the gap between *in vitro* and *in vivo* latent tuberculosis drug screening programs.

1. Introduction

Despite the prolonged chemotherapy for *Mycobacterium tuberculosis* (Mtb), a time span of 6–12 months is required to eliminate the infection and cure the disease [Dick, 2001; WHO, 2016]. It is suggested that the immune response forces the Mtb within granulomas to shift into a nonreplicating state, causing a latent tuberculosis infection (LTBI) [WHO, 2016]. Nonreplicating Mtb bacilli under *in vitro* culture conditions are characteristically tolerant to most antitubercular agents, such as isoniazid (INH) and ethambutol (EMB), usually known as dormant bacilli [Sarkar and Sarkar, 2012]. In spite of that, the pathogen acquires a phenotypically drug resistant and nonreplicating state during latent infection and creates a major barrier to curing the disease. The Mtb Bacilli are phagocytosed primarily by alveolar macrophages in the lungs and form granulomas [Manning et al., 2017]. In the active state of disease, infected macrophages and granulomas combat the infection, but bacteria can escape these defenses [Manning et al., 2017]. Since the physiological state of Mtb during infection is likely to differ from that found in standard microbiological broth, it is important to choose assay conditions that more appropriately reflect the *in vivo* conditions. Drug screening assays based on hypoxia and nutrient starvation conditions are well established for identifying dormant state inhibitors [Wayne and Hayes, 1996; Betts et al., 2002]. However, some drugs, such as pyrazinamide (PYZ) and metronidazole (MTZ), are effective at killing dormant Mtb under *in vitro* conditions, but they become less effective against dormant state intracellular mycobacteria, and *vice versa* [Wayne and Hayes, 1996; Christophe et al., 2009; Klinkenberg et al., 2008]. In addition, drugs like INH and PYZ were found to develop drug persistence of the intracellular stage of bacilli, whereas these bacilli remain viable, but unable to grow on solid agar media [Scanga et al., 1999]. Moreover, Mtb can reside in different adverse physiological microenvironments in the host, such as nutrient deprivation, hypoxia, and immune stress, in which these persistent or latent bacilli display phenotypic resistance to some antibiotics [Balganesh et al., 2008]. As a result, it is crucial to have drugs that act against all of these physiologically different intracellular states of the bacilli [Manning et al., 2017]. It is also suggested that survival within the phagosomes of macrophages requires a critical microenvironment to which Mtb adapt and enter a dormant state [Sarkar and Sarkar, 2012]. However, the validated assays available for screening the inhibitors against the intracellular dormant state include the nitrate reductase based macrophage [Sarkar and Sarkar, 2012] and high content based granuloma model [Silva-Miranda

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et al., 2015].

Recently, it has shown that infection with a hypoxia derived dormant culture maintained the nonreplicating dormant state within the intracellular environment [Iona et al., 2012; Mariotti et al., 2013]. Based on a red or green fluorescence reporter, a high content drug screening assay was developed to identify active state inhibitors against intracellular Mtb bacilli [Christophe et al., 2009; Manning et al., 2017; Silva-Miranda et al., 2015]. High content imaging coupled with high throughput screening robots and computer assisted image analysis, known as high content screening (HCS) [Ang and Pethe, 2016]. The HCS technology has several advantages over traditional phenotypic screening approaches: 1) it can be used to screen for drug candidates in physiologically relevant conditions, 2) it allows access to novel chemical entities in both microbes and host cells, 3) it provides visual input about any targeted object, and 4) it can be used to monitor multiple fluorescence based phenotypic assays [Ang and Pethe, 2016; Song et al., 2017]. However, HCS selects the best parameters for the analysis of cellular phenotypes, suggesting that the quantification process is more reliable or robust compared to conventional methods, such as colony forming unit (CFU) counting [Christophe et al., 2009; Silva-Miranda et al., 2015; Song et al., 2017]. Thus, we report here the development of a fluorescence-based high-content drug screening protocol that identifies inhibitors against active and dormant state intracellular Mtb bacilli without affecting the viability of the host macrophages. This assay is now available for large scale screening of compound libraries using physiologically relevant conditions.

2. Materials and methods

2.1. Chemicals and materials

Dubos Broth Base and Middlebrook 7H11 Agar Base were obtained from BD Difco (Sparks, MD, USA) with their respective supplements. Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). 4′,6-Diamidino-2-phenylindole (DAPI, Cat. #D1306) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Unless otherwise specified, phorbol 12-myristate 13-acetate (PMA) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions and dilutions of the inhibitors were prepared either in dimethyl sulfoxide (DMSO; Sigma-Aldrich) or distilled water, filter sterilized, and stored at −20 °C.

2.2. Cell line and bacterial cultures

Human acute monocyte leukemia cell line THP-1, obtained from the National Centre for Cell Science (NCCS) (Pune, Maharashtra, India), was maintained in a 25 or 75 cm^2 tissue culture flask (Eppendorf, Hamburg, Germany) containing an RPMI cell culture medium with 10% heat inactivated FBS. Mtb H37Ra (ATCC 25177), obtained from the Microbial Type Culture Collection (MTCC) (Chandigarh, India). The strain H37 Ra RFP (with plasmid pCHERRY3 containing the RFP gene under a constitutive P*symc*-TetO promoter system) was generated and described earlier [Yeware and Sarkar, 2018]. It was earlier reported that pCHERRY3 plasmid is stable and showed the expression of RFP gene for longer period of time in Mtb (> 3 passage). Also, all colonies derived from the macrophage-passaged bacteria were colored, confirming that the plasmid and RFP expression was stably maintained in absence of antibiotics throughout incubation [Carroll et al., 2010]. This strain was routinely maintained in Dubos albumin broth containing 50 μg/mL hygromycin under shaking at 150 rpm at 37 °C within a refrigerated shaking incubator (Model 431; Thermo Electron Corporation, Waltham, MA, USA). Dormant or nonreplicating phase-2 (NRP-2) mycobacterial cultures were obtained after 12–14 days of growing under hypoxic conditions in a glass tube with a 0.5 head spaces to broth ratio. Both active (aerobically growing) and dormant mycobacterial cultures were passed through a 10–11 μm sterile Whatman filter paper before being used to infect a macrophage.

2.3. Determination of intracellular growth of Mycobacterium tuberculosis H37Ra RFP in THP-1 macrophage cells

THP-1 cells were infected with H37Ra RFP following earlier reported methods [Sarkar and Sarkar, 2012; Iona et al., 2012]. Briefly, THP-1 cells (~5×10^4 cells/well) were seeded on a 96-microwell clear bottom plate (Corning, Sigma) in a final volume of 200 μL of RPMI media with 10–20 nM PMA, and then the plate was incubated overnight at 37 °C with 5% CO_2 and 95% humidity (Eppendorf). After differentiation, macrophages were infected with active or dormant mycobacterial cultures for 10–12 h. The uninfected bacterial cells were removed by washing three times with sterile phosphate buffer saline (PBS) (pH 7.2), and 200 μL of RPMI medium was added in each well. The plates were further incubated, and fluorescence was periodically measured using ArrayScan™ XTI (Thermo Fisher Scientific). Unless otherwise specified, the infected macrophages were lysed using 200 μL of hypotonic buffer (pH 7.4) (10 mM HEPES buffer containing 1.5 mM $MgCl_2$ and 10 mM KCl) in the microplate wells. 100 μL of the lysate was then spread on Middlebrook 7H11 albumin agar plates to obtain the CFU after three to four weeks of incubation at 37 °C.

2.4. Equipment and software

ArrayScan™ XTI Infinity High Content Reader (Cat. #NX100021NF, Thermo Fisher Scientific) was used for fluorescence based image capturing with an X1 CCD 14–1-bit camera with a resolution of 4.54 μm/pixel. The objective used for assay validation was a Zeiss Fluar 5× (0.25 NA). In order to test other magnifications, a 20× (0.75 NA) Plan Apochromat and a 40× (0.75 NA) Plan Neofluar objective lens were used. Images were captured using a wide field fluorescence module with a FITC channel of Ex. 386 nm and a red channel of Ex. 572 nm under a Zeiss Fluor 5× objective lens. The form factor was fixed for the 96-microwell clear bottom plate (Corning, Sigma) using the Thermo Scientific HCS Studio™ Cell Analysis Software (Cellomics).

A customized program was created in the Thermo Scientific HCS Studio Cellomics Scan Version 6.4.3 (Build 7207) tool to analyze the images. A simple threshold module with a range set through 0–65,535 pixel intensities (maximum intensities) was used to define the whole image as in which objects analyzed for both channels. A simple threshold mask was used to measure the sum/average of the integrated intensities of four fields per well for both channels. All plates were analyzed with the same settings through autofocus and auto exposure mode for the test, positive and negative control wells. Features were selected as total/average intensities per well for Object Channel 1 (for macrophage nuclei) and total/average intensities for CirSpot Object Channel 2 (for Mtb RFP). The images were then processed using inbuilt Cellomics Scan software (Version 6.4.3, Build 7207) and the data were exported to Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

2.5. Assay protocol for active and dormant intracellular mycobacterial inhibition

Determination of the minimum inhibitory concentration (MIC) of inhibitors against active and dormant intracellular TB bacilli was carried out in the high content format using 96-well microplates. Briefly, ~5×10^4 THP-1 cells/mL were seeded in each well in 200 μL of RPMI media and 10–20 nM PMA. After differentiation, active and dormant mycobacterial cultures were added to each well at a 1: 1 multiplicity of infection (MOI) (obtained as CFU 4.8 ± 0.04 and 4.69 ± 0.26 $\log_{10}$ bacilli for the active and dormant states, respectively). After 10–12 h of infection, the wells were washed three times with sterile PBS, followed by the addition of 198 μL of RPMI media containing 4–5% FBS. In order to check the effect of the inhibitors on the growth of intracellular

bacilli, 2μL of a solution of two fold serially diluted compounds, starting at the concentration of 100μg/mL to 0.78μg/mL, was added in triplicate and incubated at 37 °C in a CO_2 incubator with 95% humidity. After eight days, ~300–350nM DAPI was added to each well and fluorescence was measured using the ArrayScan™ XTI. The MIC value (defined as a decrease in > 88.9 ± 3.07% and > 82.5 ± 2.03% of red fluorescence intensities compared to untreated control for the intracellular active and dormant state assay, respectively) was determined from the dose response curve of percent inhibition of fluorescence *versus* the concentration of agents using the following formula:

$$\%\text{Inhibition} = \frac{\text{Control-Test}}{\text{Control-blank}} \times 100$$

where "Control" is the relative fluorescence value obtained from the culture with DMSO; "Test" is the fluorescence from a culture with an inhibitor; and "Blank" is the fluorescence signal received from the medium with an inhibitor.

3. Results

3.1. Intracellular growth kinetics of active and dormant state M. tuberculosis

Developing an HCS platform to screen a library of selected inhibitors against active and dormant state mycobacteria at the intracellular stage is necessary for hit validation. It was reported that upon infection of NRP-2 state of Mtb culture to THP-1 derived macrophages maintain the dormancy characteristics of Mtb within intracellular environment [Iona et al., 2012; Mariotti et al., 2013]. Similarly, we have observed that there was no significant increase in either the red fluorescence (relative fluorescence unit, RFU) (1245 ± 85.2 to 1365 ± 138) or the CFU (3.05 ± 0.08 to 3.27 ± 0.04 $\log_{10}$ CFU/mL) of Mtb up to eight days when macrophages were infected with NRP-2-state Mtb bacilli. However, macrophages infected with logarithmic phase of Mtb cells showed an increase in the red fluorescence (RFU) (1138 ± 76.6 to 3924 ± 215) and the CFU (3.46 ± 0.03 to 5.51 ± 0.04 $\log_{10}$ CFU/mL) values up to eight days. This clearly indicated the development of two different physiological states of Mtb in macrophages upon the use of logarithmic and NRP-2-state cultures (Fig. 1).

3.2. Robustness of the assay

The assay used for rapid drug screening of compounds must be statistically robust to guarantee a reliable outcome; this predominantly depends upon the signal to noise (S/N) ratio and the Z′ factor [Zhang et al., 1999]. The S/N ratio calculated for this assay was found to be between 21 and 29 for the active state and between 7.5 and 8.94 for the dormant state (Supplementary Fig. S1A). The Z′ values (Z′ factor = 1 – [3 × standard deviation of control + 3 × standard deviation of blank]/[mean of control − mean of blank]) were found to be varying between 0.61 and 0.81 for both states of the intracellular assay (Supplementary Fig. S1B). The S/N ratio and Z′ factor obtained from these experiments under optimized conditions indicated that the protocol is robust enough to perform HCS of any diverse compound library.

3.3. Validation of assay protocol with known antitubercular drugs

In order to validate the assay, we used standard drugs like rifampicin (RIF), an inhibitor of both active and dormant states, and INH, an inhibitor of only the active state [Lakshminarayana et al., 2015]. It was noticed that six to eight days are required to achieve a > 90% decrease of fluorescence compared to the control (only DMSO) during the incubation of standard inhibitors like RIF, INH, streptomycin (STR), and EMB with active state bacilli during the assay (Fig. 2). Interestingly, the effect of INH was shown only against the active state with an MIC value of < 0.5μg/mL, but it did not exhibit a significant inhibitory effect against dormant state mycobacteria up to 10μg/mL (~ 45.5% inhibition by RFU) (Fig. 2, Supplementary Fig. S2). Similar results were found in the case of EMB when it was incubated with the intracellular dormant state, indicating the maintenance of the dormancy phenotype used in one of the assay plates [Lakshminarayana et al., 2015]. Furthermore, when a correlation between the CFU and RFU was evaluated, we found a > 88.9 ± 3.07% and > 82.5 ± 2.03% reduction in the red fluorescence intensities equal to a 100% reduction in the viability of cells in the active and dormant states, respectively, after treatment with RIF or STR. Thus, here, we defined the MIC value as a decrease in > 88.9 ± 3.07% and > 82.5 ± 2.03% of red fluorescence intensities compared to the untreated control used in the assay (Fig. 3).

In corroboration with the current MIC definition, the MIC values were further determined against standard antibiotics. Cell wall synthesis inhibitors like INH and EMB reduced the load of actively growing bacteria inside the THP-1 cells with an MIC value determined at 0.53 ± 0.04 and 5.83 ± 0.14μg/mL, respectively, along with > 10 and > 25μg/mL obtained against the dormant state, respectively (Table 1). However, D-cycloserine (DCS) did not show any inhibition up to 25μg/mL against both states of the bacilli in this assay. Protein synthesis inhibitors like STR and chloramphenicol (CHL) showed MIC values of 5.59 ± 1.07 and 6.44 ± 0.65μg/mL as well as 11.12 ± 3.35 and 11.64 ± 0.38μg/mL against the active and dormant states, respectively (Table 1). The MIC values obtained against inhibitors of DNA synthesis and repairing enzymes, such as ciprofloxacin (CIP) and ofloxacin (OFX), were 10.18 ± 2.43 and 4.68 ± 2.29μg/mL as well as > 25 and 10.17 ± 2.29μg/mL against the active and dormant states, respectively. Inhibitors from the nitroaromatic family group, such as MTZ, nitrofurantoin (NIT), and furaltadone (FUL), did not show any inhibition till 25μg/mL applied against either of the active and dormant states of intracellular Mtb along with PYZ.

Furthermore, the growth assessed using the CFU indicated the inhibition of intracellular active state Mtb bacilli by RIF, STR, INH, and EMB when used at 2 × MIC (Fig. 2A and B). However, RIF and STR were only found to kill the dormant intracellular Mtb population (> 90%), whereas EMB and INH showed inhibition up to 50–60% even at eight days of incubation (Fig. 2C). This result is consistent with the development of drug tolerance against cell wall inhibitors as one of the characteristic features of dormant mycobacteria [Scanga et al., 1999; Lakshminarayana et al., 2015].

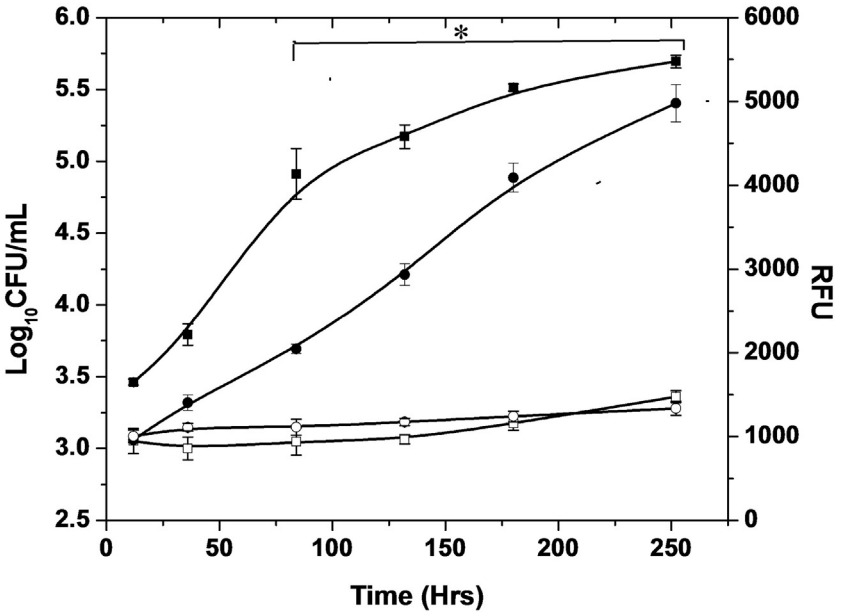
Altogether, the results indicate that RIF performed better than STR as the later acted moderately against dormant state intracellular Mtb. The MIC values of different standard drugs when applied for killing both states of intracellular Mtb confirmed the reliability of the assay protocol.

3.4. Macrophage toxicity analysis by DAPI

DAPI, a live cell permeable and high affinity blue fluorescent nucleic acid stain, is often used to determine the viability of mammalian cells [Cummings and Schnellmann, 2004]. However, we found that SYBR Green I, another DNA binding dye, efficiently stains Mtb nucleic acids (Supplementary Fig. S3), increasing unwanted noise during the cytotoxicity measurements of macrophages [Manning et al., 2017]. On the other hand, DAPI was not found to stain Mtb cells (Supplementary Fig. S3). As a result, the background for both channels remains unchanged during image capturing.

Based on the results of the DAPI staining, the standard anticancer drugs doxorubicin (Dox) (0.05μg/mL) and curcumin (Cur) (3.75μg/mL) were found to estimate < 50% survival of macrophages after incubation for eight days under the assay conditions (Fig. 4). In addition, standard anti Mtb drugs like RIF (200 × MIC) and INH (20 × MIC) showed > 90% survival of macrophages compared to untreated cells. Thus, the use of DAPI staining showed a practical application in this

A)



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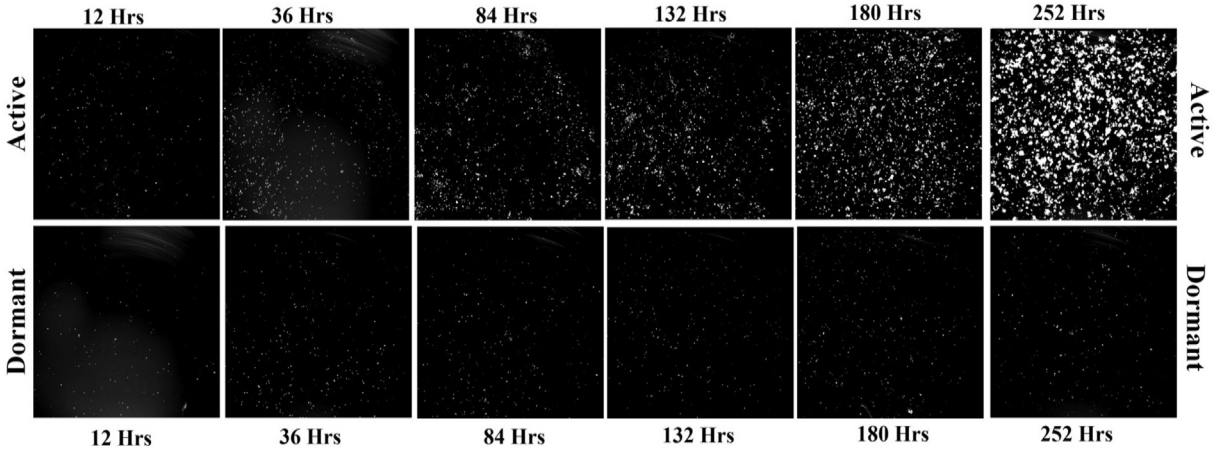

Fig. 1. (A) Kinetics of intracellular survival of active and dormant *Mycobacterium tuberculosis* within THP-1 macrophages. Differentiated macrophages were infected with actively growing and hypoxia-derived Mtb cultures with a 1:1 MOI. After 10 h of incubation, extracellular bacilli were washed with 1× PBS three times and further incubated at 37 °C under a 5% CO_2 atmosphere. Periodically, the red spot intensity (dark circle: active state, open circle: dormant state) was measured (HCS; Thermo Fisher Scientific) and CFU (dark square: active state, open square: dormant state) were determined as described in "Material and Methods" section. Data are represented as the means of triplicate results ± standard deviation (SD) ($p < .05$, $^*p < .005$). **(B) Intracellular fluorescent spot images.** Images were captured during the read and processed using Cellomics software. Each image represents one of the quadrants of the well. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assay to determine simultaneous drug toxicity of THP-1 derived macrophages while estimating any compound inhibition against infected Mtb cells (Fig. 4).

4. Discussion

Treatment of latent TB requires cidal therapeutics to completely eliminate the bacteria hiding in privileged niches (*i.e.*, either granuloma or intracellular stage) [Kaur et al., 2015]. In this report, we have developed the active and dormant states intracellular Mtb drug-screening protocol using HCS tools. In this assay protocols, the efficacy of new compounds can be determined after treating macrophages infected with either a log phase or hypoxia-derived dormant culture of fluorescent Mtb strain (Fig. 1). As reported, macrophages infected with the hypoxia derived dormant cultures also showed maintenance of the nonreplicating state up to eight days (Fig. 1) [Iona et al., 2012; Mariotti et al., 2013]. In addition, RIF showed inhibition against both active and dormant state intracellular Mtb, whereas INH showed inhibition against

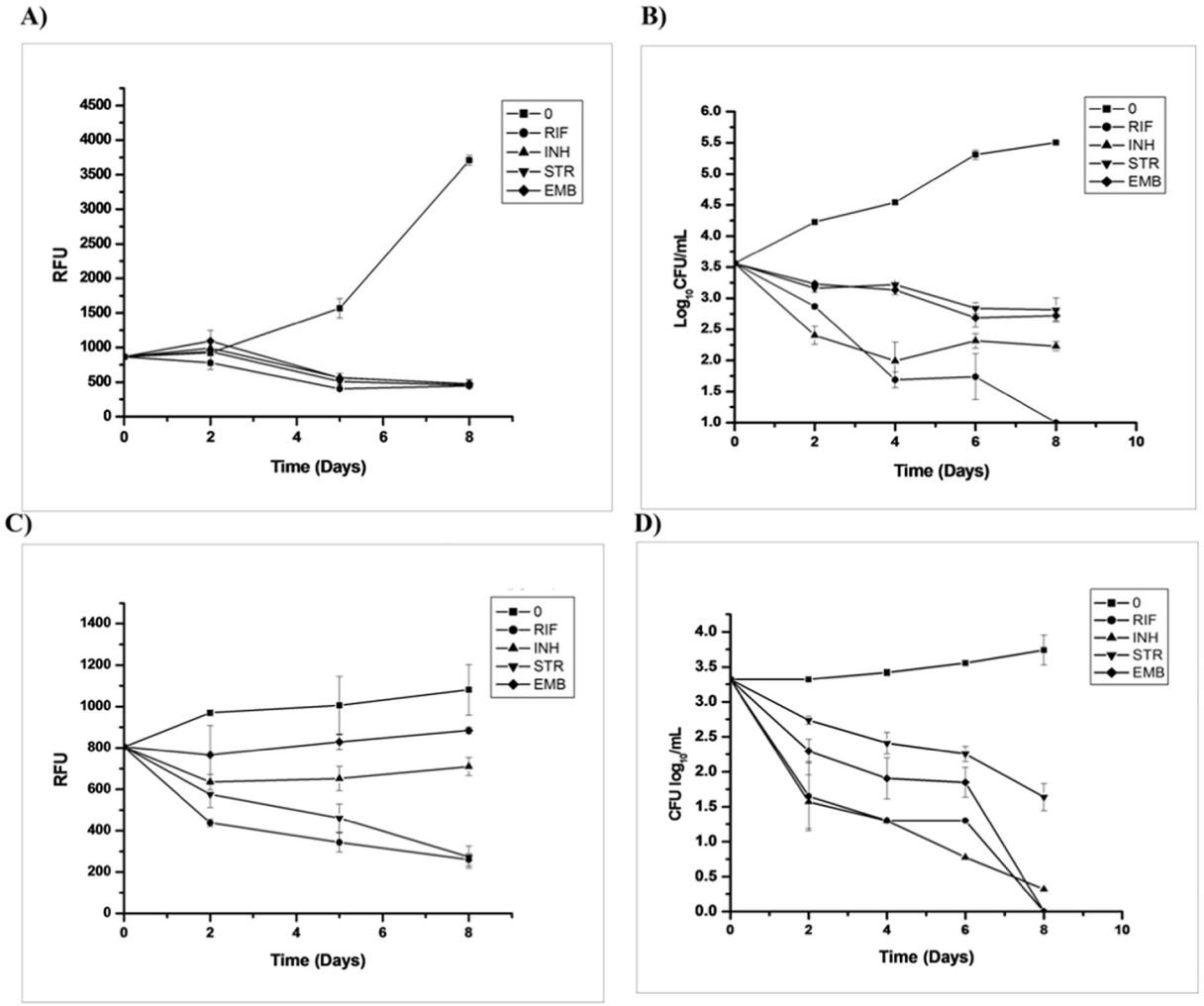

Fig. 2. Inhibition kinetics of standard antitubercular drugs against intracellular bacilli. THP-1 macrophages infected with active and dormant state Mtb, washed with PBS and 2× MIC concentration of each drug added in the respective wells. The plates were further incubated at 37 °C under a 5% CO_2 atmosphere, and the red fluorescence intensities and CFU were measured periodically as described in detail in "Material and method" section. (A) and (B) represent the active state; (C) and (D) represent the dormant state by RFU and CFU respectively. Data is shown as the mean of two identical sets of experiments with triplicates ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

only active state Mtb (Fig. 2A and B). This indicates that these dormant bacilli, even at the intracellular stage, retained their drug tolerance characteristics throughout the duration of the experiment [Lakshminarayana et al., 2015]. However, INH treated intracellular dormant mycobacteria did not show significant inhibition of fluorescence but were found to decrease in CFU counts (Fig. 2D); a similar observation was made by others, indicating conversion to a non cultivable phenotype as explained in an *in vivo* Cornell model [Scanga et al., 1999].

Inhibitors of protein synthesis machinery also showed MIC values within ~10 μg/mL, in agreement with the lesser protein synthesis during the intracellular dormant state of Mtb [Lakshminarayana et al., 2015; Yeware and Sarkar, 2018]. However, the inhibitors of DNA nicking enzymes, such as OFX, also showed inhibition against the dormant state, as reported earlier [Lakshminarayana et al., 2015; Yeware and Sarkar, 2018]. So far, metabolite interrupts like NIT and FUL were not indicated any inhibitory activity against both the states of intracellular Mtb. Nevertheless, the standard drugs RIF, INH, STR, and EMB reflect their activity against active state of Mtb as reported in previous assays [Christophe et al., 2009; Sarkar and Sarkar, 2012].

Multiple fluorescence based HCS phenotypic assays helped monitor the intracellular replication of Mtb as well as their intracellular distribution as reported [Manning et al., 2017; Song et al., 2017]. In this report, we have also investigated parallel macrophage toxicity estimation using DAPI. The emission peak of DAPI is near the blue region, which widely differs from the RFP emission. As a result, noise is significantly reduced (as DAPI does not stain Mtb, probably because of the poor permeability across the Mtb rigid cell wall) compared to SYBR Green I (Supplementary Fig. S3) [Manning et al., 2017]. Thus, using DAPI in combination with an RFP strain could provide better differentiation between the host cell toxicity and inhibition of intracellular bacilli.

High content technology has several advantages over traditional phenotypic screening approaches because it is adapted to identifying and analyzing images. We used two phenotypes to simultaneously monitor and estimate the inhibition of both states of Mtb intracellular as well as the toxicity of the macrophage. These newly developed assays are robust (S/N ratio: > 8, Z′ factor: 0.6–0.8) and can be used for any diverse chemical library in screening programs. However, validation of the intracellular dormant state hits will provide a new insight into TB drug discovery to fight against the latent phenotype of TB.

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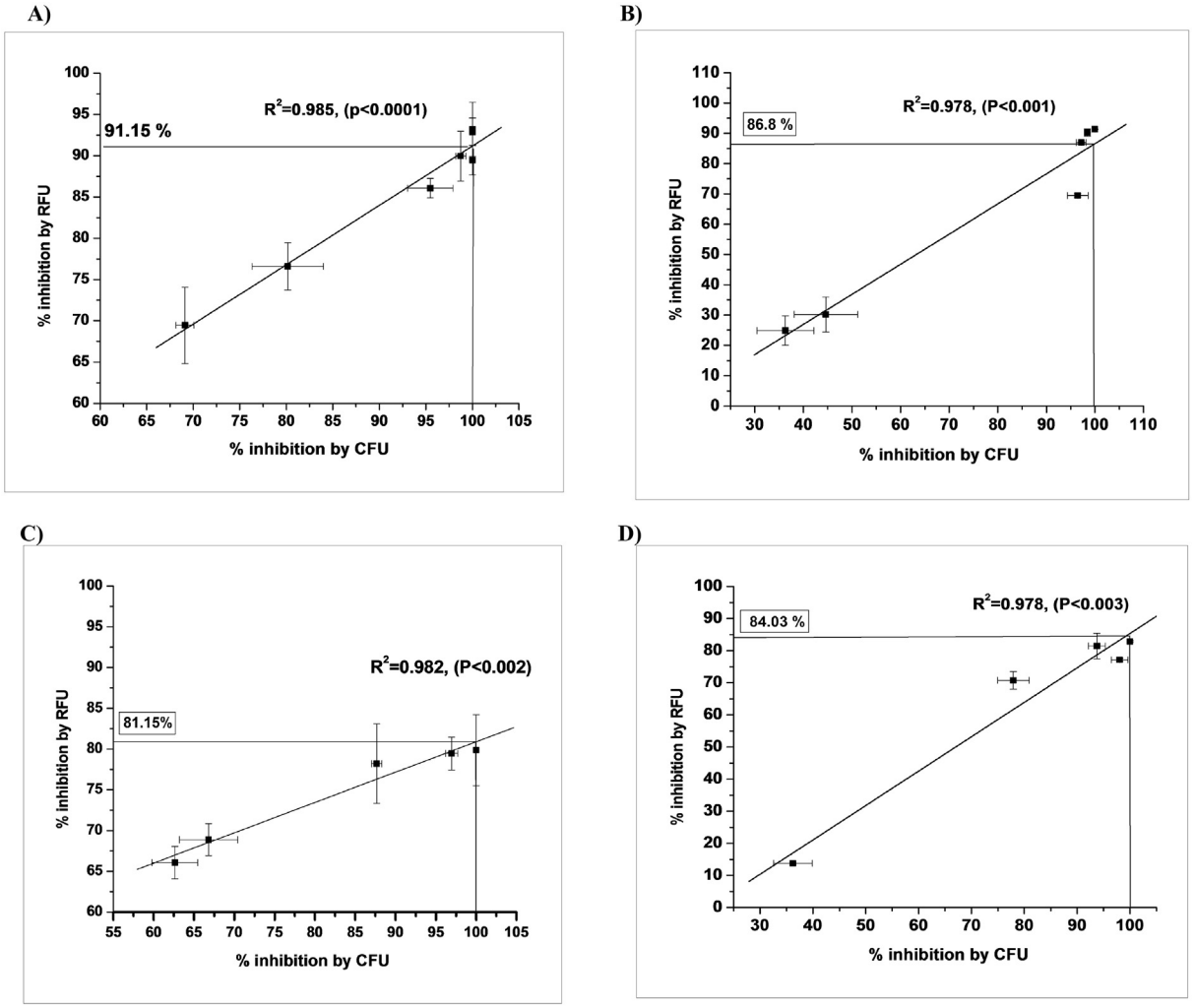

Fig. 3. Correlation between the percent inhibition values of standard drugs determined by RFU and CFU. Active and dormant state intracellular mycobacteria were treated with RIF and STR for eight days, and the percent inhibition of CFU and RFU was calculated as mentioned in "Materials and Method" section (active state: A-RIF and B-STR; dormant state: C-RIF and D-STR).

Table 1
MIC values of standard antitubercular drugs against the active and dormant state intracellular mycobacteria. The MIC value was defined as a decrease in > 88.9 ± 3.07 and > 82.5 ± 2.03% of red fluorescence intensity compared to untreated vehicle control for the intracellular active and dormant state, respectively.

	MIC (μg/mL) by RFP-HCS *ex-vivo* assay		MIC (μg/mL) by Nitrate reduction assay[a]		MIC by reported HCS assay[b]
Agents	Active state	Dormant state	Active state	Dormant state	Active state
Rifampin	0.052 ± 0.0042	0.060 ± 0.031	< 0.07	0.81	2.4
Isoniazid	0.53 ± 0.049	> 10	0.13	> 10	0.16
Streptomycin	5.59 ± 1.07	11.12 ± 3.35	1.05	9.41	> 20
Ethambutol	5.83 ± 0.14	> 25	1.66	> 50	1
Chloramphenicol	6.44 ± 0.65	11.64 ± 0.38	ND	ND	ND
Ciprofloxacin	10.18 ± 2.43	> 25	ND	ND	ND
Ofloxacin	4.68 ± 2.29	10.17 ± 2.29	ND	ND	ND
D-cycloserine	> 25	> 25	> 50	> 50	ND
Pyrazinamide	> 25	> 25	ND	ND	> 20
Metronidazole	> 25	> 25	ND	ND	ND
Nitrofurantoin	> 25	> 25	ND	ND	ND
Furaltadone	> 25	> 25	ND	ND	ND

The MIC values represent the average of three independent experiments carried out using the same protocol. ND: not determined.
[a] Determined using the reference assay of Sarkar and Sarkar, 2012.
[b] MIC value reported by Christophe et al., 2009

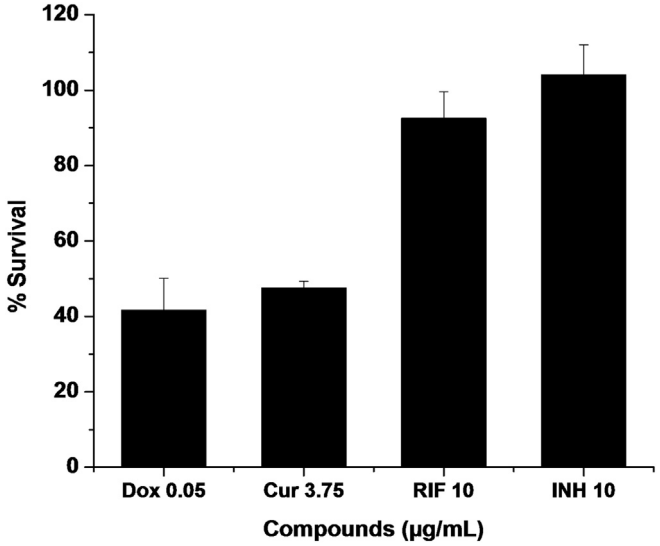

Fig. 4. Analysis of compounds toxicity on macrophages infected with *Mycobacterium tuberculosis* using DAPI staining. Dox (0.05 µg/mL), Cur (3.75 µg/mL), RIF (10 µg/mL), and INH (10 µg/mL) were added to THP-1-derived macrophages in a 96-well plate and incubated for eight days at 37 °C under a 5% CO_2 atmosphere. Fluorescence emitted after staining with DAPI from the nuclei of the macrophages was measured, and the percent survival of the macrophages was calculated and compared with the untreated control. Data are shown as the mean of triplicate results ± SD.

CSC0406.

Author contributions

This study was designed and done by AY and SA under the guidance of DS.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105687.

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