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A high content microscopy assay to determine drug activity against intracellular *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis is one of the infectious diseases with the greatest global burden, affecting millions of people. The rise of multi- and extensively-drug resistant forms of *Mycobacterium tuberculosis* over the last few decades has highlighted the urgent need for development of new drugs to treat the disease. Many drug development pipelines are based on *in vitro* assays examining a compound's effect on *M. tuberculosis* alone. These do not account for the effect of a compound on mammalian cells nor the interaction between host and pathogen. We therefore developed a live-cell fluorescence-based screen utilizing high content microscopy of mammalian macrophages infected with *M. tuberculosis* to screen for compounds with both substantial inhibition of *M. tuberculosis* growth and low cytotoxicity. Isoniazid, a first line tuberculosis drug, and staurosporine, a compound with well documented cytotoxic activity, were used to validate the assay. These and other control compounds showed results for *M. tuberculosis* growth consistent with the field. Together, this method of screening allows for high throughput testing of potential tuberculosis drugs while capturing more information per compound in a physiologically relevant context.

1. Introduction

Tuberculosis is one of the deadliest infectious diseases on a global scale, affecting millions of people predominantly in developing countries [1]. Current disease therapies require patients to take multiple drug combinations for at least 6 months, and often much longer. Due to the difficulty of accessing and adhering to such a demanding regimen, multi-drug and extensively-drug resistant (MDR and XDR) strains have become increasingly prevalent. Treating these resistant forms of tuberculosis requires therapies that are prohibitively expensive for many people and which take multiple years to complete. Unfortunately, some of the newer drugs shown to be effective in treating tuberculosis work on the same pathways as older drugs, making them less beneficial in combating drug resistance [2]. Due to these factors there is a great need for new drugs that target different bacterial proteins and pathways, act faster than current options, and are less toxic to patients.

Mycobacterium tuberculosis, the causative agent of tuberculosis, most often infects humans through inhalation. In the lungs, the bacteria are phagocytosed primarily by alveolar macrophages [3]. In most people the bacteria are cleared or remain in a latent state due to the immune system containing the infection; however, active disease results in about 10% of infected people. Granulomas may be formed to contain bacteria, comprising T- and B-cells surrounding both infected and uninfected macrophages, and a necrotic center where bacteria reside [4]. During active disease, infected macrophages and granulomas combat the infection but bacteria are able to escape these defenses [5]. During these different stages of infection and disease *M. tuberculosis* survives and divides both intracellularly and extracellularly.

Given that *M. tuberculosis* can exist in the body in different physiological states and microenvironments, it is crucial to have drugs that act against all of these [6]. Many high-throughput drug discovery efforts begin by testing compounds *in vitro* against replicating *M. tuberculosis*. This is the easiest method to test large numbers of unknown compounds, but is not always effective at predicting good drug candidates. For example, some well-known antibiotics, such as amikacin, effectively inhibit the growth of extracellular *M. tuberculosis* and have little to no effect on intracellular bacteria at non-cytotoxic concentrations [7]. Since the physiological state of *M. tuberculosis* during infection is likely to differ from that found in standard microbiological broth, it is important to choose assay conditions that reflect the *in vivo* setting. In axenic

Abbreviations: MDR, multi-drug resistant; XDR, extensively drug resistant; INH, isoniazid; STA, staurosporine; CV, coefficient of variation; MOI, multiplicity of infection.

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culture, variations such as changes in carbon source, pH, oxygen tension and immunological mimics such as nitric oxide have been used to identify novel chemotypes [8–10]. In extension from these, the use of intracellular models can be used as a more sophisticated and multifactorial approach than modifying individual medium components. These differences make it crucial to develop more complex screening paradigms to incorporate co-cultures of mammalian cells with bacteria and to maximize the data gathered from one experiment. Additionally, cytotoxicity must always be assessed for any potential drugs. Including it as a criterion during the earliest screening stage has the potential to increase the efficiency of the downstream drug development pipeline.

We developed a screening regimen utilizing high content microscopy that captures multifaceted information without sacrificing throughput. We developed a fluorescence-based, live-cell, high content assay with simultaneous readouts of drug efficacy and cytotoxicity (Fig. 1A). In this assay, the intracellular efficacy of new compounds is determined after treating macrophages infected with fluorescent *M. tuberculosis* for three days with a single concentration of test compound. A live-cell dye is used to stain macrophage nuclei; thus fluorescent bacteria and macrophage nuclei can be imaged with a high-content microscope. Images are processed via an automated scheme and used to calculate inhibition of *M. tuberculosis* and macrophage growth. Using this method, previous results for several cytotoxic and bactericidal reference compounds were recapitulated. This assay is now available for large-scale screening of compound libraries using physiologically relevant conditions.

2. Materials and methods

2.1. RAW 264.7 cell culture

RAW 264.7 murine macrophages were obtained from ATCC (ATCC TIB-71) and maintained at 37 °C with 5% CO_2 and high humidity. Cells were passaged in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate solution, and 2 mM GlutaGro supplement (Fisher Scientific). Cells were recovered from flasks by incubating in Accumax (Milipore) for 20 min at RT with sharp rapping at 5 min intervals. Prior to infection, 2.5×10^7 cells were seeded into a T225 flask and incubated for a minimum of 2 h.

2.2. M. tuberculosis culture

M. tuberculosis H37Rv [20] constitutively expressing DsRed was cultured at 37 °C in Middlebrook 7H9 medium containing 10% v/v OADC (oleic acid, albumin, dextrose, catalase) supplement (Becton Dickinson) and 0.05% w/v Tween 80 (7H9-Tw-OADC) plus 100 μg/mL hygromycin B (Hyg; Roche Diagnostics).

2.3. Preparation of compound plates

Compounds were obtained as powders and solubilized in DMSO (Honeywell). DMSO was used as a minimum inhibition control. For maximum inhibition controls, 10 mM isoniazid (INH; Sigma) and 100 μM staurosporine (STA; Santa Cruz Biotechnology) were prepared in DMSO. Compounds were dispensed into 384-well polypropylene plates (Fisher) using a Biomek 3000 Laboratory Automation Workstation (for control compound and dose response plates; Beckman Coulter) or MultiMek 384 Liquid Handler (for minimum and maximum inhibition plates; Beckman Coulter). Control compound plates were laid out with DMSO in columns 2 and 23, 10 mM INH in wells 1A-1H and 24A-24H, and 100 μM STA in wells 1I-1P and 24I-24P. Dose response plates were made by dispensing compounds in column 3 and 13 and preparing threefold serial dilutions in columns 4–12 or 14–22. Minimum and maximum inhibition plates were made by adding the appropriate compound to each well in columns 3–22.

2.4. Preparation of assay plates

Greiner μClear bottom, black 384-well plates were barcoded. A Multidrop Combi Reagent Dispenser (Thermo) was used to dispense 30 μL cRPMI (RPMI, 5% FBS, 1 mM sodium pyruvate, 2 mM GlutaGro) to each well of the plates. The final FBS concentration

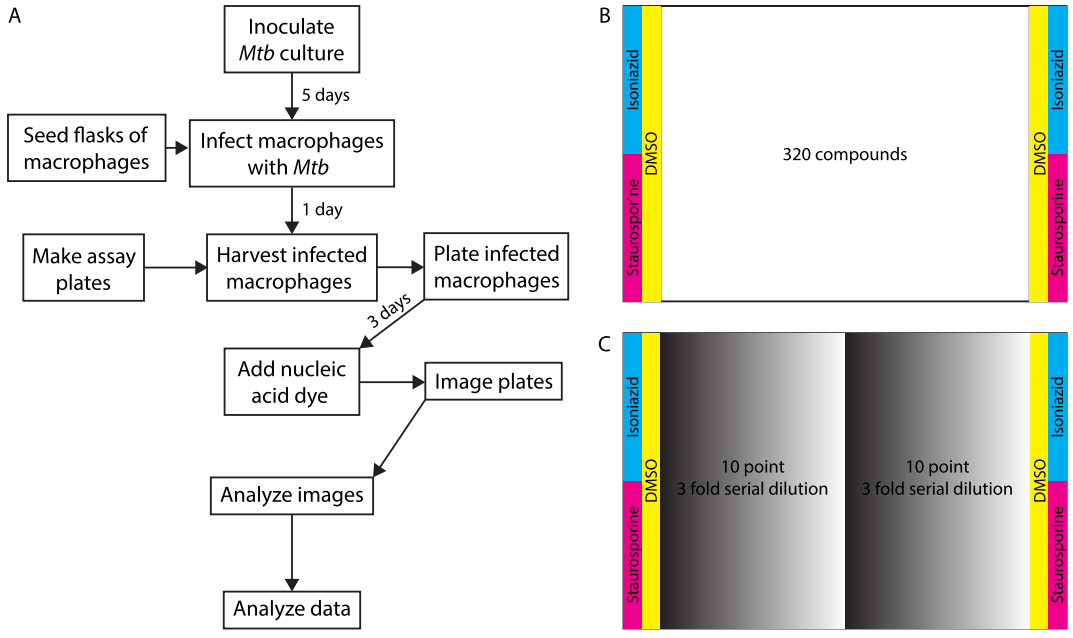

Fig. 1. Assay summary. A. Work flow diagram. B and C. Assay plate maps. All plates have isoniazid and staurosporine controls in two half-columns and DMSO control in two full columns. B. Single point plate map. The 320 middle wells have single concentrations of the same compound. C. Dose response plate map. Columns 3–12 and 13–22 have ten-point threefold serial dilutions of compound with the highest concentration in columns 3 and 13.