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Detection of Inhibitors of Phenotypically Drug-tolerant *Mycobacterium tuberculosis* Using an *In Vitro* Bactericidal Screen

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Many whole cell screens of chemical libraries currently in use are based on inhibition of bacterial growth. The goal of this study was to develop a chemical library screening model that enabled detection of compounds that are active against drug-tolerant non-growing cultures of *Mycobacterium tuberculosis*. An *in vitro* model of low metabolically active mycobacteria was established with 8 and 30 day old cultures of *M. smegmatis* and *M. tuberculosis*, respectively. Reduction of resazurin was used as a measure of viability and the assay was applied in screens of chemical libraries for bactericidal compounds. The model provided cells that were phenotypically-resilient to killing by first and second-line clinical drugs including rifampicin. Screening against chemical libraries identified proteasome inhibitors, NSC310551 and NSC321206, and a structurally-related series of thiosemicarbazones, as having potent killing activity towards aged cultures. The inhibitors were confirmed as active against virulent *M. tuberculosis* strains including multi- and extensively-drug resistant clinical isolates. Our library screen enabled detection of compounds with a potent level of bactericidal activity towards phenotypically drug-tolerant cultures of *M. tuberculosis*.

Keywords: *Mycobacterium tuberculosis*, phenotypic drug tolerance, chemical library screening

Introduction

Tuberculosis (TB) is a leading cause of infectious mortality worldwide, killing approximately 1.4 million people each year (World Health Organisation, 2012). Part of the infection cycle of *Mycobacterium tuberculosis* can involve a prolonged period known as latency, in which the pathogen persists indefinitely in the absence of clinical signs or symptoms of disease (Parrish *et al.*, 1998; Honer zu Bentrup and Russell, 2001; Kaufmann, 2001). Attempts have been made to estimate the number of people who may be latently infected with *M. tuberculosis* with one estimate putting it as high as one third of the world's inhabitants (Dye *et al.*, 1999). This represents a vast reservoir for the pathogen from which it can re-emerge and cause active TB. Reactivation is normally associated with immuno-compromisation of the host and can occur in up to 12% of latently-infected individuals over their lifetime (Vynnycky and Fine, 2000). A major obstacle to the eradication of TB is that during latency, *M. tuberculosis* becomes tolerant to currently-available chemotherapeutics (Gillespie, 2002; Gomez and McKinney, 2004). The availability of drugs which sterilised both latent infections and active cases would help prevent reactivated and recurrent TB.

Research has been performed to gain an understanding of the physiological state of *M. tuberculosis* during latency and enable experimental models of latent TB to be developed. *M. tuberculosis* has been reported to continue replicating, at reduced levels, during chronic infection of mice with a calculated 4 day generation time at 16 weeks post inoculation (Gill *et al.*, 2009). Whether this finding is also observed in other animal models such as guinea pigs or non-human primates which produce hypoxic granulomas (Via *et al.*, 2008), remains to be established. A study in humans which examined sequence variation in paired clinical isolates indicated a low likelihood of replication based on a small number of mutation events during latent TB (Yang *et al.*, 2011). In contrast, a study in non-human primates found a rate of mutation during latency that was indicative of continued growth (Ford *et al.*, 2011). Until further evidence is available, it may only be possible to deduce at this stage that the rate of replication of *M. tuberculosis* decreases to either low, or to negligible, levels during latency in humans.

Much of the whole-cell screening for new TB agents performed to date has involved the use of assays that detect the most optimum inhibitors of mycobacterial proliferation. Many of the TB drugs that are in clinical use are efficient in preventing mycobacterial growth but have limited efficacy against so-called dormant, or non-replicating persistent, mycobacteria. Screening models to detect drugs for treating latent TB need to encompass experimental conditions which produce a low-replicative state. In this work, an *in vitro* low-metabolically active model was developed to produce cells resilient to killing by first- and second-line TB drugs. The cells were screened against chemical libraries using the es-

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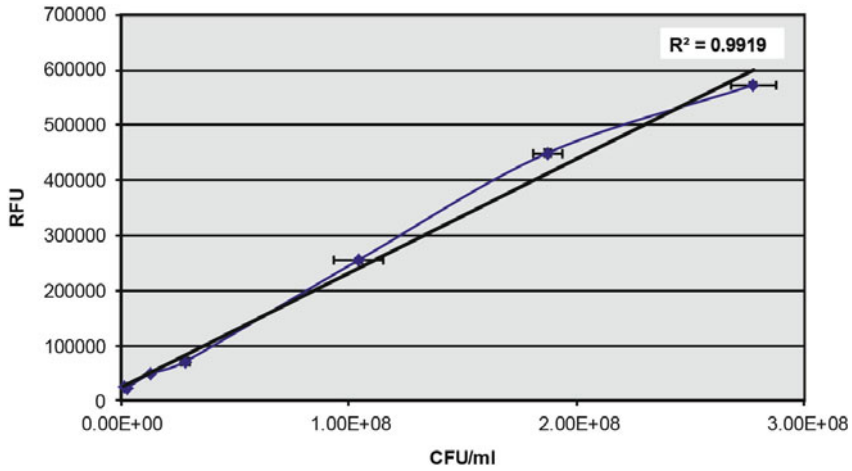

Fig. 1. Correlation of resazurin relative fluorescence units (RFU) and colony-forming units (CFU) in the determination of mycobacterial viability in *M. smegmatis*. R^2 (correlation coefficient).

tablished resazurin microplate assay (REMA) (Taneja and Tyagi, 2007) and new inhibitors of stationary phase mycobacteria were identified. The inhibitors were also tested for activity against virulent strains of *M. tuberculosis* including drug-resistant isolates.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

50 ml cultures of *M. smegmatis* carrying the pSHIGH+hsp60 plasmid (Miller *et al.*, 2009) were grown in 250 ml flasks, at 37°C, with agitation of 200 rpm, in Luria Bertani (LB) broth supplemented with 0.1% (v/v) Tween 80 and 50 μg/ml kanamycin. To achieve carbon, nitrogen or phosphorous starvation conditions, cultures were grown in modified Hartmann de Bont's (HdeB) minimal media as previously described (Smeulders *et al.*, 1999; O'Toole *et al.*, 2003). 50 ml cultures of *M. tuberculosis* H37Ra were cultured in 250 ml flasks, at 37°C, with agitation of 200 rpm in Middlebrook 7H9 broth (Becton Dickinson Ltd.) supplemented with 10% (v/v) OADC (0.06% oleic acid, 5% BSA, 2% Dextrose, 0.85% NaCl), glycerol (0.5% v/v) and Tween 80 (0.05% v/v).

Establishment of low metabolically-active cultures of *M. smegmatis* and *M. tuberculosis*

Metabolic reduction of resazurin is well established from a number of studies as an indicator of viability in mycobacteria with respect to colony-forming unit (CFU) counts (Taneja and Tyagi, 2007; Sala *et al.*, 2010; Zhang *et al.*, 2012). The correlation of resazurin relative fluorescence units (RFU) and CFU was also confirmed in our work. Serial dilution from 10^{-1} to 10^{-8} of a triplicate set of cultures of *M. smegmatis* was performed and the viability of the diluents was measured using resazurin fluorescence and viable cell plating. A correlation coefficient (R^2) of 0.9919 was obtained for RFU and CFU (Fig. 1).

To enable simultaneous harvesting of differently aged cultures, ten 50 ml cultures of *M. smegmatis* were set up in a staggered manner over a 35 day period. The cells were pelleted by centrifugation at 4,000 rpm for 10 min, supernatant removed, and the cell pellet resuspended in phosphate buffered saline (PBS) to an optical density at 600 nm (OD_{600}) of 1.0 (10 mm path length). The cells were seeded into 96 well plates, 30 μl of 0.02% (w/v) resazurin sodium salt solu-

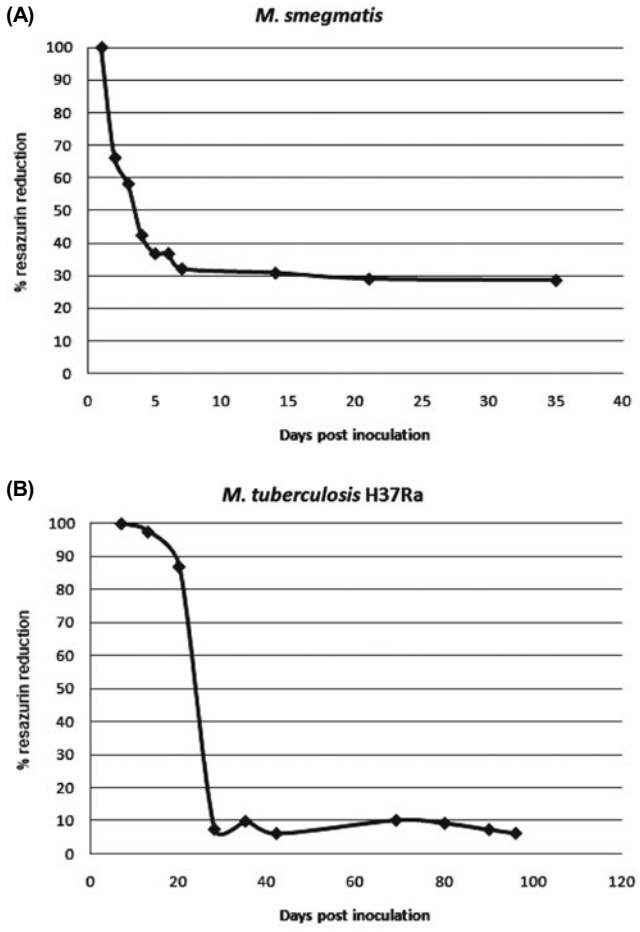

Fig. 2. Relative metabolic activity of different aged cultures of (A) *M. smegmatis* and (B) *M. tuberculosis* grown in rich media. Resazurin reduction activity is expressed as a percentage of resazurin reduction measured in logarithmic growth phase cells.

tion added, and the plates incubated for 3 h. Fluorescence was read at 530 nm (excitation) and 590 nm (emission) using a Perkin-Elmer Envision™ Multi-label Plate Reader, and metabolic activity, as determined by fluorescence readings, of aged cultures was expressed as a percentage of the activity displayed by logarithmic phase cultures (24 h post inoculation for *M. smegmatis*) (Fig. 2). For further experiments, cultures of *M. smegmatis* were incubated for 8 days to establish stationary phase in rich media. Metabolic activity of the culture did not significantly decrease with continued incubation beyond this point as indicated by no further change in resazurin reduction. Flasks were sealed with a sterile cotton wool bung and aluminium foil which allowed continued oxygenation of the cultures. This procedure was performed over the same time scale under nutrient-limited culture conditions. To achieve carbon or nitrogen limitation, cultures were grown in modified Hartmann de Bont's (HdeB) minimal media as previously described (Smeulders *et al.*, 1999; O'Toole *et al.*, 2003). For *M. tuberculosis* H37Ra, the cultures were monitored over 95 days. *M. tuberculosis* H37Ra required 30 days of incubation before no further significant reduction in metabolic activity was observed (Fig. 2).

Resazurin-based bactericidal dose response assay

Established stationary phase cultures of *M. smegmatis* and *M. tuberculosis* H37Ra were harvested by centrifugation at 4,000 rpm for 10 min, the supernatant removed, and the cell pellet resuspended in phosphate buffered saline (PBS) to an optical density at 600 nm (OD_{600}) of 1.0 (10 mm path length). The central 80 wells of a 96 well plate were set up to contain in triplicate, a 2-fold dilution series of test compound in 50 µl PBS. 50 µl of culture suspension were added to the central 80 wells of the 96 well plates to give a final assay volume of 100 µl. Rows A and H contained 200 µl of sterile distilled water (SDW) to minimise evaporation from culture wells during incubation. Wells B-F of column 1 contained 100 µl of PBS. Wells B-F of column 12 contained 50 µl of media and 50 µl of resuspended culture with no additional compound as positive controls. The plates were sealed with clear plastic plate seals, followed by replacement of the lid and sealing again with parafilm. The plates were then incubated for 96 h at 37°C after which 30 µl of 0.02% (w/v) resazurin sodium salt solution were added to control and test wells and incubated for 24 h. Fluorescence was read at 530 nm (excitation) and 590 nm (emission) to determine % viability in test and control wells. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a positive control in the bactericidal assays.

Chemical library screens for bactericidal compounds

Columns 2-11 of 96 well plates were set up to contain 48 µl of PBS, to which 2 µl of 1 mM library compound was added using a CyBio liquid handling robot. Column 1 contained 200 µl of sterile distilled water (SDW) to minimise evaporation. Column 12 contained positive and negative controls, including solvent, media and known anti-TB drug controls. 50 µl of resuspended culture was added to all test and control wells to give a final assay volume of 100 µl and a final library compound concentration of 20 µM. The plates were sealed and incubated for 96 h. 30 µl of 0.02% (w/v) resazurin sodium salt solution were added and after 24 h, the fluorescence of the wells was measured. Screens of the Library Of Pharmacologically Active Compounds, LOPAC (Sigma-Aldrich, n=1280 compounds), the NIH Diversity Set (National Institutes of Health, USA, n=1990 compounds) and the Spectrum Collection (Microsource Discovery Systems Inc., USA, n=2,000 compounds) for bactericidal compounds were performed in triplicate. Compounds, which caused a greater than 50% decrease in viability under at least one growth condition, were acquired and re-tested in dose response assays where available from commercial sources.

Testing inhibitors for bacteriostatic activity against virulent strains of *M. tuberculosis*

The inhibitors were tested in bacteriostatic assays with drug-susceptible (H37Rv, V4207), multi-drug resistant (KZN494, V2475) and extensively-drug resistant strains (R506, TF274) of *M. tuberculosis* (Ioerger *et al.*, 2009). 50 ml cultures of the *M. tuberculosis* strains were grown to mid-log phase and an aliquot diluted to an OD_{600} of 0.001 in 7H9 without Tween 80. 100 µl of the diluted culture (corresponding to approximately 10^4 CFU) were added to each well of a 96 well plate containing either serially diluted inhibitor or rifampicin control. Plates were incubated at 37°C for 7 days for re-growth of the cultures followed by the addition of 30 µl of resazurin (0.02%) to each well and incubation for a further 16–24 h. Plates were read at 544ex/590em with an Optima microplate reader (BMG) and 90% minimum inhibitory concentration (MIC_{90}) values were determined.

Testing inhibitors for bactericidal activity against virulent strains of *M. tuberculosis*

Bactericidal assays in virulent *M. tuberculosis* were performed with drug-susceptible (H37Rv, V4207), multi-drug resistant (KZN494, V2475) and extensively-drug resistant strains (R506, TF274) of *M. tuberculosis*. 50 ml cultures of the *M. tuberculosis* strains were maintained in stationary phase for 30 days. The cells were washed and resuspended in PBS to an optical density at 600 nm (OD_{600}) of 1.0 (10 mm path length). 100 µl of the cell suspension were added to each well of a 96 well plate. Plates were incubated at 37°C for 7 days followed by addition of 30 µl of resazurin (0.02%) to each well and incubation for a further 16–24 h. Plates were read at 544ex/590em with an Optima microplate reader (BMG) and 90% minimum bactericidal concentration (MBC_{90}) values were determined.

Measurement of compound cytotoxicity

4×10^4 Vero cells (ATCC no: CCL-81™) in 0.1 ml of DMEM+ 5% fetal calf serum (FCS) were seeded to wells of a 96-well plate. Plates were incubated for 16 h at 37°C with 5% CO_2. For treatment, media was replaced by 0.16 ml of two-fold serially diluted compounds, with compound concentration ranging from 100 µM to 0.2 µM. Controls included cell growth control (no compound), known low toxicity compound control (isoniazid and rifampin) and blank control (media only). Plates were incubated for 72 h at 37°C with 5% CO_2. Cells were then washed twice with PBS. After

washing, 0.1 ml of DMEM + 5% FCS containing 14 μl of 0.02% resazurin in water was added to all wells. Plates were incubated for 2 h at 37°C and read with a microplate reader (Optima, BMG). Percentage inhibition was calculated with reference to growth control and 50% inhibition (IC_{50}) values were determined.

Results

Establishment of stationary-phase cultures of mycobacteria

The first step in the application of the resazurin assay was to identify a low-replicative population of mycobacterial cells with reduced metabolic activity for the bactericidal experiments. As the reduction of resazurin relies upon ongoing metabolic activity in order to detect viability (von Groll *et al.*, 2010), this assay could be used to compare the metabolic activity of cultures of different ages as they progress through logarithmic growth into prolonged stationary phase. Cultures of *M. smegmatis*, grown in rich media and of different ages ranging from day 0 to day 35 post inoculation, were adjusted to the same optical density and assayed for their metabolic activity. *M. smegmatis* exhibited a decrease in resazurin reduction from 100% on day 0 to approximately 30% on day 7 in stationary phase after which no significant decrease was detected (Fig. 2). This indicated that the cultures, after 1 week incubation under the conditions used, maintained a steady but lower level of metabolic activity. For *M. tuberculosis*, cultures grown in rich media and of different ages, from day 0 to day 95 post inoculation, were adjusted to the same optical density. *M. tuberculosis* cultures exhibited a decrease in resazurin reduction from 100% on day 0 to approximately 10% on day 28 (Fig. 2). This indicated that after 4 weeks incubation under the conditions used, a steady but basal level of metabolic activity was maintained. Day 8 and day 30 were chosen as the time-points at which to challenge cultures of *M. smegmatis* and *M. tuberculosis*, respectively, grown under nutrient-rich conditions in the bactericidal assay. For the challenge of *M. smegmatis* grown under carbon or nitrogen limitation conditions, 10 day old cultures were used.

Testing of first and second line anti-tubercular agents in the bactericidal assay

Established stationary phase cultures of *M. smegmatis* grown under nutrient-rich conditions were challenged with standard first and second line anti-TB compounds to ascertain the level of drug tolerance exhibited by the bacilli. None of the 8 standard anti-TB compounds tested were able to achieve complete inhibition of resazurin reduction with respect to the control, CCCP, following 96 h of drug exposure, up to a maximum tested concentration of 500 μM under nutrient

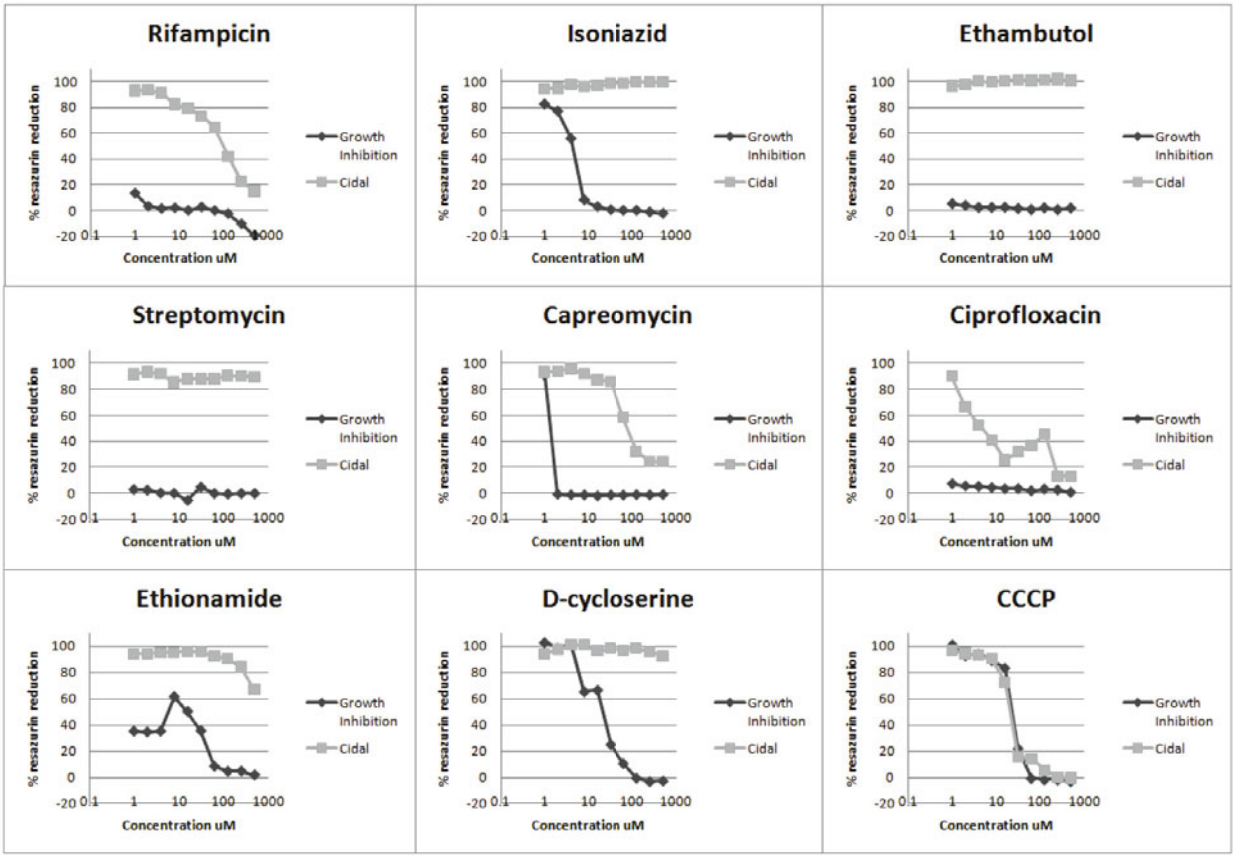

Fig. 3. Comparison of growth inhibitory and bactericidal activity of first and second line TB drugs in *M. smegmatis*. CCCP, carbonyl cyanide m-chlorophenylhydrazone, was included as a positive control.

rich conditions (Fig. 3). Rifampicin produced a decrease in viability but it failed to achieve complete loss in viability as measured by resazurin reduction at the highest concentration tested of 500 μM. Ciprofloxacin was more effective against stationary phase cultures but again, complete loss in viability was not achieved at 500 μM with respect to CCCP. This was in contrast to the bacteristatic assays where all clinical TB drugs tested produced complete growth inhibition (Fig. 3). The apparent trend of tolerance to compounds used to treat TB seen in this model was not restricted to *M. smegmatis*. Similar tolerance to rifampicin was observed in *M. tuberculosis* (see below).

Screening chemical libraries in *M. smegmatis* for bactericidal compounds

Established stationary phase cultures of *M. smegmatis* grown under nutrient-rich conditions were screened against the LOPAC, Spectrum Collection and NIH libraries for bactericidal compounds. A total of 60 compounds causing a greater than 50% decrease in viability, at a concentration of 20 μM, under nutrient-rich conditions were identified. One of the compounds detected was the anti-helminthic compound niclosamide which has previously been reported to be bactericidal towards stationary phase *M. tuberculosis* (Sun and Zhang, 1999). The bactericidal screens were also performed under carbon and nitrogen limitation as culture conditions have been shown to affect the activity profile of chemical libraries (Miller *et al*., 2009). This resulted in the detection of an additional 29 compounds. The dormacidal screen hits were examined for suitability as anti-TB agents based on information available in the literature. Compounds with already well-characterised anti-TB activity, such as the fluoroquinolones, were omitted from further studies as were compounds with documented cytotoxicity, such as antineoplastics and mercuric compounds. Two of the remaining bactericidal hits, NSC310551 and NSC321206 (Fig. 4), have been identified as inhibitors of the proteaseome in a lung carcinoma cell line (Lavelin *et al.*, 2009), however, their bactericidal activity towards mycobacteria has not been described previously.

Validation of anti-mycobacterial hits in bactericidal dose-response experiments

The pyridinyl dithiocarbamates, NSC310551 and NSC321206, and an additional 4 structurally-related pyridinyl thiosemicarbazones (NSC635448, 641296, 647132, 647133) (Fig. 4) were tested in bactericidal dose response assays in *M. smegmatis*. NSC310551 and NSC647132 had MBC_{90} values of 50 μM and 25 μM, respectively, in *M. smegmatis*. The remaining compounds, NSC321206, 635448, 641296, 647133, had an MDC_{90} of 3.125 μM in *M. smegmatis*. A similar pattern was observed in *M. tuberculosis* H37Ra where compounds NSC321206, 635448, 641296, 647133 were the most active. Rifampicin did not achieve 90% bactericidal activity at the highest concentration tested of 100 μM in either *M. smegmatis* or *M. tuberculosis*. This indicates that these strains exhibit phenotypic tolerance to rifampicin in our *in vitro* stationary phase model.

Bacteriostatic activity of inhibitors towards virulent strains of *M. tuberculosis*

To examine whether the inhibitors identified were active towards virulent *M. tuberculosis*, bacteriostatic dose response assays were performed against strains H37Rv, V4207 (drug susceptible); KZN494, V2475 (multi-drug resistant); and R506, TF274 (extensively-drug resistant). As expected, ri-

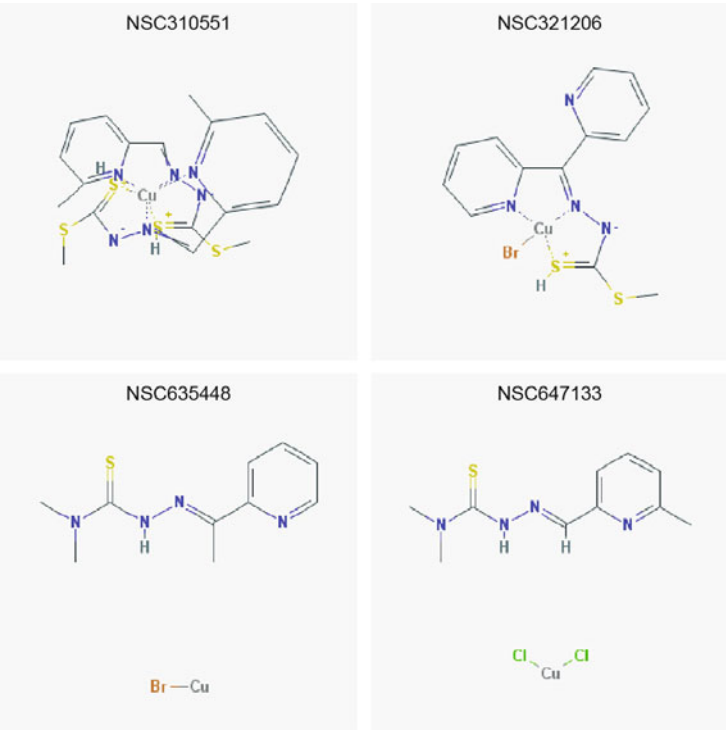

Fig. 4. Chemical structures of the pyridinyl dithiocarbamates, NSC310551 and NSC321206, and the pyridinyl thiosemicarbazones, NSC635448 and NSC647133. Images reproduced from PubChem (http://pubchem.ncbi.nlm.nih.gov) with permission.

Table 1. **Growth inhibitory activity of bactericidal compounds against drug-susceptible and drug-resistant strains of virulent *M. tuberculosis***

Compound	MIC_{90}[a]					
	H37Rv	V4207	KZN494	V2475	R506	TF274
Rifampicin	<0.19	<0.19	>100	0.39	100	100
310551	1.56	1.56	1.56	0.78	1.56	1.56
321206	12.5	25	12.5	6.25	6.25	6.25
635448	12.5	6.25	6.25	6.25	3.13	1.56
641296	3.13	1.56	0.78	0.78	0.78	0.78
647132	6.25	3.13	3.13	12.5	3.13	3.13
647133	3.13	1.56	0.78	0.78	0.78	0.78

[a] 90% minimum inhibitory concentration (MIC_{90}) values were determined following 7 days of compound treatment and are expressed in μM. Mycobacterial cell viability was determined using the resazurin microplate assay (REMA) (Taneja and Tyagi, 2007). The drug resistance phenotypes of the *M. tuberculosis* strains are as follows: H37Rv, V4207 (drug susceptible); KZN494, V2475 (multi-drug resistant); R506, TF274 (extensively-drug resistant).

fampicin exhibited a low MIC_{90} value against the drug-susceptible strains (Table 1). The MIC_{90} values for the drug-resistant strains on the other hand were greater than the highest concentration tested of 100 μM. The dormacidal compounds from this work inhibited the growth of all of the virulent *M. tuberculosis* strains tested with MIC_{90} values ranging from 0.78 μM for NSC641296 and 647133 to 25 μM for NSC321206 (Table 1).

Bactericidal activity of inhibitors towards clinical strains of *M. tuberculosis*

Dormacidal dose response assays were performed for the virulent *M. tuberculosis* strains. As with the earlier results for *M. smegmatis* mc^2155 and *M. tuberculosis* H37Ra, rifampicin did not achieve sterility (MBC_{90} greater than highest concentration tested of 100 μM) against any of the virulent *M. tuberculosis* strains tested including drug-susceptible H37Rv (Table 2). In contrast, the compounds detected in our bactericidal screen exhibited MDC_{90} values ranging from 1.56 to 25 μM across all of the strains tested including multi- and extensively-drug resistant *M. tuberculosis* (Table 2).

Determination of level of toxicity mycobacterial inhibitors

The compounds were tested for toxicity towards Vero cells and 50% inhibitory concentration (IC_{50}) values were determined. The proteasome inhibitors NSC310551 and 321206 exhibited IC_{50} values of 3.13 and 1.56 μM, respectively. The thiosemicarbazones, NSC635448, 641296, 647132, and 647133, exhibited IC_{50} values of 6.25, 0.78, 0.39, and 1.56 μM, respectively.

Discussion

There are several different models used to study latent TB. *In vitro* models often focus on inducing entry into a persistent state using a specific stress believed to be encountered by the bacterium in its natural environment, the human host. The most widely used of these is the Wayne dormancy model. This model entails gradual self-induced depletion of oxygen in a deep liquid medium combined with gentle stirring to maintain even dispersal of bacterial cells (Wayne and Hayes, 1996). It was hypothesised that *M. tuberculosis* encounters hypoxic conditions *in vivo* which induce entry into a non-replicating persistent state resulting in latent TB (Wayne and Sohaskey, 2001). This hypothesis is supported by work which has found that necrotic tuberculosis lesions in humans (Tsai *et al.*, 2006), guinea pigs, rabbits, and non-human primates (Via *et al.*, 2008) exhibit hypoxia. Another *in vitro* environmental condition used to induce entry into stationary phase is limitation of nutrients. Here consumption of finite nutrients during growth (Smeulders *et al.*, 1999) or resuspension of growing cells in a buffer devoid of nutrients (Betts *et al.*, 2002) are used. There is also a body of evidence which indicates that *M. tuberculosis* may be deprived of certain nutrients in the host lung (O'Toole, 2010).

In this work, an *in vitro* model of adaptation to stationary phase in response to limitation of nutrients was applied to a library screen for bactericidal compounds. Mycobacterial cultures were incubated in liquid culture in unsealed flasks to allow free oxygen diffusion. The cultures were monitored for metabolic activity through logarithmic growth and long-term stationary phase. The REMA assay was used to measure

Table 2. **Bactericidal activity of hit compounds against drug-susceptible and drug-resistant strains of virulent *M. tuberculosis***

Compound	MIC_{90}[a]					
	H37Rv	V4207	KZN494	V2475	R506	TF274
Rifampicin	>100	ND	>100	>100	>100	>100
310551	12.5	ND	6.25	12.5	6.25	6.25
321206	25	ND	12.5	12.5	25	6.25
635448	25	ND	25	25	25	50
641296	1.56	ND	3.13	6.25	6.25	6.25
647132	12.5	ND	12.5	25	6.25	6.25
647133	6.25	ND	6.25	6.25	6.25	3.13

[a] 90% minimum bactericidal concentration (MBC_{90}) values were determined following 96 h of compound treatment and are expressed in μM. Mycobacterial cell viability was determined using the resazurin microplate assay (REMA). The drug resistance phenotypes of the *M. tuberculosis* strains are as follows: H37Rv, V4207 (drug susceptible); KZN494, V2475 (multi-drug resistant); R506, TF274 (extensively-drug resistant). ND, not determined.

metabolic reduction of resazurin, an established marker of mycobacterial viability (Taneja and Tyagi, 2007). Reduction of resazurin was seen to decrease following entry into stationary phase and reach a plateau by 8 and 30 days for *M. smegmatis* and *M. tuberculosis*, respectively (Fig. 2). When we tested cells from cultures with static resazurin reduction activity, we found that they exhibited resistance to the eight clinical TB drugs tested with none of the drugs eliminating viability completely, as measured by resazurin reduction at the highest concentration tested of 500 μM (Fig. 3). Most noteworthy was the high level of resistance seen in these cells to isoniazid and rifampicin which are both used in the treatment of latent TB infection (Health Protection Surveillance Centre Ireland, 2010; New Zealand Ministry of Health, 2010).

Application of our assay to screening of chemical libraries identified two compounds in particular, pyridinylated dithiocarbamates NSC310551 and 321206, as being active against stationary phase *M. smegmatis* mc^2155 and *M. tuberculosis* H37Ra. While their molecular target in mycobacteria remains to be established, NSC310551 and 321206, have previously been shown to inhibit the proteaseome in a lung carcinoma cell line (Lavelin *et al.*, 2009). Interestingly, inhibition of the proteasome of *M. tuberculosis* using antisense gene silencing blocks persistence of the pathogen during the chronic stage of infection in mice (Gandotra *et al.*, 2007). The above findings highlight the proteasome as a potential bactericidal drug target. In terms of other dithiocarbamates that have been reported previously as being active against mycobacteria, diethyldithiocarbamate (DETC) and pyrrolidine dithiocarbamate (PDTC) are highly active against *M. tuberculosis* including aged cultures (Byrne *et al.*, 2007). Furthermore, a dithiocarbamate sugar derivative, 2-acetamido-2-deoxy-β-D-glucopyranosyl N,N-dimethyldithiocarbamate (OCT313, Glc-NAc-DMDC), has been found to exhibit anti-TB activity towards clinical isolates including MDR and XDR strains of *M. tuberculosis* (Horita *et al.*, 2011).

We also examined whether compounds structurally similar to NSC310551 and 321206 exhibit bactericidal activity towards *M. tuberculosis*. Four pyridinylated thiosemicarbazones (NSC635448, 641296, 647132, 647133) were found to kill stationary phase cells of *M. smegmatis* and *M. tuberculosis*. They also exhibited potent growth inhibitory and bactericidal activity towards both drug-susceptible and drug-resistant clinical strains of *M. tuberculosis* (Tables 1 and 2). Thiosemicarbazones have previously been examined with regard to treatment of TB. Their observed toxic effects in patients have limited their development as standard TB drugs (Shane *et al.*, 1951). Nevertheless, a thiosemicarbazone-containing pro-drug, thioacetazone, is currently used a second-line TB drug, particularly in developing countries due to its low cost (Alahari *et al.*, 2007). Thioacetazone requires activation by the FAD-containing monooxygenase EthA (Rv3854c) (Dover *et al.*, 2007) and has been shown to inhibit cyclopropanation of cell mycolic acids in mycobacteria (Alahari *et al.*, 2007, 2009).

While the specific compounds detected in this work displayed cytotoxicity in a cell line assay, our experimental set-up nevertheless establishes a population of cells that exhibit a high level of phenotypic drug tolerance. The screen enables identification of compounds which have a higher level of sterilising activity, with respect to existing clinical TB drugs, towards both phenotypically-tolerant and genetically-resistant *M. tuberculosis*. *M. tuberculosis* has a well documented ability to persist in the host for many years in a dormant-like state. In latently-infected individuals, the bacterium is present in a state that is refractory to the action of most current antibiotics. This may call into question a reliance on classical growth inhibition assays to discover new TB drugs. The development and more widespread application of assays that detect inhibitors of non-growing mycobacteria should aid the discovery of compounds with greater sterilising activity over currently-available anti-TB agents.

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