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Full Paper

Evaluation of *Mycobacterium smegmatis* as a possible surrogate screen for selecting molecules active against multi-drug resistant *Mycobacterium tuberculosis*

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New and better drugs are needed for tuberculosis (TB), particularly for the multi-drug resistant (MDR) disease. However, the highly infectious nature of MDR *Mycobacterium tuberculosis* restricts its use for large scale screening of probable drug candidates. We have evaluated the potential of a screen based on a 'fast grower' mycobacterium to shortlist compounds which could be active against MDR *M. tuberculosis*. Sensitivity profiles of *M. smegmatis*, *M. phlei* and *M. fortuitum* as well as MDR clinical isolates of *M. tuberculosis* were determined against anti-TB drugs isoniazid and rifampicin. Among the three fast growers, *M. smegmatis* was found to display a profile similar to MDR *M. tuberculosis*. Subsequently we evaluated the performance of *M. smegmatis* as a 'surrogate' screen for 120 compounds which were synthesized for anti-TB activity. Fifty of these molecules were active against *M. tuberculosis* $H_{37}Rv$ at a minimum inhibitory concentration (MIC) cutoff of $\leq$12.50 μg/ml. The *M. smegmatis* based screen showed 100% specificity and 78% sensitivity vis-à-vis MDR *M. tuberculosis*. These results highlight the utility of *M. smegmatis* as a primary screen to shortlist compounds for advanced screening against MDR *M. tuberculosis*.

Key Words——multi-drug resistance; *Mycobacterium smegmatis*; *Mycobacterium tuberculosis*; TB drug development

Introduction

Worldwide, tuberculosis (TB) is responsible for nearly 3 million deaths and 8 million new patients every year (Corbett et al., 2003). The long duration of chemotherapy with multiple drugs frequently leads to poor patient compliance and hence poor cure rates. The situation has worsened due to a rapid emergence of multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis* which show resistance to at least two of the frontline drugs—isoniazid and rifampicin (Espinal, 2003). There is therefore an urgent need to identify new drugs which have a good therapeutic potential against drug resistant pathogens and also allow a reduction in the duration of treatment.

Identification of candidate drugs generally requires screening of large compound collections (chemical libraries) using appropriate 'high-throughput' methods. Discovery groups across the world rely mainly on cell viability based screens for primary identification of molecules which could be active against TB. For this purpose, several methods including Mycobacterium Growth Indicator Tube (Walters and Hanna, 1996), E-test (Wagner and Mills, 1996), uracil uptake (Chung

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et. al., 1995), BACTEC 460 system (Collins and Franzblau, 1997), microplate Alamar blue assay (Collins and Franzblau, 1997), and nitrate reduction (Panaiotov and Kantardjiev, 2002) have been developed. Nonetheless, a common problem with most available methods is that they require the use of virulent *M. tuberculosis* which precludes their adoption for high-throughput screening. The risk gets further enhanced when one has to screen molecules against drug resistant strains of the pathogen.

In this study we have explored the possibility of identifying a non-pathogenic, fast-growing mycobacterium which, when used in a cell viability based screen, could serve as a 'surrogate' for MDR *M. tuberculosis*. In this pursuit, we have compared the drug susceptibility profiles of three 'fast grower' mycobacterial species—*M. smegmatis*, *M. phlei* and *M. foruitum* —and found *M. smegmatis* to show the profile closest to that of MDR clinical isolates of *M. tuberculosis*. In the follow-up, we have evaluated the potential of *M. smegmatis* as a primary screen to shortlist compounds which could be active against the drug resistant *M. tuberculosis*.

Materials and Methods

Mycobacteria. *M. tuberculosis* H_{37}Rv was procured from ATCC (ATCC 27294). The 'fast grower' mycobacteria used in this study were *M. fortuitum* (TMC 1529), *M. phlei* (ATCC 1724), and *M. smegmatis* (ATCC 607).

Three multi-drug resistant (MDR) clinical isolates of *M. tuberculosis* were provided by the Department of Biotechnology (Government of India) Mycobacterium Repository, located at National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India.

M. tuberculosis was defined by its growth rate, niacin accumulation and HPLC pattern of cell-wall associated mycolic acids (Butler et al., 1996). The fast growers were defined by their respective biochemical tests and growth patterns at different temperatures.

E. coli. *E. coli* strain DE3 (Novagen) was used as a 'control' to determine the non-specific antibacterial activity of test compounds.

Working inocula. The mycobacteria were cultured on Lowenstein-Jensen (L-J) medium slants and *E. coli* was cultured in Luria Bertani (LB) broth. Working inocula were prepared by suspending the cultures in normal saline containing 0.05% Tween-80. To make single cell suspensions, the mycobacteria were agitated vigorously with glass beads. Dilutions of the suspensions were plated on Middlebrook (MB) 7H10 agar medium for counting the colony forming units (CFU) after incubating for the required durations (described below).

Anti-tubercular drugs and test compounds. Isoniazid (INH) and rifampicin (RFM) were purchased from Sigma (USA). The test compounds were 120 molecules synthesized in-house for anti-tubercular activity. Stock solutions of the drugs and test compounds were prepared in dimethyl sulfoxide (DMSO) and stored at $-80°C$.

Susceptibility testing. The proportion method (McClachy, 1978) was followed using MB 7H10 agar medium. For tests against *M. tuberculosis*, the medium was supplemented with OADC (oleic acid, albumin fraction IV, dextrose and catalase). One hundred microliters of serial twofold dilutions of the stock solutions (2 mg/ml) of standard drugs (INH and RFM) or test compounds were incorporated in the medium (final volume: 2 ml/tube) and the tubes were kept in slanting position till the medium solidified.

Working inocula of mycobacteria or *E. coli* (2×10^7 CFU/ml) were spread on the surface of the medium (10 µl/tube) and tubes were incubated at 37°C. Results were available in 24 h with *E. coli*, 3 days with *M. smegmatis*, 5 days with *M. fortuitum*, 7 days with *M. phlei*, and 21 days with *M. tuberculosis*. The least concentration of a drug or compound that completely inhibited the growth of the mycobacteria was recorded as its minimum inhibitory concentration (MIC) with respect to the used inoculum.

Sensitivity or resistance towards a drug or compound was determined on the basis of 'Resistance Ratio (RR)' or 'R factor'(=MIC of a compound against a given mycobacterium/MIC against *M. tuberculosis* H_{37}Rv). An RR value of 4 or less is taken to denote sensitivity and that of 8 or more denotes resistance (Canetti et al., 1963).

Statistical analysis. Positive and negative predictive values as well as specificity and sensitivity of the surrogate screen were determined by using a two-by-two contingency table (Blakely and Salmond, 2002). From the same table, 'significance of association' was calculated by Chi square test (with Yates' correction) using the web software Graphpad (www.graphpad.com).

Results

Susceptibility of mycobacteria to anti-TB drugs

Initially, sensitivities of *M. tuberculosis* H_{37}Rv, MDR clinical isolates of *M. tuberculosis*, and the fast grower mycobacteria—*M. smegmatis*, *M. phlei* and *M. fortuitum* were determined against standard anti-TB drugs INH and RFM. Results are depicted in Fig. 1. MICs of these drugs for *M. tuberculosis* H_{37}Rv were 0.02 and 0.2 μg/ml, respectively (Fig. 1A). All three MDR isolates showed identical sensitivities (MIC= 25 μg/ml for INH, and 100 μg/ml for RFM) hence only one of them was used for further experiments.

Among the fast growers, *M. smegmatis* was most resistant to both drugs with a sensitivity profile identical to that of MDR *M. tuberculosis* in terms of MIC (Fig. 1A) as well as Resistance Ratio (Fig. 1B). For *M. phlei*, the MICs of INH and RFM were 12.5 and 0.39 μg/ml; and for *M. fortuitum*, they were 12.5 and 3.12 μg/ml, respectively. Considering the Resistance Ratios, *M. fortuitum* was only marginally resistant to RFM (RR=15.6, compared to 500 with *M. smegmatis*) whereas *M. phlei* was sensitive to RFM (RR=1.95). Based on these results, we selected *M. smegmatis* for evaluation as a possible surrogate screen for compounds which could be active against MDR *M. tuberculosis*.

Performance of M. smegmatis as a surrogate screen

All test compounds (n=120) were inactive (non-inhibitory) against *E. coli* at 50 μg/ml, which was the highest tested concentration in sensitivity assays. Fifty compounds were considered 'active' against *M. tuberculosis* H_{37}Rv at a cutoff MIC value of ≤12.50 μg/ml, the lowest recorded MIC being <3.12 μg/ml (Table 1). MDR *M. tuberculosis* and *M. smegmatis* were sensi-

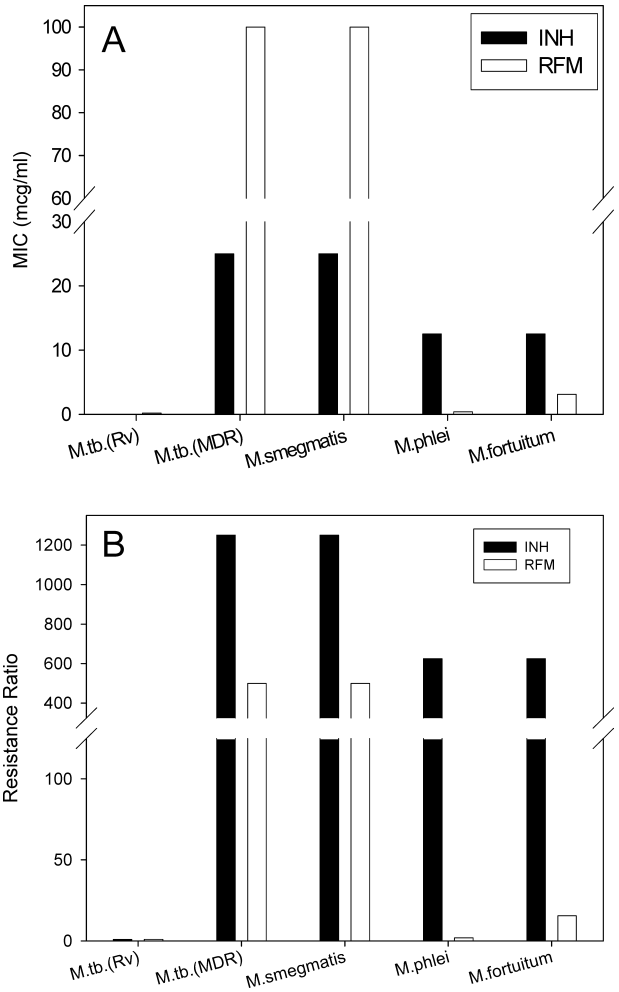

Fig. 1. Sensitivity or resistance of *M. tuberculosis* (H_{37}Rv or MDR), and the three fast grower mycobacteria *M. smegmatis*, *M. phlei* and *M. fortuitum* towards isoniazid (INH) and rifampicin (RFM).

Results are depicted as (A) minimum inhibitory concentrations (MIC) and (B) Resistance Ratio (RR).

Table 1. Sensitivity profile of the active compounds.

	Compounds active against *M. tuberculosis* H_{37}Rv	Compounds to which MDR *M. tuberculosis* showed sensitivity (RR≤4)[b]	Compounds to which *M. smegmatis* showed sensitivity (RR≤4)
	16 (≤3.12)[a]	10	5
	11 (6.25)	8	7
	23 (12.5)	9	9
Total	50	27	21

[a] Number and (MIC, μg/ml).

[b] Resistance Ratio.

Table 2. Activity against MDR *M. tuberculosis* and *M. smegmatis* of the 50 molecules which were active against *M. tuberculosis* $H_{37}Rv$.

		MDR *M. tuberculosis*		
		Sensitive	Resistant	Total
M. smegmatis	Sensitive	21	0	21
	Resistant	6	23	29
	Total	27	23	50

tive (RR≤4) to 27 and 21 of the active compounds, respectively. The overlap between sensitivities of the two microbes was highest (9 out of 9) for compounds having a MIC of 12.5 μg/ml against *M. tuberculosis* $H_{37}Rv$, followed by those having a MIC of 6.25 μg/ml (7 out of 8) and <3.12 μg/ml (5 out of 10). The strength of association between MDR *M. tuberculosis* and *M. smegmatis* with respect to these results, as calculated from a 2 ×2 table (Table 2), was highly significant ($\chi^2=27.733$, $p<0.0001$).

The specificity of the *M. smegmatis* based screen was 100% and its sensitivity was 78%, as calculated from the data in Table 2. 'Positive Predictive Value' of the screen (=probability that sensitivity of *M. smegmatis* to a compound actually means sensitivity of MDR *M. tuberculosis*) was 100% and its 'Negative Predictive Value' (=probability that resistance of *M. smegmatis* to a compound actually means resistance of MDR *M. tuberculosis*) was 79%. However, the Negative Predictive Value was 100% for the group of 70 compounds which were 'inactive' against *M. tuberculosis* $H_{37}Rv$ (MIC>12.5 μg/ml), since they were also inactive against both MDR *M. tuberculosis* and *M. smegmatis*.

Sensitivity of M. smegmatis in relation to chemical class of compounds

To have an insight into the lesser sensitivity of *M. smegmatis* (compared with MDR *M. tuberculosis*) towards some of the test compounds, we considered their chemical structures. All 50 active compounds (except one—a thiourea derivative) fell into two broad categories: (i) amino alkyls and (ii) cyclopropyl methanols. Earlier also our group had reported that amino alkyls having alkyl chains of 8–16 carbon atoms along with aryl/glycofuranosyl/galactopyranosyl moieties are potential anti-tubercular molecules (Katiyar et al., 2005). The present analysis revealed that 4 out of 6 compounds to which MDR *M. tuberculosis* was sensitive and *M. smegmatis* was resistant (Table 2) were amino alkyls with a chain of 12–16 carbon atoms and no point of unsaturation. One of the remaining two compounds was a cyclopropyl methanol and the other, a thiourea derivative.

The 70 test compounds which were inactive against *M. tuberculosis* $H_{37}Rv$ (and also against MDR *M. tuberculosis* and *M. smegmatis*) were derivatives of glycosylamine or cyclopropyl methane.

Discussion

There are several reports on the use of *Mycobacterium smegmatis* as a primary screen to select compounds which could be active against *M. tuberculosis* (Andries et al., 2005; Lu and Drlica, 2003; Murdock et al., 1978). However, its utility in screening for compounds active against MDR *M. tuberculosis* was not explored. We found that the susceptibility of *M. smegmatis* for the two frontline anti-TB drugs isoniazid and rifampicin was identical to that of MDR clinical isolates of *M. tuberculosis*. The MICs of the two drugs for *M. smegmatis* were similar to those reported by Wallace et al. (1988).

M. smegmatis is known to show a natural resistance towards both INH and RFM (Chung et al., 1995; Li et al., 2004; Quan et al., 1997; Trias and Benz, 1994). Our results further suggest that the microbe could be naturally resistant to compounds of certain chemical classes. This resistance could be occurring through more than one mechanism. Expression of alkyl hydroperoxide reductase C (ahpC) and INH sensitivity are inversely correlated in mycobacteria. This gene was found inducible in *M. smegmatis* but not in *M. tuberculosis* $H_{37}Rv$ (Dhandayuthpani et al., 1996). On the other hand, RFM is inactivated by *M. smegmatis* through ribosylation. Disruption of the gene responsible for this process resulted in mutants having much increased susceptibility to RFM (Dhandayuthpani et al., 1996). Resistance of *M. smegmatis* to INH and RFM has also been shown to be contributed by efflux mechanisms (Choudhuri et al., 1999; Li et al., 2004; Piddock et al., 2000).

To conclude, our results demonstrate that *M. smegmatis* may serve as a highly specific yet stringent screen for selecting molecules which could be active against MDR *M. tuberculosis*. The stringency (in terms of lower sensitivity) is not necessarily a disadvantage,

since the selected molecules are likely to stand a better chance of showing activity against MDR *M. tuberculosis* not only in vitro but also in vivo. A large number of molecules selected by in vitro screening turn out to be inactive in vivo since the latter requires greater potency of a test molecule.

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