

Tuberculosis drug tolerance: Differential drug susceptibility of intracellular and extracellular tuberculosis and the impact of time of drug exposure.

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Introduction

We and others have demonstrated a bi-exponential decline in sputum clearance of *Mycobacterium tuberculosis* (MTB) following chemotherapy [1, 2] characterised by an initial phase of rapid clearance of remaining bacilli from the sputum (early bactericidal activity - EBA), followed by a slower elimination of organisms (sterilising activity) [1-3].

The reason that bacteria killed during the sterilising phase are less drug susceptible than those killed by EBA is not fully understood. Slower bacterial multiplication, bacterial adaptation, and the compartmentalisation of bacteria in macrophages and granulomas have been suggested as possible explanations.

If tuberculosis therapy is to be shortened it is imperative that the sterilising activity of current and future tuberculosis drugs is enhanced.

Here we investigate the effect of prolonged drug exposure on log phase H37Rv bacilli in liquid culture, and whether macrophages have the potential to provide MTB with a pharmacological sanctuary site.

Methods

A modified microplate alamar blue assay (MABA) was used to assess H37Rv drug susceptibility. Briefly, H37Rv (1×10^7 cfu) were incubated in the presence of rifampicin (0-166 ng/mL), isoniazid (0-5 µg/mL) and ethambutol (0-50 µg/mL) for 1, 2 and 3 weeks. Bacterial viability was then assessed by alamar blue turnover, and terminated with paraformaldehyde (5%).

H37Rv was grown in rifampicin (166 ng/mL) and isoniazid (5 mg/mL) media to generate drug tolerant strains. Following selection, tolerant strains were grown in drug free media to determine if the bacteria reverted from (phenotypic tolerance) or remained tolerant (genetic tolerance) to the drugs. Ethionamide drug susceptibility of the isoniazid tolerant H37Rv strains was determined to assess cross resistance, and the involvement of *KatG* [5].

The drug susceptibility of intracellular bacilli was determined by a novel method. Briefly, A-THP1 cells were infected with H37Rv (multiplicity of infection: MOI=10, Fig 3) and grown in rifampicin (0-1 µg/mL), isoniazid (0-1 µg/mL) and ethambutol (0-10 µg/mL). Following incubation (1 week, 37°C), A-THP1 cell viability was determined.

MTB induced A-THP1 cell death was used as an inverse marker of intracellular H37Rv viability.

Fig 1: TB Extracellular kill over time

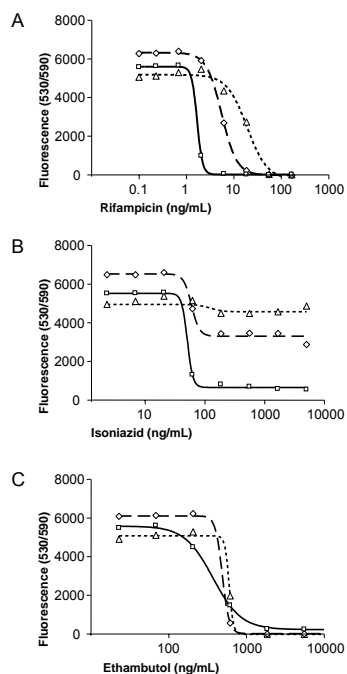


Figure 1: Representative concentration dependent H37Rv viability curves following 7 (■, solid line), 14 (◇, interrupted line) and 21 (▲, interrupted line) day drug exposure to rifampicin (A), isoniazid (B) and ethambutol (C) as measured by background corrected alamar blue fluorescence. Data represent mean (n=4).

Fig 2: H37Rv, H37Rv_{INH/MEDIA}, H37Rv_{INH/INH} drug susceptibility

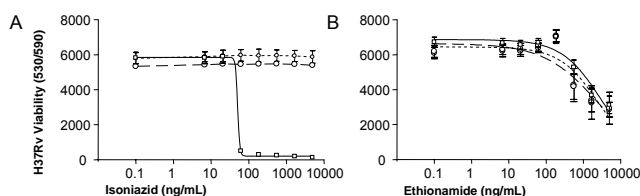


Figure 2: Parental H37Rv (□, solid line), H37Rv_{INH/MEDIA} (○, interrupted line), H37Rv_{INH/INH} (●, dashed line) viability curves following 7 day drug exposure to isoniazid (A) or ethionamide (B). Viability is expressed as the mean Alamar blue turnover (background corrected) ± SD (n=4).

Results and Discussion

The extracellular MABA showed a significant increase in rifampicin EC₅₀ from day 7 to 14 to 21 (mean (n=4) [range]; 1.6 [0.7-2.0] to 7.3 [2.0-17.8] to 24.7 [5.6-45] ng/mL respectively, p<0.05 (paired t-test), Fig 1A), and isoniazid EC₅₀ (47 [36-55] to 53 [35-71] to >5000 ng/mL respectively, p<0.05, Fig 1B). In contrast ethambutol EC₅₀ did not change over time (Fig 1C).

The drug susceptibility of rifampicin tolerant (data not shown) and isoniazid tolerant H37Rv strains (Fig 2A) did not revert back to that of wild type following growth in drug free media. This suggests that the drug tolerance that develops with time in the MABA assay is genotypic. Additionally, isoniazid tolerant H37Rv showed no cross resistance to ethionamide (Fig 2B), suggesting a *KatG* mutation[4].

The rate at which this drug tolerance developed exceeded that anticipated from data presented on MTB growing on solid media [5] and is to be further investigated..

MTB at an MOI of 10 was found to kill 100% of A-THP1 cells 7 days after infection.

Rifampicin, isoniazid and ethambutol were able to protect A-THP1 cells from MTB mediated kill (Fig 4).

A higher concentration of rifampicin was required to kill intracellular (148±32 ng/mL) versus extracellular bacilli (1.3±0.02 ng/mL; Fig 4A). The observed difference in concentration of rifampicin required to kill intracellular vs extracellular bacilli may be related to different experimental conditions used between the assays (e.g. Protein binding).

The intracellular drug activity of isoniazid (mean EC₅₀±SD: 37±2.2 ng/mL) and ethambutol (243±95) were similar to that of extracellular kill (57±2.5 ng/mL and 263±12 ng/mL respectively) (Fig 4B, 4C).

Conclusions

MTB drug susceptibility measured by MABA is time dependant for rifampicin and isoniazid, presumably associated with a greater rate in genetic resistance.

A-THP1 viability may be a useful for looking at the impact of proteins and other interactions on intracellular anti-TB drug activity.

MTB drug susceptibility testing is affected by media conditions, time of drug exposure, and whether MTB is internalised.

References

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Fig 3: Multiplicity of Infection

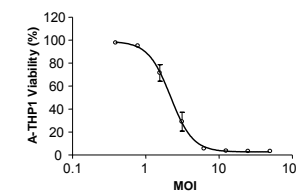


Figure 3: Percentage A-THP1 viability following infection with H37Rv at different multiplicity of infections (MOIs) for 7 days. Data are mean ± SD (n = 4).

Fig 4: Extra and Intra-cellular TB kill

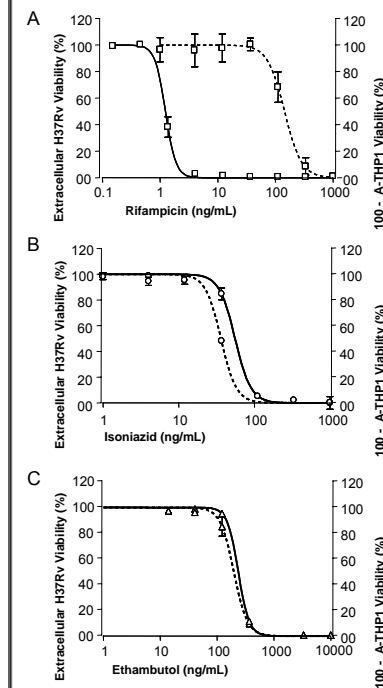


Figure 3: The affect of 7 days of A) rifampicin, B) isoniazid and C) ethambutol exposure on the percentage viability of extracellular H37Rv (—, left hand axis) and on the inverse (100 -) percentage viability of A-THP1 cells infected by H37Rv (-----, right hand axis, taken as a marker for intracellular H37Rv viability). Data represent mean ± SD (n=4).