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A luciferase-based assay for rapid assessment of drug activity against *Mycobacterium tuberculosis* including monitoring of macrophage viability

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ABSTRACT

The intracellular (IC) effect of drugs against *Mycobacterium tuberculosis* (Mtb) is not well established but increasingly important to consider when combining current and future multidrug regimens into the best possible treatment strategies. For this purpose, we developed an IC model based on a genetically modified Mtb H37Rv strain, expressing the *Vibrio harvei* luciferase (H37Rv-lux) infecting the human macrophage like cell line THP-1. Cells were infected at a low multiplicity of infection (1:1) and subsequently exposed to isoniazid (INH), ethambutol (EMB), amikacin (AMI) or levofloxacin (LEV) for 5 days in a 96-well format. Cell viability was evaluated by Calcein AM and was maintained throughout the experiment. The number of viable H37Rv-lux was determined by luminescence and verified by a colony forming unit analysis. The results were compared to the effects of the same drugs in broth cultures. AMI, EMB and LEV were significantly less effective intracellularly (MIC90: >4 mg/L, 8 mg/L and 2 mg/L, respectively) compared to extracellularly (MIC90: 0.5 mg/L for AMI and EMB; 0.25 mg/L for LEV). The reverse was the case for INH (IC: 0.064 mg/L vs EC: 0.25 mg/L). In conclusion, this luciferase based method, in which monitoring of cell viability is included, has the potential to become a useful tool while evaluating the intracellular effects of anti-mycobacterial drugs.

1. Introduction

Tuberculosis (TB) is a global infectious disease which is associated with a significant mortality in high-endemic areas (Glaziou et al., 2013). During the last years, a troublesome increase in multi- (MDR TB) and extensively (XDR TB) drug-resistant strains was observed. In order to design the best possible combination of current and newly developed drugs for such patients, new tools, which assess both the intra- and extracellular efficacy, are important.

In vivo, *Mycobacterium tuberculosis* (Mtb) mainly interacts with the macrophage. Inside host cells, the bacterial metabolism alters to better resist the antimicrobial mechanisms of the cell, and this phenotypic shift has been linked to reduced susceptibility to drugs (Ehrt and Rhee, 2013; Schnappinger et al., 2003). Additionally, factors such as the level of intracellular penetration of the drugs as well as interaction with the immune defense mechanisms may affect the intracellular activity. Assays for investigating the intracellular activity of drugs against Mtb have been limited by a lack of efficient methods for evaluation of mycobacterial viability. Colony forming unit (CFU)-based methods are time-consuming and associated with considerable variability (Eklund et al., 2010a). Therefore, there is a need to develop new methods allowing rapid intracellular testing of novel and currently used anti-mycobacterial compounds.

In clinical practice, drug susceptibility testing (DST) for Mtb is performed by using the reference liquid method BACTEC 960 MGIT according to the indirect proportion method (Lin et al., 2009). When novel methods for evaluating the activity of drugs are introduced, it is important to compare them to standardized methods using validated principles for DST against Mtb. However, in all routine DST performed, only the extracellular effect of the drug is tested. This may be of relevance during the first stage of infection and correspond to the early bactericidal effect (EBA) commonly investigated when novel TB drugs are introduced (Diacon et al., 2012; Donald and Diacon, 2008). On the contrary, extracellular testing would be of less relevance to

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measure the intracellular and sterilizing effect for which the clinical correlate is the relapse rate after treatment (Donald and Diacon, 2008; Mitchison, 2000). Thus, assessment of the intracellular activity of drugs may be important for tailoring the best possible regimens in the follow-up stage of TB.

Recently, a novel method has been presented, in which luminescence-labeled Mtb was transformed with luciferase to produce light as a sensitive indicator of viability in both the intra- and extracellular compartments (Eklund et al., 2010a). This enables experiments with a large number of exposures at a lower cost and at a considerably shorter time than the 2–3 weeks required for a CFU count. Previously, methods such as the whole blood bactericidal assay (Wallis et al., 2001), GFP-expressing mycobacteria (Collins LA and Franzblau, 1998) and more recently luminescence based strategies have been described (Andreu et al., 2010; Andreu et al., 2012). However, in these assays, the viability of the host cells is of importance for accurate measurement of the intracellular activity but this has seldom been considered.

Thus, the aim of this study was to investigate a novel method for rapid intracellular susceptibility testing where cell viability is monitored. The findings are expected to be of value when investigating the intracellular effects of anti-mycobacterial compounds.

2. Methods

2.1. Preparation of M. tuberculosis and antibiotics

The *M. tuberculosis* strain H37Rv (American Type Culture Collection 27294) harboring the pSMT1 plasmid (Andreu et al., 2012) carrying the gene for *Vibrio harveyi* luciferase was cultured in Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase (ADC) for 2–3 weeks at 37 °C with 100 μg/mL hygromycin for selection of the luciferase positive isolates and then re-inoculated for 7 days in fresh broth to reach the early exponential phase. The bacteria (hereafter labeled as H37Rv-lux) were centrifuged in phosphate-buffered saline (PBS) with 0.05% Tween 80 and the bacterial pellet was washed twice. A single-bacillus suspension was obtained by 10 passages through a sterile syringe equipped with a 27-gauge needle as described (Eklund et al., 2010a). A significant proportion of the (>95%) of the bacteria was single cells as confirmed with microscopical examination. The bacterial concentration was determined by measurement of optical density at 600 nm against a previously validated standard curve (Eklund et al., 2010a). Stock solutions of the antibiotics isoniazid (INH), ethambutol (EMB), amikacin (AMI) and levofloxacin (LEV) (Sigma-Aldrich) were prepared by dilution in sterile, deionized water and stored until use at −20 °C.

2.2. Macrophage culture

The human acute monocytic leukemia cell line, THP-1 (Sigma-Aldrich, Stockholm, Sweden), was grown in RPMI 1640-medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum. Differentiation to macrophage-like cells was induced overnight by addition of 100nM Phorbol 12-myristate 13-acetate (PMA) (Sigma). The cells were seeded in 96-well plates at the density of about 100 000 cells per well.

2.3. Intracellular exposure to anti-mycobacterial drugs

Infection of THP-1 cells with the H37Rv-lux strain was performed in 96-well culture plates at a multiplicity of infection (MOI) of 1 at 37 °C during 1 h. By washing three times with PBS at room temperature, the non-phagocytosed bacteria were removed before addition of fresh serum-containing medium (10% fetal calf serum in RPMI) with or without antibiotics (INH, EMB, AMI or LEV) in triplicates in at least 4 separate experiments. For each drug five dilutions were tested once to investigate the presence of concentration-dependent intracellular killing. Five days post-infection, cells were washed and lysed with ultra clean H_20 (Millipore) during 10 min. The intracellular fraction was measured by luminescence reading following addition of the substrate 1% *n*-decanal (Sigma) in a GloMax®-Multi Detection System (Promega). The macrophage viability was monitored with or without the addition of antibiotics and comparing day 0 and 5 cultures, by addition of Calcein acetoxymethyl (Calcein AM, 4 μg/mL) in the dark at room temperature during 30 min ($n = 4$). Cells were washed three times with room-tempered PBS. The fluorescence was then measured in a GloMax®-Multi Detection System (Promega).

2.4. Determination of colony forming units (CFU) in comparison to luminometry experiments

In parallel with the IC luminescence assay, one part of the cellular lysates containing Mtb was inoculated on Middlebrook 7H10 (7H10) medium for the detection of CFU by plating ($n = 2$–3). Ten-fold dilutions in PBS in triplicates were cultured on 7H10 for each drug exposure (INH, EMB, AMI and LEV). The 7H10 plates were sealed with parafilm and incubated at 37 °C in 5% CO_2 during two weeks before colony counting was performed.

2.5. Extracellular exposure to anti-mycobacterial drugs

In each separate experiment ($n = 7$ to 19 for each antibiotic concentration), a bacterial concentration of 10^5 CFU/mL was used. A 1:100 dilution of the initial inoculum as well as an unexposed 1:1 control was used in all experiments to evaluate the minimum inhibitory concentration (MIC). The MIC was defined as the lowest antibiotic concentration where there was less growth compared with the 1:100 diluted controls of the corresponding strain, i.e. the lowest concentration of drug that inhibited >99% of the bacterial population. In order to evaluate the MIC-level, the 1:100 diluted control was required to achieve an at least 100-fold increase from the baseline. Measurement of flash luminescence was performed in white 96-well plates (Sarstedt) during one second with addition of 25 μl 1% *n*-decanal (Sigma) per well in a GloMax®-Multi Detection System (Promega). Initial kinetic experiments for each drug were performed once with measurements of luminescence on days 0, 3, 5 and 7. All experiments ($n = 7$ to 19 for each antibiotic concentration) were performed in triplicates in 96-well plates (Sarstedt) after seven day incubation at 37 °C. Results are expressed as logarithmic values of arbitrary light units (ALU).

3. Results

3.1. Determination of the minimal inhibitory concentrations of first and second line drugs in Middlebrook 7H9 broth by luminescence detection of M. tuberculosis H37Rv-lux

In initial experiments, the growth kinetics during days 0, 3, 5 and 7 was investigated for INH, EMB, LEV and AMI (Fig. 1a–d). From these experiments it was observed that the 1:100 diluted control needed one week of growth in order for the MIC to be read, which is similar to the timing for reading of susceptible isolates in BACTEC 960 MGIT and the previously used BACTEC 460 system (Snewin et al., 1999). At seven days, the 1:100 diluted control showed a stable growth of more than 100-fold compared to the initial inoculum (Fig. 1a–d). The median MICs for INH was 0.25 mg/L, and 0.5 mg/L for LEV, EMB and AMI (Fig. 2 and Table 1a).

3.2. Macrophage viability during intracellular experiments

After five days of infection with MOI 1, the viability was not significantly altered in the infected cells as compared to uninfected control cells. Treatment of infected cells with the concentrations of antibiotics used did not influence cell viability (Fig. 3).