GREEN FLUORESCENT PROTEIN-BASED DRUG SCREENING ASSAY AGAINST INTRACELLULAR *MYCOBACTERIUM TUBERCULOSIS*

Kanchana Dokladda[1] and Prasit Palittapongarnpim[1,2]

[1]Tuberculosis Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani Province;
[2] Pornchai Matangkasombut Center for Microbial Genomics , Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand

Abstract. Novel anti-tuberculosis drugs are urgently needed. In addition to killing extracellular *Mycobacterium tuberculosis*, an ideal drug needs to be able to eliminate *M. tuberculosis* residing in macrophages. Determining viability of this intracellular organism is cumbersome, thereby making testing of drugs against macrophage-resident *M. tuberculosis* laborious. In order to examine macrophage-resident bacteria, *M. tuberculosis* H37Rv carrying a plasmid expressing a red-shifted green fluorescent protein (GFP) under control of an icl promoter that allows expression only in macrophage was constructed. Recombinant bacteria were allowed to infect U937 cells (a macrophage-like cell line) in a liquid culture for 18 hours prior to drug testing. MIC90 (minimum concentration inhibiting growth by 90%) of isoniazid, rifampin and streptomycin of 0.16, 0.08 and 2.5 μg/ml, respectively were obtained against macrophage-resident recombinant *M. tuberculosis*, while pyrazinamide was non-inhibitory. This GFP- based macrophage-resident *M. tuberculosis* drug susceptibility assay (iGFPMA) is rapid, inexpensive and suitable for a high throughput drug screening platform.

Keywords: macrophage-resident *Mycobacterium tuberculosis*, drug assay, green fluorescent protein, *icl* promoter

Correspondence: Kanchana Dokladda, Tuberculosis Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Phahonyothin Road, Amphoe Khlong Luang, Pathum Thani 12120, Thailand
Tel: + 66 (0) 2201 5881, + 66 (08) 6577 4640; Fax: + 66 (0) 2564 6701-5
E-mail: kanchana.dok@biotec.or.th

INTRODUCTION

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (*M. tb*) is a worldwide public health problem, particularly among immunocompromised individuals (Machuca *et al*, 2018). Effective therapy requires a long period of drug(s) treatment, compromised by emergence of highly multidrug-resistant *M. tb* strains.

Current armory of anti-TB drugs is are dwindling and development of new therapeutic agents are urgently needed. Traditional drug susceptibility test for tuberculosis is based on visual counting of bacteria growth (colonies on solid medium) or measurement of turbidity of bacteria in liquid medium, but owing to the slow growth of *M. tb*, the process takes weeks to complete (Beste *et al*, 2009). Screening of a large library of potential bioactive compounds depends on a reliable, rapid and high-throughput drug screening platform. Several reporter reagents suitable for high-throughput anti-TB drug screening assay have been reported, mainly based on oxidation-reduction compounds (alamar blue, resazurin, nitrate) (Banfi *et al*, 2003; Collins and Franzblau, 1997; Syre *et al*, 2003) and fluorescence or chemiluminescence (Changsen *et al*, 2003; Hazbon *et al*, 2003). However, a standard method for testing growth inhibition activity of the bioactive compounds against *M. tb* residing in macrophages still requires measurement of bacteria growth after transfer of cells to another medium, a procedure not compatible with current high-throughput assay platforms.

Aequorea victoria green fluorescent protein (GFP) is an intrinsically fluorescent protein requiring no substrate or cofactor (Kain *et al*, 1995). Heterologously expressed GFP, in general, has no toxic effect on cells and is readily detected and quantified (Chalfie *et al*, 1994). Collins *et al* (1998) explored use of a GFP-reporter microplate assay for high-throughput screening of anti-TB compounds using recombinant bacteria carrying a plasmid expressing mutant GFP with a red-shifted excitation peak and higher fluorescence signal than wild-type GFP. Changsen *et al* (2003) produced even a stronger GFP fluorescence signal by introducing heat shock protein 60 together with *M. smegmatis* acetamidase promoters into the GFP reporter plasmid, resulting larger differences between positive and background signals permitting a more sensitive assay of test compounds.

Here, *M. tb* H37Rv harboring a plasmid expressing a red-shifted GFP reporter plasmid under control of a macrophage-inducible promoter (piclGFP) (Dokladda *et al*, 2015) was employed in developing a microplate assay for drug testing of macrophage-resident transformed *M. tb*. This method should enable a more rapid and effective drug susceptibility assay of intracellular *M. tb*.

MATERIALS AND METHODS

Bacterial strains, cell lines and culture media

M. tb transgenic strain H37Rv piclGFP from previous work of Dokladda *et al*, 2015 was maintained and propagated in Middlebrook 7H9 broth (Becton, Dickinson and Company, Sparks, MD) or on 7H11 plate agar medium (Becton, Dickinson and Company, Sparks, MD) supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase) and 30 μg/ml kanamycin. Human macrophage-like U937 (courtesy of Dr Sureemas Buates, Faculty of Science, Mahidol University) and THP-1 monocyte cell lines (ATCC, Manassas, VA) were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (complete medium) at 37°C under a humidified atmosphere containing 5% CO_2. Mouse monocyte RAW264.7 (courtesy of Dr Marisa Ponpuak, Faculty of Science, Mahidol University) and J774A.1 cell lines (ATCC, Manassas, VA) were maintained in DMEM medium (GIBCO, Grand Island, NY) supplemented with 10% FBS and 2 mM L-glutamine (complete medium) as described above.

Bacteria preparation

M. tb piclGFP in culture was collected when $A_{600\ nm}$ reached 0.6-0.8, centrifuged at 3,200 g for 10 minutes, and cell pellet washed twice with PBS (P-3813; Sigma-Aldrich, St Louis, MO), resuspended in PBS containing 0.05% Tween 80 (Ajax Finechem, Thermo Fisher Scientific, Scoresby, Victoria, Australia), sonicated eight times at 15 seconds, and centrifuged at 200 g at 4°C for 10 minutes. Bacterial cells in supernatant were collected, counted in a Petroff-Hausser chamber (Electron Microscopy Sciences, Hatfield, PA) and kept at -80°C until used.

Optimal host cell determination

Aliquot of 0.5 ml of U937 and THP-1 cells ($1.2x10^6$ cells/ml RPMI 1640 complete medium) containing 10 and 50 nM phorbol myristate acetate (PMA) to induce cell differentiation respectively was added into well of a 24-well plate, incubated at 37°C for three days under a humidified atmosphere containing 5% CO_2. RAW264.7 and J774A.1 cells ($6x10^5$ in DMEM complete medium) were incubated overnight as described above. Supernatant from each cell culture was removed and 5 multiplicity of infection (MOI) of *M. tb* piclGFP in respective complete medium were added, and cell suspension incubated for 18 hours as described above, supernatant removed and fresh medium containing 100 μg/ml amikacin added, and cell suspension incubated for 3 hours to eliminate extracellular *M. tb*. Then cells were washed twice with respective complete medium and incubated for a further one day and four days as described above prior to fluorescence measurement and determination of colony forming unit (CFU) as follows.

At each time point, infected cells were washed with PBS (Sigma-Aldrich, St Louis, MO) and scraped off plate into 1 ml aliquot of PBS. One hundred µl aliquot was centrifuged at 3,200 g for 10 minutes, pellet added with 100 µl aliquot of 0.05% Napthol Blue Black (Sigma-Aldrich, St Louis, MO) in 0.1 M citric acid pH 2.2 containing 1% Triton X-100 and solution incubated for 10 minutes at 37°C. Five µl aliquots of 10-fold serial dilutions of macrophage cell lysate (10^{-1} - 10^{-4}) were spotted into slots of a Middlebrook 7H11 plate (Becton, Dickinson and Company, Sparks, MD), incubated at 37°C for 2-3 weeks or until bacteria growth could be counted. Colony count is expressed as number of viable bacteria/ 10^5 macrophages.

One hundred µl of 10% (w/v) paraformaldehyde was added to the remaining 900 µl of cell suspension, kept at 4°C for 30 minutes and then cells were subjected to fluorescence-activated cell counting using a BD FACS Canto™ instrument (Becton, Dickenson and Company, Franklin Lakes, NJ) (excitation $\lambda_{485\,nm}$, emission $\lambda_{535\,nm}$).

Optimizations of host cell concentration, MOI and infection and incubation times

For determination of optimal host cell concentration in a 96-well plate, 100 µl aliquots of host cell suspension (10^5-10^6 cells/ml) were incubated as described above and infected with 5MOI *M. tb* H37Rv piclGFP for 18 hours, killed extracellular bacteria by amikacin, further incubated for 6 days, then fluorescence was measured in a micro-plate reader (Victor^{2}V reader; Perkin Elmer, Waltham, MA) with the excitation wavelength of 485 nm and the emission wavelength of 535 nm (excitation λ_{485nm}, emission λ_{535nm}). For determination of optimal MOI, host cells were infected with *M. tb* H37Rv piclGFP at 3, 5, 7, 10 and 15 MOI for 18 hours and assayed as described above, once optimal MOI was determined, optimal infection time was determined at 4, 8, 18 and 24 hours and optimal time of infected cell incubation was determined at 3, 4, 5 and 6 days (host cells were incubated in the medium containing 5MOI *M. tb* H37Rv piclGFP for 4, 8, 18 and 24 hours before incubation in the medium containing amikacin, the infected cells were further incubated for 3, 4, 5 and 6 days before fluorescent intensity was measured).

GFP-based intracellular drug susceptibility assay (iGFPMA) procedure

One hundred µl aliquots of host cells (10^5 cells/well) in appropriate complete medium containing 10 nM PMA were incubated in a 96-well flat-bottom microplate for three days as described above, then infected with optimal MOI of *M. tb* H37Rv piclGFP and processed at the optimal conditions determined from the above experiments. Then, 100 µl aliquot of two-fold serial dilutions of test drug in RPMI 1640 medium was added to each well and incubation continued as described above for another

6 days. Controls were infected cells in the absence of drug (bacteria control) and medium with drug in absence of cells (blank control). Each experiment was conducted in triplicate. Fluorescence intensity was measured using a microplate reader as described above. MIC90 (drug concentration inhibiting 90% bacterial growth) was calculated using the following equation:

Percent inhibition = [(bacteria control fluorescence - blank control fluorescence) - (sample fluorescence - blank control fluorescence)/(bacteria control fluorescence - blank control fluorescence)] x 100

Alamar Blue assay (MABA) procedure

Activity of drugs against extracellular *M. tb* H37Rv piclGFP growth was measured using MABA (Collins and Franzblau 1997). One hundred µl aliquots of drugs at the same concentrations used in iGFPMA but dissolved in 7H9 broth (Becton, Dickinson and Company, Sparks, MD) containing 10% OADC together with 100 µl aliquots of *M. tb* ($A_{600\ nm}$ = 0.005) in same culture medium were added to wells of a 96-well plate. Controls were the same as those used in iGFPMA but blank control contained the highest concentration of test drug. Plate was incubated as described above for 5 days, then 20 µl aliquot of 10X Alamar Blue (Bio-Rad, Hercules, CA) and 12.5 µl aliquot of 20% Tween 80 were added to control wells, and plate incubated for another 24 hours. If bacterial control wells turned from blue to pink color, Alamar Blue reagent solution were added to other wells of the plate. If color in bacteria control wells was blue, then plate was incubated for periods of 24 hours until color of bacteria controls changed to pink, then the remaining wells were treated with Alamar Blue reagent solution. MIC is defined as the lowest drug concentration that prevents bacterial growth (change from blue to pink color).

RESULTS

In order to establish a GFP-based fluorescent assay for testing of drugs against intracellular *M. tb*, four cell lines (murine J774A.1, murine RAW264.7, human THP-1, and human U937), were evaluated for their appropriateness as host cell by determining optimal i) intracellular growth of *M. tb* H37Rv piclGFP and ii) host cell viability at day 4 post-infection. Based on these criteria, both RAW264.7 and U937 cells were possible candidates (Table 1). However, U937, a human monocyte-like cell line, was chosen for subsequent evaluation of cell concentration in a 96 well-

Table 1

Determination of suitable host cell for use in GFP-based intracellular anti-*Mycobacterium tuberculosis* drug susceptibility assay

Parameters	J774A.1 cell line	RAW264.7 cell line	THP-1 cell line	U937 cell line
Fluorescence intensity[a], RFU, mean ± SD	2.7 ± 0.3	27.3 ± 1.5	50.5 ± 1.2	24.7 ± 4.0
Cell viability[b] (x 10^5 cells), mean ± SD	2.6 ± 0.6	15.6 ± 1.3	0.9 ± 0.3	4.0 ± 0.5
M. tuberculosis growth within cell, CFU (x 10^4), mean ± SD	1.2 ± 2.5	24.10± 3.7	27.8 ± 2.8	20.0 ± 5.9
Fluorescence/CFU	2.2	1.1	1.8	1.2

[a]*M. tuberculosis* H37Rv piclGFP; [b]Determined on Day 4 (by counting the blue-stained macrophage nuclei in the cell lysates after napthol blue black staining)
CFU: colony forming unit; RFU: relative fluorescent unit; SD: standard deviation

microplate, and optimal MOI, infection time and incubation time, yielding 10^5 U937 cells/well, MOI of 5, infection time of 18 hours and incubation time of 6 days as being the most optimal conditions for conducting iGFPMA in a microplate format (data not shown).

Activity of several known anti- and non-anti-TB drugs were tested using the developed iGFPMA and standard MABA for intracellular and extracellular *M. tb* respectively. First-line anti-tuberculosis drugs, isoniazid, rifampin and streptomycin, demonstrated MIC90 values against intracellular *M. tb* 2 folds higher than MICs against extracellular Mycobacteria (Table 2). Inhibition by ethambutol against intracellular *M. tb* did not reach MIC90 at the highest concentration tested (40 μg/ml) [MIC80 of 5 μg/ml was observed (data not shown)] compared to MIC of 2.5 μg/ml against extracellular Mycobacteria. Inhibition by pyrazinamide did not reach MIC90 and MIC values against both extracellularly and intracellularly *M. tb* at highest concentration tested (1,600 μg/ml). As regards second-line anti-TB drugs (amikacin, capreomycin and ofloxacin) MIC90 values for intracellular *M. tb* were 8-80 folds higher than MICs for extracellular Mycobacteria, while inhibitory activity of ethionamide and gentamycin did not reach MIC90 at highest concentration tested, 16 and 32 μg/ml respectively. Non-anti-TB drug amoxicillin failed to reach MIC90 value at highest concentration tested (32 μg/ml) but co-trimoxazole demonstrated MIC90 of 16 μg/ml (Table 2).

Table 2

MIC90 (minimum inhibitory concentration for 90% inhibition of growth) and MIC of anti-*Mycobacterium tuberculosis* drugs and amoxicillin determined by a GFP-based intracellular drug susceptibility (iGFPMA) and microplate Alamar Blue (MABA) assay respectively

Drug	iGFPMA MIC90 (μg/ml)	MABA MIC (μg/ml)
Isoniazid	0.16	0.075
Rifampin	0.08	0.04
Ethambutol	>40	2.5
Streptomycin	2.5	1.25
Pyrazinamide	>1,600	>1,600
Amikacin	100	1.25
Capreomycin	16	1.0
Ethionamide	>16	0.75
Ofloxacin	4	0.5
Gentamycin	>32	0.5
Co-trimoxazole	16	NA
Amoxicillin	>32	NA

μg/ml: microgram per milliliter; NA: not applicable

DISCUSSION

The standard method of intra-macrophage drug assay against *M. tb* requires lysis of macrophage and quantification of *M. tb* colonies on solid agar (Rodriguez *et al*, 2018). An iGFPMA was developed to determine inhibitory effects of anti-TB drugs against human macrophage-like U937 cell-infected *M. tb* using a 96-well plate format that could be potentially adapted to a higher throughput platform, *eg* in 384-well plate. The method utilizes recombinant *M. tb* H37Rv carrying piclGFP under the control of an intra-cellular-responsive *icl* promoter which will be highly expressed when bacteria are intracellualar (Dokladda *et al*, 2015). The method obviates the necessity of host cell lysis and subsequent tedious culture of released *M. tb* and allows direct quantification of intra-cellular Mycobacteria. Having GFP gene under the regulation of *icl* promoter reduces background signals from extracellular

M. tb H37Rv piclGFP, although infected U937 cells were cultured in the presence of amikacin, a poorly -penetrating host cell anti-TB drug (requires more than 4 hours to enter eukaryotic cells) (Maurin and Raoult 2001).

MIC90 values of three known first line anti-TB drugs were, as expected, higher for intracellular than MICs of extracellular *M. tb*, consistent with literature reports (Table 3). Surprisingly, MIC90 and MIC of pyrazinamide could not be determined, but results from other studies are inconsistent, with one study supporting results from the present study and two studies reporting inhibitory activity (Table 3). In an early work, Rastogi and co-workers (1988) observed pyrazinamide having bacteriostatic effect against intracellular *M. tb* when J774 mouse macrophages are treated prior to infection, but no effect if the drug is added two days post-infection, a phenomenon more alike the clinical situation (Rastogi *et al*, 1988). Heifets *et al* (2000) noted pyrazinamide does not inhibit *M. tb* growth in either TNF/IFN-activated or non-activated human monocyte-derived macrophages. Hence, demonstration of inhibitory activity of pyrazinamide (and probably other anti-TB drugs) depends on nature of intracellular bacteria within different host cells.

There are few previous reports on high-throughput methods for screening new compounds against intracellular pathogenic bacteria. Methods using reporter enzymes, such as beta-galactosidase or luciferase, are rapid and sensitive, but they require addition of substrates to fully infected cultures before signal measurements, with potential risk of infection to investigators (Sorrentino *et al*, 2016; Srivastava *et al*, 1997). The use of intrinsic fluorescent markers such as GFP allows simpler and safer screening methods to be employed with minimal risk of infection to investigators as results can be measured from a sealed culture containment.

In conclusion, iGFPMA method developed provides a safe and more rapid screening assay of drugs against intracellular *M. tb*, with results comparable with published values. In addition, the GFP reporter with red-shifted emission wavelength generates a stronger signal enabling improved sensitivity compared to conventional reporter GFP. The method needs to be validated with drugs that act exclusively on intracellular *M. tb*.

ACKNOWLEDGEMENTS

The authors thank Drs Sureemas Buates and Marisa Ponpuak, Faculty of Science, Mahidol University for kindly providing U937 and RAW264.7 cell line respectively, Pamaree Billamas, Tada Jathayothin and Sarinya Jaitrong for assistance in conducting the study, and Drs Philip James Shaw and Piyanun Harnpicharnchai for kindly reviewing the manuscript. The study was supported by a grant from National Center for Genetic Engineering and

Table 3

Comparison of anti-tuberculosis drug assays against intracellular *Mycobacterium tuberculosis*

Reference	*Mycobacterium tuberculosis* strain	Host cell (MOI)	Measurement method	Unit	INH (μg/ml)	RMP (μg/ml)	EMB (μg/ml)	SM (μg/ml)	PZA (μg/ml)
This study	H37Rv piclGFP	U937 cell line (5)	Fluorescence	MIC90	0.16	0.08	>40 MIC80 = 5	2.5	>1,600
Sorrentino (2016)	H37Rv luciferase/ Erdman GFP	THP-1 cell line (1)	Luciferase activity and/or fluorescence	MIC90	0.62/0.43	0.02/0.06	16.67/14.63	-	>50/>50
Chanwong et al (2007)	H37Rv	Guinea pig macrophage (10)	Bacterial colony count	MIC	0.1	0.1	-	-	-
Hartkoorn et al (2007)	H37Rv	THP-1 cell line (10)	Host cell viability	EC50	0.04	0.15	0.24	-	-
Takii et al (2002)	H37Rv	Fibroblast MRC-5 cell line (50)	Viability of host cell	MIC	0.428	0.013	3.46	1.82	3.85
Dhillon and Mitchison (1989)	*M. microti* OV254	Murine peritoneal macrophage (1)	Bacterial colony count	MIC	0.05	0.05	-	5	20

EC50: effective dose to inhibit 50% viability; EMB: ethambutol; INH: isoniazid; MIC: minimum inhibitory concentration; MIC90: minimum concentration to inhibit 90% growth; MOI: multiplicity of infection; RPM: rifampin; SM: streptomycin; PZA: pyrazinamide

Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

REFERENCES

Banfi E, Scialino G, Monti-Bragadin C. Development of a microdilution method to evaluate *Mycobacterium tuberculosis* drug susceptibility. *J Antimicrob Chemother* 2003; 52: 796-800.

Beste DJ, Espasa M, Bonde B, Kierzek AM, Stewart GR, McFadden J. The genetic requirements for fast and slow growth in mycobacteria. *PLoS One* 2009; 4: e5349.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science* 1994; 263: 802-5.

Changsen C, Franzblau SG, Palittapongarnpim P. Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter. *Antimicrob Agents Chemother* 2003; 47: 3682-7.

Chanwong S, Maneekarn N, Makonkawkeyoon L, Makonkawkeyoon S. Intracellular growth and drug susceptibility of *Mycobacterium tuberculosis* in macrophages. *Tuberculosis (Edinb)* 2007; 87: 130-3.

Collins L, Franzblau SG. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob Agents Chemother* 1997; 41: 1004-9.

Collins LA, Torrero MN, Franzblau SG. Green fluorescent protein reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1998; 42: 344-7.

Dhillon J, Mitchison DA. Activity and penetration of antituberculosis drugs in mouse peritoneal macrophages infected with Mycobacterium microti OV254. *Antimicrob Agents Chemother* 1989; 33: 1255-9.

Dokladda K, Billamas P, Palittapongarnpim P. Different behaviours of promoters in *Mycobacterium tuberculosis* H37Rv and H37Ra. *World J Microbiol Biotechnol* 2015; 31: 407-13.

Hartkoorn RC, Chandler B, Owen A, *et al*. Differential drug susceptibility of intracellular and extracellular tuberculosis, and the impact of P-glycoprotein. *Tuberculosis (Edinb)* 2007; 87: 248-55.

Hazbon MH, Guarin N, Ferro BE, *et al*. Photographic and luminometric detection of luciferase reporter phages for drug susceptibility testing of clinical *Mycobacterium tuberculosis* isolates. *J Clin Microbiol* 2003; 41: 4865-9.

Heifets L, Higgins M, Simon B. Pyrazinamide is not active against *Mycobacterium tuberculosis* residing in cultured human monocyte-derived macrophages. *Int J Tuberc Lung Dis* 2000; 4: 491-5.

Kain SR, Adams M, Kondepudi A, Yang TT, Ward WW, Kitts P. Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques* 1995; 19: 650-5.

Machuca I, Vidal E, De La Torre-Cisneros J, Rivero-Roman A. Tuberculosis in immunosuppressed patients. *Enferm Infecc Microbiol Clin* 2018; 36: 366-74.

Maurin M, Raoult D. Use of aminoglycosides in treatment of infections due to intracellular bacteria. *Antimicrob Agents Chemother* 2001; 45: 2977-86.

Rastogi N, Potar MC, David HL. Pyrazinamide is not effective against intracellularly growing *Mycobacterium tuberculosis. Antimicrob Agents Chemother* 1988; 32: 287.

Rodriguez DC, Ocampo M, Salazar LM, Patarroyo MA. Quantifying intracellular *Mycobacterium tuberculosis*: an essential issue for in vitro assays. *Microbiologyopen* 2018; 7: e00588.

Sorrentino F, Gonzalez Del Rio R, Zheng X, *et al*. Development of an Intracellular screen for new compounds able to inhibit *Mycobacterium tuberculosis* growth in human macrophages. *Antimicrob Agents Chemother* 2016; 60: 640-5.

Srivastava R, Kumar D, Subramaniam P, Srivastava BS. β-galactosidase reporter system in mycobacteria and its application in rapid antimycobacterial drug screening. *Biochem Biophys Res Commun* 1997; 235: 602-5.

Syre H, Phyu S, Sandven P, Bjorvatn B, Grewal HM. Rapid colorimetric method for testing susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin in liquid cultures. *J Clin Microbiol* 2003; 41: 5173-7.

Takii T, Yamamoto Y, Chiba T, *et al*. Simple fibroblast-based assay for screening of new antimicrobial drugs against *Mycobacterium tuberculosis. Antimicrob Agents Chemother* 2002;46: 2533-9.