Journal of Antimicrobial Chemotherapy (2008) **62**, 1037–1045
doi:10.1093/jac/dkn307
Advance Access publication 7 August 2008

JAC

A microbiological assessment of novel nitrofuranylamides as anti-tuberculosis agents

Julian G. Hurdle[1], Robin B. Lee[1], Nageshwar R. Budha[1], Elizabeth I. Carson[1], Jianjun Qi[1], Michael S. Scherman[2], Sang Hyun Cho[3], Michael R. McNeil[2], Anne J. Lenaerts[2], Scott G. Franzblau[3], Bernd Meibohm[1] and Richard E. Lee[1]*

[1]*Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN, USA;*
[2]*Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA;*
[3]*Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, USA*

Received 16 May 2008; returned 13 June 2008; revised 20 June 2008; accepted 3 July 2008

***Objectives*: Nitrofuranylamides (NFAs) are nitroaromatic compounds that have recently been discovered and have potent anti-tuberculosis (TB) activity. A foundational study was performed to evaluate whether this class of agents possesses microbiological properties suitable for future antimycobacterial therapy.**

***Methods*: Five representative compounds of the NFA series were evaluated by standard microbiological assays to determine MICs, MBCs, activity against anaerobic non-replicating persistent *Mycobacterium tuberculosis*, post-antibiotic effects (PAEs), antibiotic synergy and the basis for resistance.**

***Results*: The antimicrobial activity of these compounds was restricted to bacteria of the *M. tuberculosis* complex, and all compounds were highly active against drug-susceptible and -resistant strains of *M. tuberculosis*, with MICs 0.0004–0.05 mg/L. Moreover, no antagonism was observed with front-line anti-TB drugs. Activity was also retained against dormant bacilli in two *in vitro* low-oxygen models for *M. tuberculosis* persistence. A long PAE was observed, which was comparable to that of rifampicin, but superior to isoniazid and ethambutol. Spontaneous NFA-resistant mutants arose at a frequency of 10^{-5}–10^{-7}, comparable to that for isoniazid (10^{-5}–10^{-6}). Some of these mutants exhibited cross-resistance to one or both of the nitroimidazoles PA-824 and OPC-67683. Cross-resistance was associated with inactivation of the reduced F_{420}-deazaflavin cofactor pathway and not with inactivation of the Rv3547, the nitroreductase for PA-824 and OPC-67683.**

***Conclusions*: Based on these studies, NFAs have many useful antimycobacterial properties applicable to TB chemotherapy and probably possess a unique mode of action that results in good activity against active and dormant *M. tuberculosis*. Therefore, the further development of lead compounds in this series is warranted.**

Keywords: *M. tuberculosis*, nitrofuran, nitroaromatic antibiotics, latency

Introduction

Tuberculosis (TB) is one of the leading causes of death worldwide due to an infectious disease agent. According to the World Health Organization (WHO), there are ~8 million new cases of active TB disease each year, with an estimated 2 million deaths.[1] In addition, one-third of the world's population is latently infected with *Mycobacterium tuberculosis*, presenting a large reservoir from which new TB infections will arise.[2] In recent years, the lethal synergy of TB and HIV epidemics[3] and the emergence of multidrug-resistant (MDR) *M. tuberculosis* have further complicated the global fight against TB.[4,5] These trends are predicted to continue for at least the next two decades[6] and so exemplify the urgent need for new anti-TB drugs.

In an effort to develop novel drugs to treat TB, a library of compounds was screened for inhibition of *M. tuberculosis* UDP-Gal

*Corresponding author. Tel: +1-901-448-6018; Fax: +1-901-448-6828; E-mail: relee@utmem.edu

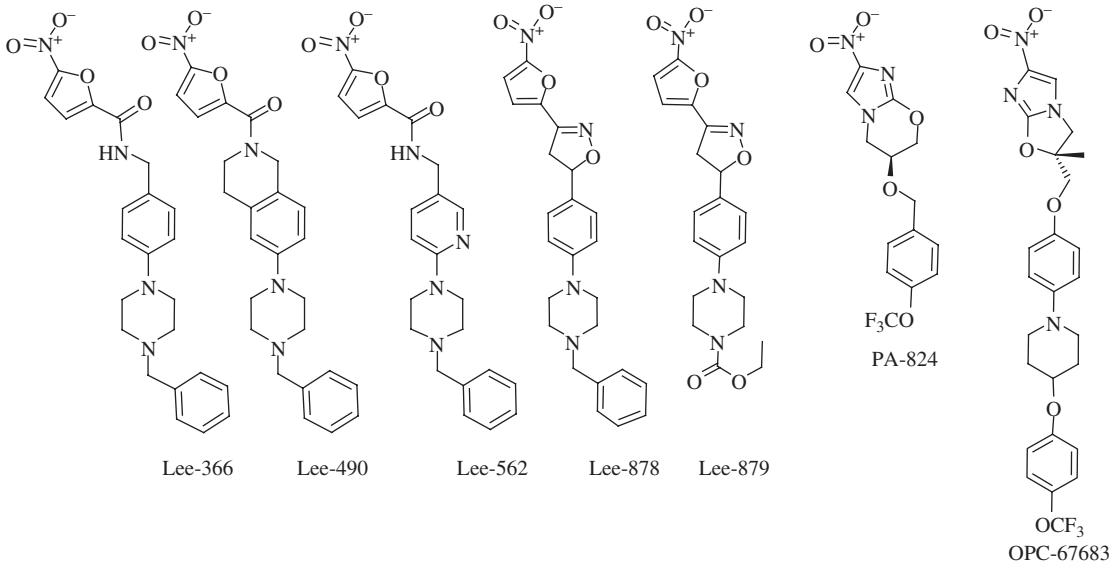

Figure 1. Chemical structures of nitrofuranylamides and comparator nitroimidazoles used in this study.

mutase.[7] This led to the discovery of the nitrofuranylamides (NFAs), which exhibited good activity against *M. tuberculosis*, MIC $\leq$ 1.6 mg/L.[7] Although UDP-Gal mutase was later disproved as the primary cellular target, subsequent generations of novel nitrofuranyl compounds were optimized based on anti-tubercular MICs and therapeutic properties.[8–11] However, a comprehensive evaluation of the antibacterial effects of these compounds has not been reported. Since early microbiological characterization of emerging antibiotic leads is an important part of the drug development cycle,[12] five representative NFAs were selected for this study (Figure 1).

The nitroaromatic class of antibiotics is a successful part of any infectious disease physician's repertoire and is mostly used to treat anaerobic infections. However, these antibiotics are generally not utilized to treat *M. tuberculosis* infections due to the low anti-TB activity of the currently approved members of this class. We believe that nitroaromatic antibiotics represent an understudied class for TB therapy. Members of this class include: the related nitrofuranyl species furaltadone, nitrofurantoin and nitrofurazone, which demonstrate weak activity against *M. tuberculosis*,[13] the nitroimidazole metronidazole and the nitrothiazole nitazoxanide. The recent discovery of the nitroimidazole PA-824 as an anti-TB agent first suggested the potential of this class of antibiotics to treat *M. tuberculosis* infections.[14] PA-824 and the newer nitroimidazole OPC-67683 (Figure 1) are in current clinical trials as anti-TB agents.[15] These compounds are all prodrugs requiring the bio-reduction of their nitro group to form reactive species, which subsequently exhibit antibacterial properties.[16–18] In the case of PA-824 and OPC-67683, activation requires the action of the reduced deazaflavin cofactor F_{420}, its reductively activating enzyme F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD1) and the nitroreductase gene product of *Rv3547*. Mutations within these determinants have been shown to confer resistance to nitroimidazoles that require their function for activation[16,18] The mechanism by which NFAs are activated in *M. tuberculosis* is, however, unknown.

Much recent attention has been given to developing nitroimidazoles as treatments for TB, since these agents are one of the few drug classes with demonstrated activity against latent bacilli.[13,18,19] As a new analogous chemical class of nitroaromatics, NFAs may therefore represent a new important approach with which to counteract both active and latent TB disease. Herein, we report a microbiological assessment of representative compounds from our NFA optimization programme, which was performed to evaluate whether this class of compounds demonstrates characteristics suitable for future antimycobacterial therapy and to establish baseline information to guide subsequent stages of drug development.

Materials and methods

Antibiotics

The synthesis of all nitrofuranyl antibiotics used in this study (Lee-366, -490, -562, -878, and -879; Figure 1) has been described previously.[8–10] The solubility and pharmacokinetics of these compounds have also been described.[20] The reference compound OPC-67683 was synthesized using the synthetic scheme of Sasaki *et al.*[21] and PA-824 was a gift from Dr Clifton Barry of the National Institutes of Health. The antibiotics isoniazid, rifampicin and ethambutol were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Stock solutions of antibiotics were prepared at 10 mg/mL in DMSO and stored at -80°C.

Growth of bacteria

Unless otherwise stated, all mycobacterial species were grown in Middlebrook 7H9 broth (Difco Laboratories, MI, USA) supplemented with 10% albumin–dextrose complex and 0.05% (v/v) Tween 80 or on Middlebrook 7H11 agar (Difco Laboratories) supplemented with 10% oleic acid–albumin–dextrose complex. Methicillin-resistant *Staphylococcus aureus* ATCC 33591, *Enterococcus faecalis* ATCC 33186, *Bacillus anthracis* Sterne 34F2, *Escherichia coli* K12 and *Pseudomonas aeruginosa* PAO1 were grown in Mueller–Hinton

(MH) broth (Remel Inc., Lenexa, KS, USA), while *Streptococcus pyogenes* MGAS1 was cultured in MH broth supplemented with 5% (v/v) lysed horse red blood cells (BD Diagnostic Systems, USA).

MIC determination

MICs were determined using the microbroth dilution method according to the CLSI[22] and were read by visual inspection. Two-fold serial dilutions of antibiotic in 100 μL of the appropriate broth medium were first prepared in 96-well round-bottomed microtitre plates (Nunc, USA). An equivalent volume (100 μL) of bacterial broth containing $\sim 10^5$ bacterial cfu/mL was added to each well to give final concentrations of drug starting at 200 mg/L, and the plates were incubated aerobically at 37°C. *M. tuberculosis*, *Mycobacterium bovis* BCG and *Mycobacterium avium* ATCC 25291 microtitre plates were incubated for 7 days, and all other strains were incubated overnight. The MIC was recorded as the lowest concentration of drug that prevented visible growth. MICs for *M. tuberculosis* were also evaluated by a modified agar proportion method based on the CLSI standards. Briefly, 24-well plates were prepared with 2-fold serial dilutions of antibiotic in 2 mL of 7H11 agar and were inoculated with $\sim 10^5$ cfu. After 3 weeks of incubation, the MIC was recorded as the lowest concentration that prevented growth.

MBC determination

MBC tests were performed in duplicate, directly from the microtitre plates used to determine drug MICs for *M. tuberculosis* H37Rv. After recording the MICs, the cell pellets were resuspended and the contents of each well transferred to separate microfuge tubes before removing the antibiotic by centrifugation (21 000 **g**, 2 min). Pellets were washed twice with 7H9 broth, resuspended in 200 μL of 7H9 and 100 μL aliquots plated onto 7H11 agar. Plates were incubated for 4 weeks at 37°C. For compounds Lee-366, -490 and -562, the MBC was recorded as the lowest concentration of drug that killed 99% of cells present in the starting inocula (determined by initial viable counts). For Lee-878 and -879, a 99% killing could not be observed since compounds at higher concentrations precipitated during the 7 day incubation and could not be removed by washings. This resulted in precipitated drug being transferred to the agar plates causing subsequent growth inhibition. Therefore, at concentrations that did not precipitate, the MBC was recorded as the lowest concentration of drug demonstrating maximum killing (this corresponded to 97% killing of cells for Lee-878 and -879).

Activity against hypoxic non-replicating persistent M. tuberculosis

Two *in vitro* models, both based on the well-established principle of culturing hypoxic persister cells under gradual oxygen depletion, were used to evaluate: (i) if NFAs affect the metabolic recovery of non-replicating persisters (NRPs); and (ii) their ability to kill hypoxic NRP cells.

Determination of activity in a low-oxygen recovery assay (LORA). The method for the LORA was performed as recently described by Cho *et al.*[23] In brief, a low-oxygen adapted culture of recombinant H37Rv (pFCA-*luxAB*), expressing a *Vibrio harveyii* luciferase gene driven by an acetamidase promoter, was grown in a BiostatQ fermentor. Cells were collected on ice, washed in PBS and stored at −80°C. About 10^5 cfu/mL of thawed NRP cells were exposed to 2-fold serial dilutions of antibiotic in 7H9 broth in black 96-well plates, which were incubated anaerobically at 37°C for 10 days. Cultures were allowed to recover in an aerobic environment (5% CO_2) for 28 h before luminescence readings were obtained. The data were analysed graphically, and the lowest concentration of antibiotic preventing metabolic recovery was determined as described previously.[23]

Eradication of cells in a low-oxygen Wayne model. The NFAs were tested in duplicate in the low-oxygen Wayne model system as described by Lenaerts *et al.*[24] Briefly, mid-log cultures of *M. tuberculosis* H37Rv were diluted 100-fold in Dubos medium and grown at 37°C with slow stirring for 24 days in tubes sealed with silicone rubber septa. After *in situ* anaerobic conditions were established, drugs were injected through the septa of 24-day-old cultures at final concentrations of 10 and 50 mg/L and incubation continued for another 4 days; these concentrations have been previously used to evaluate several anti-TB drugs in this model and were adopted here for direct comparison.[24] The numbers of cfu were subsequently determined on diluted cultures by plating onto Middlebrook 7H11 agar.

Chequerboard synergy assay

The activities of NFAs in combination with rifampicin, isoniazid and ethambutol were evaluated in duplicate against *M. tuberculosis* H37Rv by the chequerboard titration method in 96-well round-bottomed plates. Plates contained bacterial inocula (10^5 cfu/mL) and 2-fold serial dilutions of each antibiotic in total volumes of 200 μL of broth. The maximum and minimum concentrations of each diluted drug were at least ±4-fold their MIC. Following 7 days of incubation at 37°C, MICs of drug combinations were read by visual inspection and fractional inhibitory concentration (FIC) indices for *M. tuberculosis* H37Rv were calculated as described by Eliopoulos and Moellering.[25] FIC indices were interpreted as follows: ≤ 0.5, synergy; $>0.5-4$, no interaction; and >4, antagonism.[26]

Post-antibiotic effect (PAE)

The PAEs for NFAs were determined using previously described approaches,[27] which were modified to accommodate the doubling time of *M. bovis* BCG (24 h). *M. bovis* BCG was grown from a −80°C glycerol stock to mid-log phase, subcultured to an $OD_{600} \sim 0.001$ and grown to early log phase (OD_{600} 0.2). Aliquots (50 mL) were exposed to NFAs at their peak serum concentrations ($C_{max} \approx 5$ mg/L) as well as fractional doses (0.5 and 0.05 mg/L). Experimental C_{max} values for NFAs were previously reported and the value 5 mg/L represents the average peak concentration in serum following oral administration in rats dosed at 100 mg/kg.[11] Control drug experiments were conducted with C_{max} concentrations in humans for rifampicin (10 mg/L), isoniazid (4 mg/L) and ethambutol (5 mg/L).[28,29] After incubation at 37°C for 2 h, antibiotic was removed by centrifugation (3600 **g**, 10 min, 37°C) and the cell pellets were washed three times in 50 mL of pre-warmed 7H9. Finally, the washed pellets were resuspended in 50 mL of pre-warmed 7H9 and incubated at 37°C until they reached growth saturation (OD_{max}). The OD_{600} and the number of cfu were determined for each culture before drug exposure, after drug removal and at 24 h intervals thereafter. The duration of PAE was calculated according to Odenholt-Tornqvist[27] as the time taken for the antibiotic-treated culture to reach 50% of the OD_{max} of the drug-free culture minus the time taken for the drug-free control to reach the same point. A PAE value of ≥ 24 h was considered significant, since this value indicates the ability of a drug to retard the growth of *M. bovis* BCG by one doubling time after its removal, and also reflects a daily dose interval for most TB drug regimens. *M. bovis* BCG was adopted as a surrogate for these experiments in view of the laborious requirements and potential health risks for PAE studies in *M. tuberculosis*.[28]

Selection and phenotypic characterization of NFA-resistant mutants

Spontaneous mutation frequencies were determined for *M. tuberculosis* H37Rv by plating 100 μL of a saturated culture onto 7H11 agar plates containing drug at 2, 4, 8, 16 and 32× the agar MIC. Plates were incubated for 3 weeks at 37°C and the mutation frequency was recorded as the number of resistant colonies divided by the total number of viable cells. Ten isolated colonies were then picked at random from the 4 and 8× selection plates and inoculated into 7H9 broth containing the concentration of antibiotic on which they were selected. Colonies able to regrow were then grown to mid-log phase in the absence of drug and stored at −80°C. MICs were determined for 25 NFA-resistant mutants (5 mutants per NFA) against rifampicin, isoniazid, ethambutol, PA-824 and OPC-67683 by microbroth dilution.

Cytotoxicity

Vero epithelial cells (from African green monkey; ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator (37°C, 5% CO_2). Cells were dislodged with a cell scraper, collected by centrifugation, resuspended in fresh medium at $\sim 10^6$ cells/mL, dispensed into 96-well microtitre plates (100 μL/well) and incubated for 18 h at 37°C. Two-fold serial dilutions of test compounds (800–0.4 mg/L) in DMEM with FBS were subsequently added and cells incubated for another 72 h. From triplicate studies, the cytopathic effects of compounds were evaluated colorimetrically using the MTT cell proliferation assay (ATCC). IC_{50} data were obtained from dose–response curves plotted using Graphpad prism 5.

Bacterial reverse mutation (BRM) test

The mutagenic potential of NFAs (Lee-366, -562 and -878) was evaluated by the BRM (Ames) test as described previously,[30,31] using *Salmonella typhimurium* TA98 and its daughter strain TA98NR. Strain TA98NR lacks the classical bacterial nitroreductase system and was included to characterize the mutagenicity of compounds in the absence of nitro reduction.[32] NFAs were tested against TA98 at 0.05, 0.1, 0.5, 1 and 5 μg/plate and TA98NR at 0.5, 1, 5, 10 and 50 μg/plate. Both bacterial strains were cultured overnight in nutrient broth and $\sim 2 \times 10^8$ cells were added to each assay mixture containing NFA (dissolved in 100 μL of DMSO) in the absence or presence of rat liver 10% S9 mix (500 μL, a cofactor supplemented post-mitochondrial fraction for metabolic activation). The cultures were incubated for 20 min at 37°C, with shaking. An aliquot of 2.0 mL of top agar was subsequently added and the entire mixture then poured onto minimal agar plates. The number of revertant colonies was counted after 48–72 h of incubation at 37°C. A compound was considered mutagenic when the number of revertant colonies on plates containing the test compound was more than three times the drug-free control. Positive controls included: TA98 (+S9), 2-amino anthracene (2-AA, 1 μg/plate); TA98 (−S9), 2-nitro fluorene (2-NF, 1 μg/plate); and TA98-NR (±S9), 1,8-dinitro pyrene (1,8-DNP, 0.1 μg/plate).

Results and discussion

Anti-TB activity

The antimycobacterial activities of NFAs are summarized in Table 1. Only strains belonging to the TB complex were significantly affected by NFAs. Furthermore, NFAs did not demonstrate

Table 1. Antibacterial activities of five nitrofuranylamide antibiotics

	Antibiotic activity (mg/L)							
Organism	366	490	562	878	879	RIF	EMB	INH
M. tuberculosis H37Rv								
broth MIC	0.012	0.012	0.006	0.006	0.006	0.024	0.8	0.024
agar MIC	0.012	0.003	0.003	0.003	0.0015	0.024	0.8	0.003
activity in LORA	0.17	0.85	0.71	0.2	0.3	1.8[a]	>128[a]	>128[a]
MBC[b]	0.4	1.6	0.4	0.1	0.1	0.05	NA	0.1
M. tuberculosis CDC1551[c]	0.024	0.1	0.05	0.012	0.1	0.2	6.25	0.05
M. tuberculosis HN878[c]	0.05	0.05	0.05	0.012	0.006	0.4	12.5	0.024
M. tuberculosis UT15	0.012	0.012	0.012	0.0008	0.0004	0.012	0.4	0.024
M. tuberculosis UT18	0.012	0.006	0.012	0.0008	0.0008	0.024	0.8	0.024
M. tuberculosis UT26	0.012	0.012	0.012	0.006	0.003	0.012	0.8	0.012
M. tuberculosis UT29	0.024	0.024	0.024	0.012	0.012	0.024	0.8	0.024
M. tuberculosis UT30[d]	0.006	0.012	0.012	0.003	0.003	0.024	0.8	0.024
M. bovis BCG	0.012	0.006	0.0015	0.0008	0.003	0.012	0.8	0.05
M. avium ATCC 25291	200	25	100	12.5	100	1.6	25	3.12
M. smegmatis mc^2155	100	50	100	100	25	25	1.6	0.24

NA, not assessed; RIF, rifampicin; EMB, ethambutol; INH, isoniazid.
[a]Values obtained from Cho *et al.*[23]
[b]MBCs for Lee-366, -490 and -562 reflect 99% killing of cells. Due to drug precipitation at higher concentrations, MBCs for Lee-878 and -879 reflect 97% killing.
[c]With the exception of strains HN878 and CDC1551, all other MICs were determined in broth.
[d]*M. tuberculosis* UT30 is streptomycin-resistant at 4 mg/L.

significant activity (6.12 to >200 mg/L) against the other clinically relevant non-mycobacterial species tested. This indicates that the antibacterial action of NFAs is specific to the TB complex. This finding is consistent with the unique mode of action displayed by other recent nitroaromatics against *M. tuberculosis*.[16,18,24] NFAs retained their potency against a panel of clinical TB strains, including a streptomycin-resistant isolate and four pan-susceptible isolates from unrelated disease clusters recently isolated in the US state of Tennessee, the highly transmissible strain CDC1551[33] and the drug-resistant strain HN878.[34] The finding of activity against HN878 is important as this strain belongs to the widely disseminated W-Beijing family of isolates that show a propensity for developing multidrug resistance and disease outbreaks worldwide.[35] To evaluate whether the anti-TB activity shown by NFAs was bacteriostatic or cidal, MBCs were determined for strain H37Rv. As expected, both rifampicin and isoniazid were found to be bactericidal (Table 1). However, data obtained for NFAs indicated that these agents induced bacteriostasis at concentrations equivalent to their MIC and were bactericidal at higher concentrations ranging from 0.1 to 1.56 mg/L (Table 1). NFAs therefore appear to be primarily bacteriostatic, although bactericidal killing occurs at concentrations within the experimentally determined C_{max}.[11]

Activity against dormant TB in two hypoxic in vitro models

Activity against NRP-TB in LORA. The activity of NFAs was evaluated against NRP cells of *M. tuberculosis* using a recently validated luminescence LORA.[23] In this assay, cells exposed to drug during the low-oxygen stage, which subsequently express lower luminescence compared with drug-free controls after recovery, are indicative that the drug is effective against NRP-*M. tuberculosis*. In this way, several classes of antibiotics have recently been successfully evaluated for anti-NRP action.[23] In this study, the lowest concentrations of NFA that prevented metabolic recovery of NRP-*M. tuberculosis* ranged from 0.17 to 0.85 mg/L. Although this represents a 14- to 118-fold increase over the MICs for aerobic cultures, these values still provide a clear indication that NFAs affect essential cellular events within NRP-*M. tuberculosis* (Table 1). Indeed, the activity was found to be comparable to the action of rifampicin (1.8 mg/L),[23] which is the only current front-line TB therapy with the ability to sterilize latent infections.[36] In contrast, the anti-TB drugs isoniazid and ethambutol were both shown to be inactive (Table 1). The NFAs were also superior to several other drug classes that were previously characterized using the LORA model.[23]

Eradication of M. tuberculosis in the Wayne model system. To further investigate the anti-dormancy activities shown by NFAs, the antibiotics Lee-366 and -490 were examined in a traditional Wayne-type model system. The Wayne model determines the cidality of drugs against NRP cells cultured under constant anaerobic conditions, which is in contrast to the LORA system that requires a brief exposure of cells to ambient air to generate robust luminescence signals during metabolic recovery.[23] The Wayne model data were consistent with the data obtained in the LORA system indicating that isoniazid lacked activity against NRP cells, whereas rifampicin was highly bactericidal, killing 99.6% of the cell population at 10 mg/L. The representative NFA antibiotics Lee-366 and -490 were also active against the NRP cells with respective kill rates of 86.2% and 86.8% at 10 mg/L and 99.4% and 99.5% at 50 mg/L, respectively. These results suggest that the mode of action of NFAs is independent of cell growth, under *in vitro* conditions that mimic TB latency.

Antibiotic synergy

In combination with rifampicin or ethambutol, NFAs were either synergistic or indifferent against *M. tuberculosis* H37Rv having FIC indices of 0.5–0.75 and 0.31–0.75, respectively. Although no synergy was observed with isoniazid, this combination was also not antagonistic since the FIC index ranged from 1 to 1.5. Combinations of rifampicin and ethambutol (FIC = 0.37–0.63) and rifampicin and isoniazid (FIC = 0.56–0.75) were in accordance with previous reports.[37,38] Therefore, NFAs would most likely be suitable for use in combination treatments with front-line agents for TB.

PAE in M. bovis

The PAE is a well-established desirable pharmacodynamic parameter that reflects delayed bacterial growth upon removal of antibiotic from the bacterial growth environment. Following 2 h of pulse exposure to 5, 0.5 and 0.05 mg/L of each NFA antibiotic, the growth of log-phase *M. bovis* BCG cells was severely retarded in a dose-dependent manner as shown by long PAE recovery times (Table 2 and Figure 2). These values were comparable to the PAE induced by rifampicin, but were far superior to those of ethambutol and isoniazid. Viability counts determined before exposure and immediately after washings were in all cases similar to drug-free controls, indicating that substantial cell death did not occur during pulse dosing (data not shown). Previous studies of antibiotic-induced PAEs in slow-growing mycobacteria have been performed using the Bactec T100 and colony counting assays.[28,39] However, in this study, OD_{600} readings were found to provide a reasonable, rapid method for the assessment of delayed bacterial growth. The data from viable counting were relatively consistent with optical density readings (Figure 2), and the PAE results for rifampicin, isoniazid and ethambutol were in keeping with previously published information. These results suggest that in theory antibiotics such as NFAs that impose long PAEs could improve clinical outcomes for TB therapy since they could

Table 2. Comparison of the PAEs induced by nitrofuranylamides (NFAs) and other antibiotics against *M. bovis* BCG after 2 h pulse dosing; PAEs were determined from OD_{600} data[27]

	PAE (h)		
Antibiotic	C_{max}[a]	$^1/_{10}$ C_{max}[a]	$^1/_{100}$ C_{max}[a]
Lee-366	148	135	79
Lee-490	230	164	56
Lee-562	169	149	67
Lee-878	119	95	51
Lee-879	136	111	46
Rifampicin	156		
Ethambutol	8		
Isoniazid	22		

[a]C_{max} for NFAs = 5 mg/L, ethambutol = 5 mg/L, isoniazid = 4 mg/L and rifampicin = 10 mg/L.

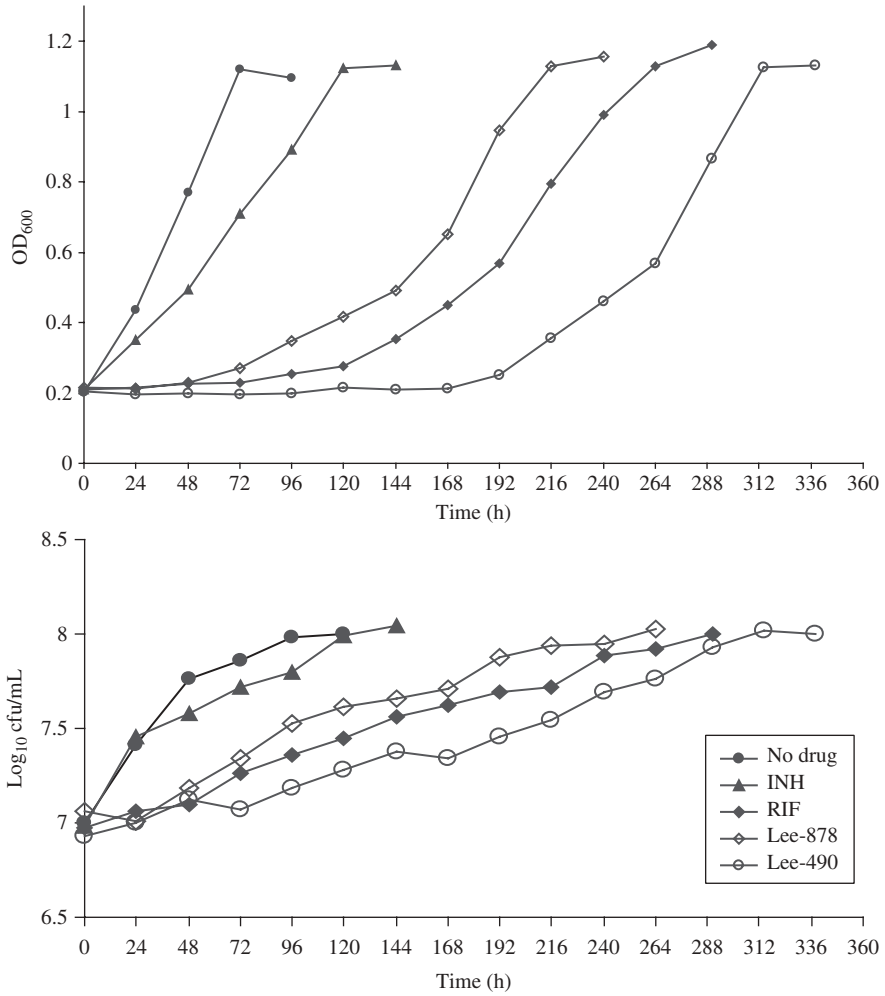

Figure 2. Growth of *M. bovis* BCG after pulse dosing with nitrofuranylamides (NFAs), isoniazid (INH) and rifampicin (RIF) at C_{max} concentrations of 5, 4 and 10 mg/L, respectively. Compounds Lee-878 and -490 demonstrate the lowest and highest PAEs found for NFAs.

(i) enable fewer overall doses and (ii) limit drug toxicity as a result of decreased periods of exposure.[40]

Development of resistance to NFAs

NFA-resistant mutants of *M. tuberculosis* H37Rv were selected *in vitro* to quantify the proportion of spontaneous resistant mutants and to assess the potential for cross-resistance with other front-line anti-TB agents. In summary, mutants arose at frequencies between 0.73×10^{-6} and 10×10^{-6} on agar containing 2–32× the MICs of respective NFAs. Individual mutation frequencies were as follows: Lee-366 ($4.0–9.8 \times 10^{-6}$); Lee-490 ($5.1–8.0 \times 10^{-6}$); Lee-562 ($7.1–8.7 \times 10^{-6}$); Lee-878 ($3.3–15 \times 10^{-6}$); and Lee-879 ($0.73–10 \times 10^{-6}$). In comparison, the mutation frequencies for resistance to established drugs were as follows: ethambutol ($2.2 \times 10^{-6}–4.4 \times 10^{-7}$); isoniazid ($2.3 \times 10^{-5}–1.4 \times 10^{-6}$); and rifampicin ($1.7 \times 10^{-6}–3.8 \times 10^{-7}$). The potential for the development of resistance to NFAs, *in vitro*, is therefore analogous to current major front-line anti-TB drugs.

Phenotypic characterization of NFA-resistant mutants

A panel of 25 NFA-resistant mutants was established by choosing five of the resistant mutants for each NFA drug described above. As expected, the entire mutant panel was susceptible to ethambutol, isoniazid and rifampicin. Owing to their structural similarity to nitroimidazoles PA-824 and OPC-67683, the panel was additionally screened against these compounds. Eight mutants (32%) were found to be cross-resistant to PA-824 and 11 mutants (44%) were cross-resistant to OPC-67683 (Table 3). Low-level resistance to OPC-67683 was seen in one mutant each for Lee-366, -490 and -879 (MICs $\geq$ 0.05 mg/L) and only one mutant of Lee-879 displayed low-level resistance to PA-824 (MIC = 0.8 mg/L). No cross-resistance to PA-824 was observed for mutants of Lee-366 and -490. Conversely, high-level resistance to both OPC-67683 and PA-824 was detected for mutants selected with compounds Lee-562 and Lee-878. Four of the five Lee-562 mutants were resistant to both OPC-67683 and PA-824. In the case of Lee-878, four of the five mutants were cross-resistant to OPC-67683 at >200 mg/L and three of these exhibited cross-resistance to PA-824 (Table 3). These preliminary results for our representative NFAs suggest that cross-resistance to PA-824 and OPC-67683 may readily occur for some (e.g. Lee-562 and -878), but not all (e.g. Lee-366, -490 and -879), NFA compounds. Moreover, this mixture of susceptible and cross-resistant mutants, even on a small sample set of NFAs and their corresponding mutants, presented some interesting questions regarding the mechanisms by which NFAs are activated and

Table 3. Cross-resistance of 25 *M. tuberculosis* H37Rv nitrofuranylamide-resistant mutants to PA-824 and OPC-67683

Mutant[a]	MIC (mg/L) NFA[b]	OPC-67683[c], when R = >0.025 mg/L	PA-824[c], when R = >0.4 mg/L
H37Rv-wt		0.006	0.1
Lee-366-1	12.5	0.0015	0.2
Lee-366-2	12.5	0.0002	0.2
Lee-366-3	12.5	0.0002	0.2
Lee-366-4	25	**0.05**	0.05
Lee-366-5	25	0.025	0.1
Lee-490-1	6.25	0.025	0.006
Lee-490-2	6.25	**0.2**	0.05
Lee-490-3	6.25	0.025	0.006
Lee-490-4	6.25	0.025	0.025
Lee-490-5	6.25	0.025	0.025
Lee-562-1	6.25	**0.05**	**50**
Lee-562-2	3.125	**3.12**	**100–200**
Lee-562-3	6.25	**>200**	**>200**
Lee-562-4	1.6	**0.1**	**100**
Lee-562-5	6.25	0.003	0.2
Lee-878-1	25	**>200**	**>200**
Lee-878-2	6.25	**>200**	0.4
Lee-878-3	6.25	0.0002	0.4
Lee-878-4	200	**>200**	**6.25**
Lee-878-5	25	**>200**	**3.125**
Lee-879-1	6.25	0.025	0.2
Lee-879-2	6.25	0.025	0.4
Lee-879-3	6.25	0.025	0.05
Lee-879-4	6.25	**0.05**	0.2
Lee-879-5	6.25	0.025	**0.8**

[a]Mutants are labelled according to the nitrofuranylamide (NFA) compound used in their selection.
[b]MIC of the selecting NFA is shown.
[c]Resistance (R) to PA-824 and OPC-67683 was defined as an MIC >4× the MIC for wild-type (wt) H37Rv and is shown in bold.

Table 4. Susceptibility to nitrofuranylamides of strains with defined resistance mechanisms to PA-824

Strain	Phenotype	MIC (mg/L) PA-824	366	490	562
H37Rv-wt	$FGD1^+$, F_{420}^+, $Rv3547^+$	<0.05	0.003	0.003	0.0008
H37Rv-T3	$FGD1^-$, F_{420}^+, $Rv3547^+$	100	3.12	0.39	1.56
H37Rv-5A1	$FGD1^+$, F_{420}^-, $Rv3547^+$	50	0.78	0.19	0.39
H37Rv-14A1	$FGD1^+$, F_{420}^+, $Rv3547^-$	50	0.006	0.012	0.0015

With the exception of H37Rv-T3, genetic changes conferring PA-824 resistance have been reported.[16] PA-824-resistant mutants and their wild-type (wt) progenitor were gifts from Dr C. Barry, NIH, USA.

which gene products for this process are mutual to the activation pathway for nitroimidazoles. To explore some of these questions, we evaluated the MICs of Lee-366, -490 and -562 for three well-characterized PA-824-resistant mutants and their progenitor strain *M. tuberculosis* H37Rv (Lee-878 and -879 were unavailable at the time of testing). These resistant mutants contained disruptions in three genes required for nitroimidizole activation, i.e. those encoding FGD1, its F_{420} deazaflavin cofactor or the Rv3547 nitroreductase. Mutants of FGD1 and F_{420} were both resistant to NFAs (Table 4), with MICs from 0.39 to 3.12 mg/L and 0.19 to 0.78 mg/L, respectively. Conversely, the strain H37Rv-14A1 carrying a nucleotide deletion in *Rv3547* was highly susceptible to all three NFAs with MICs ranging from 0.0015 to 0.012 mg/L. Based on these early findings, we suggest that although prodrug NFAs are structurally distinct from nitroimidazoles, reductive activation likely occurs in an analogous manner via the action of $FGD1/F_{420}$. However, the actual electron-transferring nitroreductase used by NFAs is not encoded by *Rv3547*. A similar conclusion has already been drawn to explain the activity of CGI-17341 against H37Rv-14A1 and other mutants disrupted in Rv3547.[16] CGI-17341 is a nitroimidazole, but is sufficiently structurally different from PA-824 and OPC-67683, not to be activated by Rv3547. Nevertheless, at this point, we cannot exclude unknown genetic determinants that may be responsible for the nitroaromatic resistance observed in our genetically uncharacterized mutant collection. A more detailed study to determine the mechanism of action of NFAs and the reason for the differences in the spectrum of cross-resistance with nitroimidazoles for mutants of Lee-366, -490 and -879 compared with those of Lee-562 and -878 will be the focus of future reports.

Evaluation of NFA cytotoxicity

The cytotoxicities of the five NFAs were evaluated against the Vero epithelial cell line. Mean IC_{50} values from three experiments were: 65.8, 45.9, 47.7, 65.3 and 48.4 mg/L for compounds 366, 490, 562, 878 and 879, respectively. In contrast, the cytotoxicity values determined for isoniazid, rifampicin and ethambutol were significantly higher (>1000 mg/L). However, the selectivity indices obtained by dividing the cytotoxic IC_{50} values by the broth MICs were high for the NFAs (5483, 3975, 6667, 10 883 and 8067, respectively), suggesting that there is a large therapeutic window for the selective killing of *M. tuberculosis* with this class.

Mutagenicity of NFAs

The BRM (Ames) test is a widely used procedure to probe the mutagenic potential of candidate therapeutic agents. It primarily determines whether a compound has general mutagenic properties and whether metabolic activation of the compound by a mammalian S9 liver fraction results in products with increased mutagenicity. The mutagenic potential of NFAs Lee-366, -562 and -878 was evaluated using *S. typhimurium* TA98 and its nitroreductase-deficient daughter strain TA98NR. During preliminary BRM testing of NFAs, growth inhibition by NFAs was observed at concentrations >5 and 50 μg/plate for *S. typhimurium* TA98 and TA98NR, respectively. This observation is most likely the result of the increased cell wall permeability of these strains due to an *rfa* mutation that makes the cell walls of BRM test strains, including TA98 and TA98NR, more permeable compared with wild-type strains.[30] This strategy removes the permeability limiting factor in mutagenicity assessment of polar

Table 5. Bacterial reverse mutation (Ames) test for nitrofuranylamide compounds

Strain	Compound	S9	Number of revertants per plate containing drug (μg)						
			5	1	0.5	0.1	0.05	0	positive control[a]
TA-98	Lee-878	+	59[b]	36	22	22	22	18	133
		−	>200[b]	15	19	14	14	9	108
	Lee-562	+	31	37	33	37	37		
		−	8	12	8	14	14		
	Lee-366	+	28	23	25	20	22		
		−	7	17	17	11	13		
			50	10	5	1	0.5	0	positive control[a]
TA98-NR	Lee-878	+	84	48	36	49	44	39	271
		−	253[b]	133[b]	55	53	27	39	>300
	Lee-562	+	27	45	38	44	30		
		−	71	48	44	46	18		
	Lee-366	+	51	46	47	46	35		
		−	71	55	51	38	45		

[a]Positive (+ve) controls: TA98 (+S9), 2-amino anthracene (2-AA, 1 μg/plate); TA98 (−S9), 2-nitro fluorene (2-NF, 1 μg/plate); and TA98-NR (±S9), 1,8-dinitro pyrene (1,8-DNP, 0.1 μg/plate).
[b]Statistically significant difference between no drug control and treatment based on criteria that drug-treated cells produce at least three times more revertant colonies than drug-free controls.

and poorly permeable compounds. However, as in this case, increasing permeability can result in increased antimicrobial activity, which may limit the highest concentration of test compound that can be used in the BRM assay. Therefore, NFAs were tested at five concentrations below the inhibitory concentration for each strain, but with the maximum test concentrations (Table 5) either above or close to the previously determined C_{max} values, which are also above that needed to be cidal against non-replicating and replicating *M. tuberculosis*. No mutagenic activity was observed for Lee-366 and -562 under the conditions tested. Only Lee-878 was adversely mutagenic against TA98 and TA98NR. In the absence of metabolic activation, Lee-878 was mutagenic at 5 μg/plate to TA98 and at 10 μg/plate to TA98NR. With metabolic activation, Lee-878 was only mutagenic to TA98 at 5 μg/plate and was not found to be mutagenic to TA98NR at the highest concentration tested, i.e. 50 μg/plate. Due to the antimicrobial activity of the NFAs against the BRM test strains, some caution must be used when interpreting these results and alternative eukaryotic test systems will be required to suitably evaluate the genotoxic potential of NFAs. However, the following preliminary conclusions can be derived from the data in this study: not all NFAs have general mutagenic properties; BRM is most associated with the *S. typhimurium* nitroreductase status; and most importantly, metabolic activation by the mammalian S9 liver fraction results in metabolic products that are either not mutagenic or are less mutagenic than the parent compound.

This microbiological assessment of NFAs has revealed several positive factors that warrant their further development as anti-TB drugs, including: a narrow spectrum of activity that is restricted to bacteria of the TB complex; a long PAE that is relevant to antibiotic dosing regimens; a lack of cross-resistance or antagonism to front-line TB drugs appropriate for combination treatment; and importantly a unique mode of action against active and latent bacteria, which could potentially lead to drug candidates that shorten the current time to treat TB disease.

Acknowledgements

This work was presented in part at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy as a Poster entitled '*In vitro* characterization of nitrofuranylamide antibiotics as potent anti-tuberculosis agents' (Abstract F1-304).

We thank Dr Claudia A. F. Aiub of Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, for the supply of Salmonella NR strains; Dr Clifton Barry from NIH, MD, USA, for providing strain HN878 and PA824-resistant mutants; and Dr David Smalley from the Tennessee Department of Health, TN, USA, for providing the UT clinical isolates.

Funding

Financial support was provided by National Institutes of Health Grant AI062415.

Transparency declarations

None to declare.

References

1. World Health Organization Global Tuberculosis Programme. WHO Report on the Tuberculosis Epidemic, Geneva, 2005.

2. Stewart GR, Robertson BD, Young DB. Tuberculosis: a problem with persistence. *Nat Rev* 2003; **1**: 97–105.

3. Corbett EL, Watt CJ, Walker N *et al*. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med* 2003; **163**: 1009–21.

4. Blondal K. Barriers to reaching the targets for tuberculosis control: multidrug-resistant tuberculosis. *Bull World Health Organ* 2007; **85**: 387–90.

5. Chakrabarti B, Davies PD. Key issues in multidrug-resistant tuberculosis. *Future Microbiol* 2007; **2**: 51–61.

6. Murray CJ, Salomon JA. Modeling the impact of global tuberculosis control strategies. *Proc Natl Acad Sci USA* 1998; **95**: 13881–6.

7. Tangallapally RP, Yendapally R, Lee RE *et al*. Synthesis and evaluation of nitrofuranylamides as novel antituberculosis agents. *J Med Chem* 2004; **47**: 5276–83.

8. Tangallapally RP, Lee RE, Lenaerts AJ *et al*. Synthesis of new and potent analogues of anti-tuberculosis agent 5-nitro-furan-2-carboxylic acid 4-(4-benzyl-piperazin-1-yl)-benzylamide with improved bioavailability. *Bioorg Med Chem Lett* 2006; **16**: 2584–9.

9. Tangallapally RP, Yendapally R, Daniels AJ *et al*. Nitrofurans as novel anti-tuberculosis agents: identification, development and evaluation. *Curr Top Med Chem* 2007; **7**: 509–26.

10. Tangallapally RP, Yendapally R, Lee RE *et al*. Synthesis and evaluation of cyclic secondary amine substituted phenyl and benzyl nitrofuranyl amides as novel antituberculosis agents. *J Med Chem* 2005; **48**: 8261–9.

11. Tangallapally RP, Sun D, Rakesh *et al*. Discovery of novel isoxazolines as anti-tuberculosis agents. *Bioorg Med Chem Lett* 2007; **17**: 6638–42.

12. O'Neill AJ, Chopra I. Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opin Investig Drugs* 2004; **13**: 1045–63.

13. Murugasu-Oei B, Dick T. Bactericidal activity of nitrofurans against growing and dormant *Mycobacterium bovis* BCG. *J Antimicrob Chemother* 2000; **46**: 917–9.

14. Stover CK, Warrener P, VanDevanter DR *et al*. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature* 2000; **405**: 962–6.

15. Spigelman MK. New tuberculosis therapeutics: a growing pipeline. *J Infect Dis* 2007; **196** Suppl 1: S28–34.

16. Manjunatha UH, Boshoff H, Dowd CS *et al*. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2006; **103**: 431–6.

17. Manjunatha UH, Lahiri R, Randhawa B *et al*. *Mycobacterium leprae* is naturally resistant to PA-824. *Antimicrob Agents Chemother* 2006; **50**: 3550–4.

18. Matsumoto M, Hashizume H, Tomishige T *et al*. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis *in vitro* and in mice. *PLoS Med* 2006; **3**: e466.

19. Barry CE 3rd, Boshoff HI, Dowd CS. Prospects for clinical introduction of nitroimidazole antibiotics for the treatment of tuberculosis. *Curr Pharm Des* 2004; **10**: 3239–62.

20. Budha N, Mehrotra N, Tangallapally R *et al*. Pharmacokinetically-guided lead optimization of nitrofuranylamide anti-tuberculosis agents. *AAPS J* 2008; **10**: 157–65.

21. Sasaki H, Haraguchi Y, Itotani M *et al*. Synthesis and anti-tuberculosis activity of a novel series of optically active 6-nitro-2,3-dihydroimidazo[2,1-b]oxazoles. *J Med Chem* 2006; **49**: 7854–60.

22. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Seventh Edition: Approved Standard M7-A7*. CLSI, Wayne, PA, USA, 2006.

23. Cho SH, Warit S, Wan B *et al*. Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2007; **51**: 1380–5.

24. Lenaerts AJ, Gruppo V, Marietta KS *et al*. Preclinical testing of the nitroimidazopyran PA-824 for activity against *Mycobacterium tuberculosis* in a series of *in vitro* and *in vivo* models. *Antimicrob Agents Chemother* 2005; **49**: 2294–301.

25. Eliopoulos GM, Moellering RC Jr. Antimicrobial combinations. In: *Antibiotics in Laboratory Medicine*. Baltimore, MD, USA: Williams and Wilkins, Co., 2000; 432–49.

26. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother* 2003; **52**: 1.

27. Odenholt-Tornqvist I. Studies on the postantibiotic effect and the postantibiotic sub-MIC effect of meropenem. *J Antimicrob Chemother* 1993; **31**: 881–92.

28. Chan CY, Au-Yeang C, Yew WW *et al*. Postantibiotic effects of antituberculosis agents alone and in combination. *Antimicrob Agents Chemother* 2001; **45**: 3631–4.

29. Peloquin C. Using therapeutic drug monitoring to dose the antimycobacterial drugs. *Clin Chest Med* 1997; **18**: 79–87.

30. Maron DM, Ames BN. Revised methods for the salmonella mutagenicity test. *Mutat Res* 1983; **113**: 173–215.

31. OECD. *Section 4 of the OECD Guidelines for the Testing of Chemicals: Bacterial Reverse Mutation Test*, *Guideline 471, adopted 21 July 1997*. http://www.oecd.org/dataoecd/18/31/1948418.pdf (16 July 2008, date last accessed).

32. Rosenkranz EJ, McCoy EC, Mermelstein R *et al*. Evidence for the existence of distinct nitroreductases in *Salmonella typhimurium*: roles in mutagenesis. *Carcinogenesis* 1982; **3**: 121–3.

33. Valway SE, Sanchez MP, Shinnick TF *et al*. An outbreak involving extensive transmission of a virulent strain of *Mycobacterium tuberculosis*. *N Engl J Med* 1998; **338**: 633–9.

34. Sreevatsan S, Pan X, Stockbauer KE *et al*. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci USA* 1997; **94**: 9869–74.

35. Barczak AK, Domenech P, Boshoff HI *et al*. *In vivo* phenotypic dominance in mouse mixed infections with *Mycobacterium tuberculosis* clinical isolates. *J Infect Dis* 2005; **192**: 600–6.

36. Mitchison DA. The search for new sterilizing anti-tuberculosis drugs. *Front Biosci* 2004; **9**: 1059–72.

37. Goldstein E, Eagle MC, LaCasse ML. *In vitro* chemotherapeutic combinations against isoniazid-resistant *Mycobacterium tuberculosis* and *Mycobacterium fortuitum*. *Appl Microbiol* 1971; **22**: 329–33.

38. Heifets LB. Synergistic effect of rifampicin, streptomycin, ethionamide and ethambutol on *Mycobacterium intracellulare*. *Am Rev Respir Dis* 1982; **125**: 43–8.

39. Fuursted K. Evaluation of the post-antibiotic effect of six antimycobacterial agents against *Mycobacterium avium* by the Bactec radiometric method. *J Antimicrob Chemother* 1997; **40**: 33–8.

40. Kardas P. Patient compliance with antibiotic treatment for respiratory tract infections. *J Antimicrob Chemother* 2002; **49**: 897–903.