Characterization of tetracycline resistance mediated by the efflux pump Tap from Mycobacterium fortuitum

Santiago Ramón-García¹, Carlos Martín¹, José A. Aínsa¹* and Edda De Rossi²

¹Departamento de Microbiología, Medicina Preventiva y Salud Pública, Universidad de Zaragoza, 50009 Zaragoza, Spain; ²Dipartimento di Genetica e Microbiologia, Università degli Studi di Pavia, 27100 Pavia, Italy

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Objectives: The aim of this study was to characterize the efflux pump Tap from Mycobacterium fortuitum, to test its sensitivity to well known efflux inhibitors, to study the interaction between tetracycline and these compounds and to test the ability of these compounds to overcome efflux pump-mediated tetracycline resistance. For all these studies, we produced Tap protein in Mycobacterium smegmatis.

Methods: Antibiotic susceptibility tests, tetracycline uptake/efflux experiments and chequerboard synergy tests.

Results: Tetracycline uptake/efflux experiments showed that Tap protein from M. fortuitum uses the electrochemical gradient across the cytoplasmic membrane to extrude tetracycline from the cell. This efflux activity is inhibited by carbonyl cyanide m-chlorophenylhydrazone (CCCP) and reserpine, consistent with the decrease in MIC observed in antibiotic susceptibility testing in the presence of these inhibitors. Accumulation was not inhibited in experiments in which θ-vanadate and chlorpromazine (CPZ) were tested. Inhibitor-treated cells used glycerol as a carbon source to re-establish the electrochemical gradient across the membrane and to restore efflux activity. CCCP, reserpine and CPZ reduced the MIC of tetracycline in the M. smegmatis strain expressing the Tap protein, whereas θ-vanadate increased the MIC. We also observed synergy between tetracycline and CPZ or reserpine, and antagonism with θ-vanadate.

Conclusions: The Tap for efflux pump uses the electrochemical gradient to extrude tetracycline from the cell. This efflux activity can be inhibited by several compounds. This suggests that similar compounds could be used to overcome antibiotic resistance mediated by efflux pumps.

Keywords: tetracycline efflux, antibiotic resistance, mycobacteria, efflux pump inhibitors, synergy

Introduction

Non-tuberculous mycobacteria (NTMB) are environmental species found in water, soil, milk and in fish, birds and other animals. NTMB include rapidly growing mycobacteria, such as Mycobacterium fortuitum, Mycobacterium peregrinum, Mycobacterium abscessus and Mycobacterium chelonae. M. fortuitum is an opportunistic pathogen of humans, causing abscesses and soft-tissue infections, especially at injection sites or surgical wounds, and is often associated with indwelling catheters, breast implants and other surgical devices.¹

Prolonged antibiotic therapy is generally required for the treatment of M. fortuitum infections.¹ Mycobacterial infections are generally difficult to treat because these bacteria are intrinsically resistant to most common antibiotics and chemotherapeutic agents.² Furthermore, the emergence of drug-resistant strains has become an additional handicap, especially in the case of multidrug-resistant Mycobacterium tuberculosis strains. Mutations in genes encoding drug targets or the enzymes required to activate the drug are frequently found in resistant strains. However, no mutation has been found in a number of drug-resistant strains. The resistance of these strains may be accounted for by mutations in new, as yet unidentified loci, changes in drug permeability, or the involvement of efflux pumps extruding the antibiotic. Efflux pump-mediated antibiotic resistance has been widely reported for other bacterial pathogens.³,⁴

Bacterial membrane efflux pumps form a large, heterogeneous family of energy-dependent membrane proteins capable of extruding either a single antibiotic, such as tetracycline, or a wide variety of chemically and structurally unrelated substances, allowing bacteria to adapt themselves to a hostile environment. Furthermore, as efflux pumps have been shown to be of clinical relevance in

*Corresponding author. Tel: +34-976-762420; Fax: +34-976-762604; E-mail: ainsa@unizar.es

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bacterial infections, the characterization of such pumps (mode of action, source of energy used and substrate profile) is important for the design of new therapeutic strategies.

Many compounds capable of inhibiting efflux pumps have been described. Some affect the electrochemical gradient across the membrane used by some efflux pumps as a source of energy. These compounds include the proton uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), nigericin (which dissipates the transmembrane ion gradient), the calcium channel blocker verapamil, and valinomycin (which dissipates the electrical potential). Other pumps using ATP as an energy source may be inhibited by the P-type ATPase inhibitor o-vanadate. Other inhibitors interact more specifically with the efflux pump. An example of one such inhibitor is the mammalian P-glycoprotein inhibitor reserpine, which also inhibits bacterial efflux pumps, such as NorA. Thus, efflux pump inhibitors can be used to make bacteria more susceptible to antimicrobial agents.

The first efflux pump described for the genus Mycobacterium, the LfRA protein of Mycobacterium smegmatis, confers low-level resistance to fluoroquinolones and other compounds. Many other efflux pumps have since been described. Efflux pump components also seem to play a role in mycobacterial drug tolerance and induced resistance to isoniazid. Tapfor and Tapub proteins are membrane efflux pumps of the major facilitator superfamily (MFS) isolated from M. fortuitum and M. tuberculosis, respectively. Note that in this study the tap gene and Tap protein from M. fortuitum will be designated tapfor and Tapfor, respectively. The homologous gene and protein from M. tuberculosis will be referred to as tapub and Tapub. MFS proteins extrude substrates, using the proton gradient through the membrane, in an antiporter mechanism.

These membrane proteins confer low-level resistance and have broad substrate specificity. When cloned on a plasmid, Tapfor protein increases the resistance of M. smegmatis mc\textsuperscript{2}155 to gentamicin, 2-N\textsuperscript{0}-ethylnetilmicin, 6-N\textsuperscript{0}-ethylnetilmicin, streptomycin and tetracycline. Rifampicin and ofloxacin increase expression of the Rv1258\textsubscript{c} gene, encoding Tapub, in a clinical strain of M. tuberculosis.

In this work, we describe the characterization of tetracycline resistance mediated by the Tapfor efflux pump. This membrane protein uses the electrochemical gradient as a source of energy to extrude tetracycline from the cell and is inhibited by CCCP and reserpine, but not by o-vanadate or chlorpromazine (CPZ). Cells use glycerol as a carbon source to establish the electrochemical gradient through the membrane and to restore the activity of the pump. We also describe interactions between tetracycline and the inhibitory compounds, and the ability of these compounds to overcome efflux pump-mediated tetracycline resistance.

Materials and methods

**Bacterial strains and general growth conditions**

*M. smegmatis* mc\textsuperscript{2}155 cells harbouring the cloning vector pSUM36 and its derivative pAC48 containing the tapfor gene—strains SUM36 and AC48, respectively—were grown in Middlebrook 7H9 broth (Difco) supplemented with 10% Middlebrook ADC (Difco) and 0.2% glycerol. Kanamycin was added to the cultures at a final concentration of 20 mg/L to maintain the plasmids. We used Luria-Bertani (LB) agar for colony counts. All the cultures were incubated at 37°C, with shaking.

**Chemicals and solvents**

\[^{[H]}\text{H}\]Tetracycline (0.91 Ci/mmol) was obtained from Perkin Elmer. CCCP, kanamycin, reserpine, tetracycline and o-vanadate were provided by Sigma. CPZ was obtained from Fluka. CCCP and CPZ were dissolved in DMSO; reserpine was dissolved in glacial acetic acid; and kanamycin, tetracycline and o-vanadate were dissolved in water.

**Antibiotic susceptibility testing**

The MICs of the drugs listed above for the *M. smegmatis* SUM36 and AC48 strains were determined on microplates by the Resazurin assay. Briefly, we inoculated 100 μL of 2-fold serial dilutions of antibiotic with 100 μL of a 10\textsuperscript{7} cells/mL suspension of mycobacteria in microtitre plates. The plates were incubated for 4 days at 37°C, 30 μL of the redox indicator Resazurin (1 g/L) was added to each well and the plates were incubated for an additional day. A change in colour from blue to pink indicated the growth of bacteria, and the MIC was defined as the lowest concentration of drug preventing this colour change. We prevented the formation of biofilms, which might affect drug resistance, by using polystyrene microtitre plates from TPP for antibiotic susceptibility tests.

We also determined the MIC of tetracycline in the presence of CCCP and CPZ (both at a concentration of 20 mg/L), reserpine (12 mg/L) and o-vanadate (9 mg/L). All antibiotic susceptibility tests were carried out at least three times.

**Chequerboard synergy testing**

For a given strain, we determined interactions between tetracycline and CPZ, CCCP, reserpine or o-vanadate. Using a 96-well plate, we assayed 2-fold dilutions of tetracycline against a range of 2-fold dilutions of CCCP, CPZ or reserpine by the Resazurin assay.17 The fractional inhibitory concentration of tetracycline (FICTET) in the presence of CCCP, CPZ or reserpine was calculated as follows: FICTET = (MIC of tetracycline in the presence of CCCP, CPZ or reserpine)/(MIC of tetracycline alone). Similar FIC values were calculated for CCCP, CPZ and reserpine in the presence of tetracycline. The FICTET values were plotted graphically against the FIC values for CCCP, CPZ and reserpine. A concave curve indicates synergy, whereas a convex curve indicates antagonism. The point closest to the intersection of the axes (point zero) corresponds to the most effective combination for inhibiting bacterial growth.

Given the high MIC of o-vanadate (>640 mg/L), we tested a series of 2-fold dilutions of tetracycline against a reference concentration of o-vanadate (9 mg/L), as previously described. In parallel, reference concentrations (0.5× MIC values) were fixed for the other drugs: tetracycline, 1 mg/L; CCCP, 20 mg/L; reserpine, 48 mg/L; and CPZ, 20 mg/L. We used these reference concentrations to calculate the FIC index (FICI) as follows: FICI = (MIC of CCCP, CPZ or reserpine in the presence of reference tetracycline concentration)/(MIC of CCCP, CPZ or reserpine alone) + (MIC of tetracycline in the presence of reference CCCP, CPZ or reserpine concentration)/(MIC of tetracycline alone). Synergy was defined by FICI values ≤0.5, antagonism by FICI values >4.0, and no interaction by FICI values between >0.5 and 4.0.

**Uptake experiments**

Tetracycline accumulation experiments. Uptake experiments were performed as previously described. We inoculated 20 mL of Middlebrook 7H9 broth with *M. smegmatis* SUM36 and AC48 strains, which were cultured to the exponential growth phase, harvested by centrifugation, washed twice with 0.1 M potassium phosphate (pH 7.0)
and resuspended in assay buffer [0.1 M potassium phosphate (pH 7.0); 1 mM MgCl₂]. We used several strategies to prevent aggregate formation and cell clumping. First, bacterial cultures were filtered through a syringe filter with 5 μm pores (Sartorius). Filtrates were cultured overnight at 37°C, then centrifuged, with the pellet washed and resuspended, as described above. Second, cells resuspended in assay buffer were allowed to stand for 15 min to allow clumps to sediment out. Aliquots (1 mL) were collected from the upper part of the cultures and used for accumulation assays.

Cells were incubated at 37°C (the optimal growth temperature for *M. smegmatis*) for 15 min, and the uptake assay was initiated by adding [³H]tetracycline to a final concentration of 5 μM. At various time intervals, we removed 50 μL of the suspension in triplicate, diluted it in 1 mL of ice-cold 0.1 M potassium phosphate (pH 7.0) containing 0.1 M LiCl, and immediately filtered it through a filter with 0.45 μm pores (Millipore). The filter was rapidly washed twice with 5 mL of the same buffer and dried. Radioactivity was then determined in a Beckman LS 7000 liquid scintillation counter, using Ecolume scintillation cocktail (ICN Biomedicals). Experiments were carried out at least three times, to ensure that the results were reproducible. Finally, after uptake experiments, serial dilutions of the remaining cells were plated on LB agar. Colonies were counted after 4 days of incubation at 37°C.

Inhibitor experiments. We used several compounds to assess the energy dependence of the accumulation process (40 mg/L of CCCP, 12 mg/L of reserpine, 20 mg/L of CPZ and 9 mg/L of o-vanadate). The inhibitors were added to the cells 2.5 min after the addition of [³H]tetracycline and the suspension was treated as described above. For o-vanadate experiments, HEPES buffer (pH 7.0) was used as the assay buffer.

In another set of experiments, cell suspensions were incubated with 40 mg/L of CCCP in phosphate buffer, with gentle shaking at 37°C for 60 min, to exhaust the endogenous energy reserve of the cells. The starved cells were then washed three times with phosphate buffer at room temperature to remove CCCP and resuspended in assay buffer. The assay was started by adding [³H]tetracycline and the cells were incubated for 5 min to allow antibiotic uptake. The cells were then re-energized, by adding 200 mM glycerol as an energy source, and treated as described above.

### Results and discussion

**Susceptibility profiles of *M. smegmatis* SUM36 and AC48 strains**

The MICs of CCCP, reserpine, CPZ, o-vanadate and tetracycline for the *M. smegmatis* mc²155, SUM36 and AC48 strains were determined by the Resazurin assay. The MIC values (mg/L) are shown in Table 1. The MICs of the solvents used (DMSO and glacial acetic acid) were also determined for *M. smegmatis* mc²155. The MICs of DMSO and glacial acetic acid were 12.5–25% (v/v). In a standard MIC determination, solvent concentration does not exceed 1% of the final volume. We, therefore, conclude that the solvents had no appreciable effect on the growth of *M. smegmatis* mc²155.

The MIC of tetracycline was determined for strains SUM36 and AC48, in the presence of subinhibitory concentrations of the known efflux pump inhibitors CCCP and reserpine (Table 2). Generally, susceptibility to tetracycline increased. CCCP and reserpine decreased the MIC of tetracycline by a factor of 2–4 for the *M. smegmatis* strain SUM36 carrying the cloning vector. Thus, baseline tetracycline resistance in *M. smegmatis* depends on inhibitor-sensitive efflux systems, such as the tet(V) determinant, but not excluding other putative intrinsic non-efflux systems. Tetracycline MICs in the presence of these inhibitors were always higher for AC48 than for SUM36, indicating that these two efflux pump blockers do not completely block the Tapfor efflux pump.

We also determined the MIC of tetracycline for SUM36 and AC48 in the presence of CPZ. The same MIC was obtained for both strains (0.12 mg/L; Table 2), indicating that, in the presence of this compound, the Tapfor pump confers no advantage. Moreover, CPZ decreased the MIC for SUM36 and AC48, but it is noteworthy that this decrease was larger for AC48 (factor of 128) than for CCCP (factor of only 16). There are two possible explanations for these results. First, it has been suggested that phenothiazines inhibit efflux pumps, which suggests that CPZ is a more powerful, or more selective efflux pump inhibitor than CCCP or reserpine. Second, CPZ has also been reported to bind to membranes, increasing their permeability. CPZ may, therefore, affect the integrity of the cell wall of *M. smegmatis*, allowing tetracycline to enter cells and to accumulate in large amounts, rendering Tapfor protein

<table>
<thead>
<tr>
<th>Table 1. Antimicrobial activity of tetracycline and efflux pump inhibitors against <em>M. smegmatis</em> mc²155, SUM36 and AC48</th>
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<tbody>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>TET</td>
</tr>
<tr>
<td>CCCP</td>
</tr>
<tr>
<td>Reserpine</td>
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<td>CPZ</td>
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TET, tetracycline; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CPZ, chlorpromazine.

<table>
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<tr>
<th>Table 2. Antimicrobial activity of tetracycline in the presence of several compounds against SUM36 and AC48</th>
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<tbody>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>TET</td>
</tr>
<tr>
<td>TET + CCCP</td>
</tr>
<tr>
<td>TET + reserpine</td>
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<tr>
<td>TET + CPZ</td>
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</table>

TET, tetracycline; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CPZ, chlorpromazine.

The MICs were assayed in a range of 2-fold dilutions of tetracycline in the presence of non-lethal concentrations (below the MIC for *M. smegmatis* mc²155) of the following compounds: CCCP, 20 mg/L; reserpine, 12 mg/L; CPZ, 20 mg/L; and o-vanadate, 9 mg/L. Values in brackets correspond to the increase or decrease in MIC with respect to the MIC of tetracycline alone.
activity inefficient. We carried out uptake experiments to determine whether CPZ inhibited Tapfer protein function or interacted with this protein in an unknown manner, by reducing tetracycline resistance levels (see below).

A different effect was noted when o-vanadate was tested. Unlike the other inhibitors, o-vanadate increased tetracycline resistance. For SUM36, the MIC of tetracycline increased from 2 to 32 mg/L (factor of 16), whereas for AC48, the MIC increased from 16 to 64 mg/L (factor of 4) (Table 2). It has been suggested that the influx of tetracycline into Escherichia coli involves both phosphate bond hydrolysis and proton motive force.29,30 The higher MIC of tetracycline in the presence of o-vanadate may therefore be due to...

Figure 1. Tetracycline uptake/efflux experiments with SUM36 and AC48. (a) Tetracycline uptake of SUM36 cells (open circles) and AC48 cells (closed circles). [3H]Tetracycline was added to the cells at time zero. (b) Tetracycline accumulation of AC48 in the absence (closed circles) and presence (open triangles) of CCCP. CCCP was added after 2.5 min. (c) Tetracycline accumulation in de-energized AC48 cells (closed squares) and upon re-energization (open squares) following the addition of glycerol at 5 min. (d) Tetracycline accumulation in AC48 cells (closed circles) and upon addition of reserpine after 2.5 min and glycerol after 10 min (inverted filled triangles). (e) Tetracycline accumulation in AC48 cells (closed circles) and upon addition of CPZ (inverted open triangles) after 2.5 min.
inhibition of the ATP-dependent uptake system. It has been suggested that tetracycline and vanadium compounds may form complexes, affecting the action of tetracycline.\textsuperscript{31} This provides an alternative explanation for the increase in the MIC of tetracycline in the presence of o-vanadate.

**Accumulation experiments**

We investigated the mechanism of tetracycline resistance conferred by the Tap\textsubscript{for} protein, by measuring tetracycline accumulation in *M. smegmatis* SUM36 and AC48 cells. As shown in Figure 1(a), SUM36 cells accumulated [*H]tetracycline rapidly, reaching steady-state levels within ~2.5 min of incubation. This level of accumulation is about five times higher than that in AC48 cells harbouring the tap\textsubscript{for}-containing plasmid. These results suggest that the Tap\textsubscript{for} protein has tetracycline efflux activity.

As antibiotic efflux pump systems are energy-dependent, bacterial cells accumulate larger amounts of antibiotic in the presence of uncouplers. We investigated whether this was the case in the presence of the tap\textsubscript{for} gene, by determining the effects of various compounds. Uptake experiments were carried out in the presence of CCCP, o-vanadate, reserpine or CPZ.

**CCCP.** Efflux pumps belonging to the MFS family extrude compounds, using proton motive force as a source of energy, in an antiporter mechanism.\textsuperscript{32} We investigated whether Tap\textsubscript{for} function was an energy-dependent process, using the protonophore CCCP at a concentration of 40 mg/L in uptake experiments. This concentration is lethal for *M. smegmatis* cells in antibiotic susceptibility test conditions, but had no effect on cell viability in experiments measuring accumulation over a period of 30 min.\textsuperscript{22,33}

Upon CCCP addition, [*H]tetracycline accumulation increased by a factor of ~4 in AC48 (Figure 1b), consistent with antibiotic susceptibility test results (Table 2). In contrast, under our conditions, CCCP had no detectable effect on the level of tetracycline accumulation in SUM36 (data not shown). One of the possible limitations of our experiments is that small changes in accumulation in SUM36 (carrying single-copy efflux pump genes on the chromosome) may not have been detectable following the addition of inhibitor. When efflux pump genes are present on multicopy plasmids (tap\textsubscript{for} in the AC48 strain and the tet(V) determinant described elsewhere\textsuperscript{22}), the changes in accumulation are greater and are therefore more readily detected.

A second experiment was carried out to determine whether energizing de-energized cells could restore tetracycline efflux in AC48 cells. De-energized SUM36 and AC48 cells accumulated similar amounts of [*H]tetracycline (data not shown). However, following the addition of glycerol as an energy source, AC48 cells rapidly extruded tetracycline (Figure 1c), whereas no effect was observed in SUM36 cells (data not shown). Thus, glycerol is used by cells to produce energy, which is used to re-establish the proton gradient across the membrane used by the Tap\textsubscript{for} pump for tetracycline extrusion, probably via an antiporter mechanism.

These results are consistent with the proposed mechanism of tetracycline uptake. Tetracyclines must cross one or more membrane systems, depending on whether the susceptible microorganism is Gram-positive or Gram-negative, to reach their targets. Tetracyclines cross the outer membrane of Gram-negative enteric bacteria via the OmpF and OmpC porin channels, as positively charged cation (probably magnesium)–tetracycline coordination complexes.\textsuperscript{34,35} The cationic metal ion-antibiotic complex is attracted by the Donnan potential across the outer membrane, leading to accumulation in the periplasm, in which the metal ion–tetracycline complex probably dissociates to release uncharged tetracycline, a weakly lipophilic molecule that diffuses through the lipid bilayer regions of the cytoplasmic membrane. The electroneutral, lipophilic form is presumed to be the species transferred across the cytoplasmic membrane of Gram-positive bacteria. Tetracycline uptake also depends on phosphate bond hydrolysis and proton motive force.\textsuperscript{29,30,35,36} As tetracycline is extruded primarily by proton motive force-driven efflux pumps, tetracycline efflux is abolished in the presence of a proton motive force uncoupler, such as CCCP, whereas tetracycline influx is affected only mildly, resulting in an increase in tetracycline accumulation.

**o-Vanadate.** Tetracycline accumulation was not increased by the ATPase inhibitor o-vanadate in SUM36 or AC48 (data not shown). In another report, o-vanadate also failed to increase the accumulation of substrates transported by the LmrP protein of *Lactococcus lactis* when cloned in a plasmid.\textsuperscript{37} These results rule out an ATP-dependent efflux mechanism for the Tap\textsubscript{for} protein.

**Reserpine.** We further investigated the efflux pump Tap\textsubscript{for} by carrying out additional uptake experiments in the presence of reserpine. Reserpine was added to cells 2.5 min after the addition of [*H]tetracycline. Tetracycline accumulation increased by a factor of ~4 in AC48, suggesting that reserpine inhibits the efflux pump (Figure 1d). Similar results were obtained in antibiotic susceptibility tests, in which reserpine reduced the MIC of tetracycline (Table 2). The inhibitory effect of reserpine may be owing to an interaction with the reserpine-binding site in the Tap\textsubscript{for} protein, as described for the multidrug transporter Bmr from *Bacillus subtilis*.\textsuperscript{38} We investigated whether the pump could be reactivated by adding glycerol 7.5 min after reserpine. Efflux activity was rapidly restored in AC48 cells, which extruded the drug. The mechanism by which glycerol reactivates the pump remains unknown.

**CPZ.** We also carried out accumulation experiments in the presence of CPZ. We added CPZ to cells 2.5 min after the addition of [*H]tetracycline. No difference in tetracycline accumulation

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**Table 3.** FICI of efflux pump inhibitors and CPZ when combined with tetracycline for SUM36 and AC48

<table>
<thead>
<tr>
<th>Compound</th>
<th>SUM36 (vector)</th>
<th>AC48 (tap\textsubscript{for})</th>
<th>interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCCP</td>
<td>0.75</td>
<td>0.6</td>
<td>no interaction</td>
</tr>
<tr>
<td>Reserpine</td>
<td>0.4</td>
<td>0.13</td>
<td>synergy</td>
</tr>
<tr>
<td>CPZ</td>
<td>0.075</td>
<td>0.04</td>
<td>synergy</td>
</tr>
<tr>
<td>o-Vanadate</td>
<td>50</td>
<td>4</td>
<td>antagonism</td>
</tr>
</tbody>
</table>

TET, tetracycline; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CPZ, chlorpromazine.

\*FICI was calculated as follows: FICI = [(MIC of compound in the presence of TET at the reference concentration)/(MIC of compound alone)] + [(MIC of TET in the presence of any compound at the reference concentration)/(MIC of TET alone)]. Reference concentrations (0.5× MIC values) were fixed as follows: tetracycline, 1 mg/L; CCCP, 20 mg/L; reserpine, 48 mg/L; CPZ, 20 mg/L; and o-vanadate, 9 mg/L. Synergy was defined as FICI values of ≤0.5, antagonism as FICI values >4.0 and no interaction as FICI values ranging between 0.5 and 4.0.
was observed (Figure 1e), suggesting that CPZ had no inhibitory
effect on the efflux activity of Tap_for. Despite suggestions that
phenothiazines act as efflux pump inhibitors, CPZ did not
block Tap_for protein activity under our conditions. These results
are largely consistent with those of Viveiros et al. who showed
that reserpine decreased the MIC of isoniazid for M. tuberculosis
cells with induced resistance, whereas CPZ did not, suggesting that
this putative efflux mechanism was not affected by CPZ. However,
these authors also reported that CPZ and other phenothiazines may
be useful for managing antibiotic-resistant tuberculosis.

CPZ rendered SUM36 and AC48 equally susceptible to tetracycline (0.12 mg/L) (Table 2), with both strains considerably more
susceptible to tetracycline than in the absence of CPZ. These
results, together with the lack of effect of CPZ on Tap_for efflux,
suggest that CPZ may affect tetracycline resistance levels through other, unknown, mechanisms.13–42

Chequerboard synergy testing

Compounds affecting efflux pumps would be expected to react synergistically with antibiotics with targets located within the bacterial cell (i.e. tetracycline), the internal concentrations of which depend on the efficacy of efflux pumps.43 We, therefore, studied the interaction of tetracycline with CCCP, reserpine, o-vanadate and CPZ and the ability of these compounds to affect tetracycline resistance in the SUM36 and AC48 strains. We calculated FICI values. The FICI value >4 obtained for o-vanadate indicated antagonism, whereas reserpine had an FICI of 0.4 for SUM36 and 0.13 for AC48, suggesting synergy. CCCP had FICI values between 0.5 and 1 for both strains, indicating no interaction with tetracycline. Finally, the FICI clearly indicated synergy between CPZ and tetracycline for both strains (Table 3).

The FICI values of reserpine, CPZ, CCCP and tetracycline were calculated for SUM36 and AC48, as described in the Materials and methods section. All the FICI values of each compound (reserpine, CPZ, CCCP) were then plotted against the corresponding FICI values of tetracycline (Figure 2). For reserpine (Figure 2a and d) and CPZ (Figure 2b and e), a concave curve was obtained, indicating synergy between these two compounds and tetracycline.19 For reserpine and CPZ, the most effective combinations for inhibiting bacterial growth were as follows: for the SUM36 strain, 12 mg/L of reserpine plus 0.5 mg/L of tetracycline and 1.25 mg/L of CPZ plus 0.25 mg/L of tetracycline; and for the AC48 strain, 12 mg/L of reserpine plus 1 mg/L of tetracycline and 2.5 mg/L of CPZ plus 1 mg/L of tetracycline or 5 mg/L of CPZ plus 0.5 mg/L of tetracycline. The MIC values of each drug in the combination were lower (by a factor between 8 and 32 times) than those of any single drug used alone, consistent with the synergy inferred from the FICI values obtained.

The shape of the curve for CCCP was inconclusive, suggesting no interaction (Figure 2c and f). The most effective combination would probably be 1 mg/L of tetracycline plus 20 mg/L of CCCP for SUM36 and 8 mg/L of tetracycline plus 10 mg/L of CCCP for AC48. These values are only half those for the two drugs used alone, consistent with the FICI value indicating an absence of interaction. These data support the idea that synergic combinations of drugs could be used to overcome drug resistance, especially when resistance is mediated by an efflux pump.

In conclusion, we describe here the sensitivity of the TapPtot protein to efflux pump inhibitors and the interactions of tetracycline with these compounds. The TapPtot and TapPsub proteins were initially described as increasing resistance to several compounds.14,15 More recently, the TapPsub efflux pump has been shown to be induced by drugs in a clinical strain of M. tuberculosis.16 It may be possible to devise therapeutic strategies in the future based on the use of efflux pump inhibitors to overcome efflux pump-mediated resistance.

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