Thioridazine and chlorpromazine inhibition of ethidium bromide efflux in *Mycobacterium avium* and *Mycobacterium smegmatis*

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Objectives: Therapy of AIDS patients infected with *Mycobacterium avium* is problematic due to its intrinsic resistance to antibiotics. We have characterized the efflux pump activity of *M. avium* wild-type strain through an automated fluorometric method and correlated it with intrinsic resistance to antibiotics.

Methods: *M. avium* ATCC 25291 T and *Mycobacterium smegmatis* mc²155 were evaluated for accumulation and efflux of ethidium bromide in the presence or absence of the efflux pump inhibitors (EPIs) thioridazine, chlorpromazine, verapamil and the proton uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). For this purpose, a new automated fluorometric method was used that separately assesses accumulation and extrusion of ethidium bromide.

Results: The automated fluorometric method described in this paper allowed the detection and quantification of ethidium bromide transport across *M. avium* and *M. smegmatis* cell walls. Accumulation of ethidium bromide was found to be temperature-dependent and significantly increased by EPIs thioridazine, chlorpromazine, verapamil and CCCP in a concentration-dependent manner. Efflux of ethidium bromide under optimum conditions of temperature and glucose is inhibited by the above agents. At half their intrinsic MICs, both thioridazine and chlorpromazine, similarly to verapamil and CCCP, significantly increased the susceptibility of *M. avium* to erythromycin, suggesting an effect upon an efflux pump with ethidium bromide and erythromycin as substrates. A similar effect was observed for *M. smegmatis* with verapamil only.

Conclusions: *M. avium* and *M. smegmatis* intrinsic resistance is affected by EPIs such as thioridazine or chlorpromazine, an effect that might be important in research and development of new, more effective antimycobacterial therapies.

Keywords: mycobacteria, phenothiazines, efflux pumps, efflux pump inhibitors, automated fluorometric method

Introduction

Environmental opportunistic mycobacteria are distinguished from the members of the *Mycobacterium tuberculosis* complex and from *Mycobacterium leprae* by the fact that they are not obligate pathogens and are commonly found in the environment as saprophytes, commensals and symbionts. Their need to survive in the environment, a source of numerous dangers in the form of toxic substances and wildly varying osmotic pressures, forces these microorganisms to be able to transport nutrients into the cell and, at the same time, selectively exclude toxic molecules and remove toxic metabolic products from the cell.¹ Therefore, environmental mycobacteria are intrinsically more resistant to most of the commonly used antibiotics creating

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severe difficulties in treating such infections.\textsuperscript{1,2} Their intrinsic resistance to antibiotics has been assigned almost exclusively to the nature and structure of their lipid-rich cell wall.\textsuperscript{1–3}

\textit{Mycobacterium avium}, an environmental mycobacteria found in water, soil, air, food and animals, which shares genetic and phenotypic characteristics with several other environmental mycobacteria, is of significant clinical importance because it causes severe infections in AIDS patients in whom, as a consequence of low CD4+ cell counts, the bacteria spread through vascular and lymphatic channels causing disseminated disease.\textsuperscript{2,4} Like in other environmental mycobacteria, the cell envelope relatively impermeable to antibiotics is generally assumed to be the reason for the multidrug-resistant (MDR) phenotype of \textit{M. avium} and thus makes the therapy of \textit{M. avium} infections extremely difficult.\textsuperscript{2,4,5}

Recently, it has been shown that efflux pumps of mycobacteria play an important role in their antibiotic resistance.\textsuperscript{6–8} It has been shown that mycobacterial strains can present a MDR phenotype as a consequence of increased activity of efflux pumps that prevent the compounds from reaching their intended targets.\textsuperscript{6,7,9} The activity of some of these pumps has been shown to be inhibited by different compounds such as the phenothiazines chlorpromazine and its derivative thiordanize, as well as reserpine and verapamil, which have been shown to have efflux pump inhibiting activity against mycobacteria both \textit{in vitro} and \textit{ex vivo}.\textsuperscript{6,10–13}

Several strategies and laboratory methods detect and quantify the activity of bacterial efflux pump systems using measurement of radiolabelled, fluorescent or metal-labelled substrates.\textsuperscript{14–18} However, these methods, for the most part, fail to separate accumulation of substrate from its extrusion and vice versa.

In the study to be described herein, an automated method has been developed that demonstrates and quantifies efflux pump activity on a real-time basis, therefore allowing the identification of specific efflux pump inhibitors (EPIs).

Materials and methods

Materials

Chlorpromazine, thioridazine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), verapamil, erythromycin, rifampicin, ethambutol, ciprofloxacin, amikacin, ethidium bromide and phosphate-buffered saline (PBS) in tablets (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) were purchased from Sigma Aldrich Química SA (Madrid, Spain). Clarithromycin was obtained from Abbott Laboratories (Abbott Park, IL, USA). Middlebrook 7H11 solid medium, Middlebrook 7H9 broth and the oleic acid–albumin–dextrose–catalase (OADC) supplement were purchased from Difco (Detroit, MI, USA). Microtitre plates were purchased from Nalgene (Rochester, NY, USA). All solutions were prepared in distilled, sterile water on the day of the experiment.

Bacteria

\textit{Mycobacterium smegmatis} mc\textsuperscript{2}155 and \textit{M. avium} sp. \textit{avium} ATCC 25291\textsuperscript{T} were grown at 37°C in Middlebrook 7H9 broth or Middlebrook 7H11 solid medium, both supplemented with 10% of OADC. Cultures of these strains served as the source of bacterium for the preparation of a standard inoculum in PBS to be used in determination of MICs. The number of colony-forming units (cfu) corresponding to aliquots of the inoculum was routinely calculated in order to ensure a constant number of bacterial cells from experiment to experiment.

Determination of MICs of agents employed

The determination of MICs of thioridazine, chlorpromazine, verapamil and CCCP, as well of the antibiotics studied alone and in the presence of an EPI, was conducted by the broth microdilution method adapted from previous studies and in accordance to the NCCLS guidelines.\textsuperscript{19,20} Briefly, \textit{M. smegmatis} mc\textsuperscript{2}155 and \textit{M. avium} ATCC 25291 were grown in 7H9/OADC-supplemented medium at 37°C until an optical density (OD) of 0.8 at a wavelength of 600 nm. The bacterial cultures were diluted in PBS and the suspension adjusted to equal the McFarland No. 0.5 turbidity standard.

Final inoculum was prepared by diluting the adjusted bacterial suspension at 1:100, and 0.1 mL aliquots were transferred to each well of the 96-well plate that contained 0.1 mL of each agent at concentrations prepared from 2-fold serial dilutions in 7H9/ OADC-supplemented medium. The inoculated plates were incubated at 37°C and the MIC results registered after a period of time, at the end of which growth in the agent-free control-well was evident (3 and 5 days for \textit{M. smegmatis} and \textit{M. avium}, respectively). The MIC was defined as the lowest concentration of compound that inhibited visible growth.

Automated fluorometric method

For the assessment of accumulation and extrusion of the fluorochrome ethidium bromide, a new protocol was developed using a real-time thermocycler. The method: (i) employs the most commonly used efflux pump substrate ethidium bromide, a biocompatible molecule that, under defined and controlled conditions, does not affect cell viability nor perturb cellular functions. Ethidium bromide has a low fluorescence signal outside the bacterial cell that increases once inside the cell in a concentration-dependent manner and is widely recognized as the best candidate for monitoring efflux pump activity;\textsuperscript{21–24} (ii) uses an instrument, Rotor-Gene 3000\textsuperscript{TM} (Corbett Research, Sydney, Australia), that reads and quantifies the ethidium bromide fluorescence signal; (iii) uses a methodology (see below) that distinguishes accumulation of the molecule from its extrusion as two separate transports across the cell; and (iv) provides the means by which environmental conditions and agents affecting accumulation and extrusion of substrate can be studied.

This real-time fluorometry assay allows detection of accumulation and efflux of ethidium bromide by following the evolution of fluorescence per unit period of time and provides the means by which the potential activity of a given EPI can be assessed. The ethidium bromide accumulation and efflux assays were repeated at least three times with reproducible results.

Ethidium bromide accumulation assay

Mycobacterial cells were grown in 10 mL of 7H9/ OADC-supplemented medium at 37°C until an OD of 0.8. The culture was centrifuged at 13 000 rpm for 3 min, the supernatant discarded, the pellet washed once and re-suspended in PBS. After adjusting the OD to 0.4, glucose (to yield a final concentration of 0.4%) was added to one set of microtubes containing 1.0 mL of bacterial suspension, whereas another set remained without glucose. To determine the lowest concentration of ethidium bromide that resulted in minimal accumulation, aliquots of 0.005 mL were transferred from aqueous stock solutions of ethidium bromide to the
above microtubes to yield final concentrations of ethidium bromide that ranged from 0.125 to 8 mg/L. Aliquots of 0.1 mL were distributed to replica sets of 0.2 mL PCR microtubes that were placed into a 36-well rotor and the fluorescence measured in the real-time thermocycler Rotor-Gene 3000TM, using the 530 nm band-pass and the 585 nm high-pass filters as the excitation and detection wavelengths, respectively. Fluorescence data were acquired every 60 s for 30–100 min. To determine the effect of temperature on the accumulation of ethidium bromide, accumulation assays were separately conducted at 25 and 37 °C.

The effect of the EPIs thioridazine, chlorpromazine, verapamil and CCCP on the accumulation of ethidium bromide at 37 °C, in the presence or absence of glucose, was determined as described earlier.

Ethidium bromide efflux assay

Conditions that were shown to cause maximum accumulation of ethidium bromide without causing any significant inhibition of growth, as confirmed by cfu counting, were used for the loading of both M. smegmatis mc²155 and M. avium ATCC 25291 cells with this fluorochrome. The following were the selected conditions: accumulation at 25 °C in the absence of glucose; use of an ethidium bromide concentration that caused a higher accumulation without compromising the cellular viability (3 mg/L, corresponding to half the MIC); and use of the EPI that caused the highest level of ethidium bromide accumulation (in this case, verapamil). The ethidium bromide loaded cells were centrifuged at 13 000 rpm for 3 min and re-suspended in ethidium bromide-free PBS containing 0.4% glucose. After adjusting the OD to 0.4, aliquots of 0.095 mL were transferred to replicate 0.2 mL microtubes and the EPIs added. Replica tubes that did not receive any EPI served as a control for the assessment of efflux allowed by the conditions used in the assay. The amount of fluorescence emitted was monitored on a real-time basis, using the conditions described for the ethidium bromide accumulation assay.

In order to allow a comparative analysis of the efflux, the raw data obtained from the Rotor-Gene™ instrument was normalized, establishing the ethidium bromide loaded cells as the maximum fluorescence value (relative fluorescence equivalent to 1) that can be obtained during the assay. The relative fluorescence of the tubes used for the measurement of efflux was determined as the ratio between the raw fluorescence data of the efflux and the ethidium bromide loaded cells. The efflux is thus represented as the ratio of fluorescence that remains per unit of time, relatively to the ethidium bromide loaded cells.

Results and discussion

The lowest concentrations of ethidium bromide that resulted in minimal accumulation by M. avium ATCC 25291 at 37 °C during a period of 100 min are 0.25 and 1.0 mg/L for M. smegmatis mc²155 (data not shown). These minimal concentrations of ethidium bromide are deemed to be close to those which the intrinsic efflux systems of these organisms is capable of extruding. Because accumulation of ethidium bromide takes place at a higher concentration in M. smegmatis mc²155, this suggests that it has a more effective intrinsic efflux system than M. avium ATCC 25291. Higher concentrations of ethidium bromide that exceed the capacity of the efflux pump system are expected to result in increased accumulations, which if sufficiently high, can eventually result in ethidium bromide reaching DNA where it can readily intercalate and inhibit all DNA-based processes. Accumulation of ethidium bromide by M. smegmatis or M. avium exposed to minimal concentrations of ethidium bromide of 1.0 and 0.25 mg/L, respectively, could be significantly increased with a reduction of temperature but practically not by the absence of glucose during the period of assessment (30–40 min), as shown in Figure 1.

If accumulation of ethidium bromide by the mycobacterial cells is minimal at 37 °C and in the presence of glucose, then any activity of an EPI on accumulation of ethidium bromide must be demonstrated under these optimum conditions. Concentrations of thiophidazine, chlorpromazine, verapamil and CCCP at half their MIC (Table 1), when the assay is conducted at 37 °C with glucose present in the medium, significantly increased the accumulation of ethidium bromide by M. smegmatis mc²155 and M. avium ATCC 25291 cells, as summarized in Figure 2. Lower concentrations of each EPI produced minor increases of accumulation of ethidium bromide (data not shown).
Table 1. MICs of ethidium bromide and EPIs determined for *M. smegmatis* mc²155 and *M. avium* ATCC 25291 by the broth microdilution method

<table>
<thead>
<tr>
<th></th>
<th><em>M. smegmatis</em> mc²155</th>
<th><em>M. avium</em> ATCC 25291</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>TZ</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>CPZ</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>VP</td>
<td>300</td>
<td>800</td>
</tr>
<tr>
<td>CCCP</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>

EB, ethidium bromide; TZ, thioridazine; CPZ, chlorpromazine; VP, verapamil; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

It is noteworthy that accumulation of ethidium bromide may be affected by causes other than diminished efflux pump activity; as an example, increased permeability to ethidium bromide due to physical changes of the cell envelope.\(^7,8,25\) If one is to assess efflux of ethidium bromide and conditions that affect it, one must first employ conditions which promote the accumulation of ethidium bromide. When defining the optimum ethidium bromide accumulation conditions for the efflux experiments, we found that cells could be loaded up to 3 mg/L ethidium bromide (half the MIC). This ethidium bromide concentration ensures that its accumulation is lower than that which causes ethidium bromide to reach and intercalate into DNA, for once it does so, its binding constant is sufficiently high to obviate its removal,\(^26\) while promoting the optimum conditions for efflux measurements. Therefore, the following conditions were selected for promoting significant accumulations of ethidium bromide that did not affect the replication of either of the two mycobacterial species (data not shown): a temperature of 25°C, absence of glucose and presence of verapamil at half of its MIC (the EPI for which the highest accumulation of ethidium bromide was obtained; Figure 2). These conditions were applied to both *M. smegmatis* mc²155 and *M. avium* ATCC 25291 and when accumulation was at the level desired, the PBS containing ethidium bromide and verapamil was removed and replaced with PBS containing 0.4% glucose, lacking or containing the EPIs thioridazine, chlorpromazine and verapamil at varying concentrations. Changes of fluorescence were assessed by the Rotor-Gene™ instrument at 37°C over a period of 30 min. As shown in Figure 3 for *M. smegmatis* mc²155 and Figure 4 for *M. avium* ATCC 25291, efflux of ethidium bromide readily took place at 37°C in the presence of glucose. Thoridazine, chlorpromazine and verapamil inhibited this efflux in a concentration-dependent manner during the period of the assay (Figures 3 and 4). The same effects were also noted with CCCP (data not shown).

The MICs of ethidium bromide, thoridazine, chlorpromazine, verapamil and CCCP for *M. smegmatis* mc²155 and *M. avium* ATCC 25291 are summarized in Table 1. The MICs of clarithromycin, erythromycin, rifampicin, ethambutol, ciprofloxacin and amikacin for the studied strains are summarized in Table 2. They are consistent with those reported for the *M. avium* ATCC 25291 strain by others.\(^27\) Table 2 also summarizes the effect of thoridazine, chlorpromazine, verapamil and CCCP at half their MIC. Briefly, thoridazine, chlorpromazine, verapamil and CCCP significantly increased the susceptibility of the *M. avium* ATCC 25291 strain to erythromycin. With the exception of the ability of thoridazine to reduce resistance to amikacin and of CCCP to reduce resistance to ethambutol, the remainder of the EPIs did not alter resistance of the organism to the other compounds tested.

With respect to the effect of the EPIs on the resistance of *M. smegmatis* mc²155 to the same panel of antibiotics, thoridazine and chlorpromazine were only marginally effective in increasing the susceptibility to erythromycin, and less so when compared with verapamil (Table 2).

The results suggest that the presence of an intrinsic efflux pump system for the *M. avium* ATCC 25291 strain is responsible for the intrinsic resistance of this organism to erythromycin. Since the susceptibility to amikacin and ethambutol could be increased by thoridazine and CCCP, respectively, and not by chlorpromazine and verapamil, intrinsic resistance to these antibiotics may be due to efflux pumps other than the one that extrudes erythromycin and which is affected by all of the EPIs.

Thoridazine was shown in our study to be an effective inhibitor of the intrinsic efflux pump system, which we deem is...
responsible for intrinsic resistance to erythromycin. This phenothiazine has been in use for over 35 years for the successful therapy of psychoses. When compared with its parental neuroleptic chlorpromazine, thioridazine is as effective and produces milder side effects. Because arrhythmia has been reported to occur in humans treated with thioridazine, there is some reluctance to use this phenothiazine for any pathology other than psychoses. The incidence of arrhythmia caused by thioridazine, however, is relatively small and may be further minimized with proper cardiological management.

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Transparency declarations

None to declare.

References


Table 2. Effect of the EPIs thioridazine, chlorpromazine, verapamil and carbonyl cyanide m-chlorophenylhydrazone on the MICs of different antimicrobial agents for M. smegmatis mc²155 and M. avium ATCC 25291

<table>
<thead>
<tr>
<th></th>
<th>MIC</th>
<th>MIC with TZ</th>
<th>MIC with CPZ</th>
<th>MIC with VP</th>
<th>MIC with CCCP</th>
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</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>200</td>
<td>16</td>
<td>100</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>Clarithromycin</td>
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<td>12.5</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>Rifampicin</td>
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<td>2</td>
<td>50</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Ethambutol</td>
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<td>8</td>
<td>1.56</td>
<td>8</td>
<td>1.56</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.39</td>
<td>16</td>
<td>0.39</td>
<td>16</td>
<td>0.39</td>
</tr>
<tr>
<td>Amikacin</td>
<td>0.39</td>
<td>4</td>
<td>0.39</td>
<td>0.5</td>
<td>0.39</td>
</tr>
</tbody>
</table>

TZ, thioridazine; CPZ, chlorpromazine; VP, verapamil; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

Data in bold type represent significant (at least 4-fold) reduction of resistance produced by the presence of the EPI.


