Plant phenolic compounds as ethidium bromide efflux inhibitors in *Mycobacterium smegmatis*

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**Background:** One-third of the world’s population is infected with the dormant tuberculosis bacillus, and there have been no new antimycobacterial compounds with new modes of action for over 30 years. Extensively drug-resistant tuberculosis is resistant to first- and second-line drugs, which can have severe side effects, and requires the breakthrough of new antituberculotics and resistance-modifying agents. Efflux pumps can cause multidrug resistance and have recently evoked much interest as promising new targets in antimicrobial therapy.

**Objectives:** The study was performed to set up an ethidium bromide (EtBr) efflux assay in *Mycobacterium smegmatis mc²155* for testing plant natural compounds as mycobacterial efflux pump inhibitors (EPIs).

**Methods:** After determining the MICs of the putative EPIs, they were tested for synergistic effects with EtBr prior to the efflux assay.

**Results:** We established an EtBr efflux assay in *M. smegmatis mc²155*. The isoflavone biochanin A exhibited efflux pump inhibiting activity comparable to that of verapamil. The flavone luteolin and the stilbene resveratrol were less active.

**Conclusions:** A new assay was established to observe the EtBr efflux in *M. smegmatis* and was applied to evaluate plant phenolic compounds. Our results highlighted that the isoflavonoid biochanin A exhibited better EPI activities than other flavonoids in mycobacteria.

Keywords: isoflavonoids, biochanin A, efflux pumps, mycobacteria

**Introduction**

Mycobacterial infections including *Mycobacterium tuberculosis* as well as fast-growing strains are increasing globally. The additional prevalence of multidrug-resistant (MDR) strains and extensively drug-resistant tuberculosis¹ stimulates an urgent need for the development of new drugs for the treatment of mycobacterial infections. The mycobacterial cell wall barrier and active multidrug efflux pumps are involved in intrinsic and acquired resistance of these pathogens to many commonly used antibiotics.² Efflux pumps can be specific for a class of antibiotics or responsible for MDR.³ They are attractive antibacterial targets, and the co-administration of an efflux pump inhibitor (EPI) with an antibiotic has progressed to human clinical trials.³ Effective bacterial EPIs should decrease the intrinsic resistance of bacteria to antibiotics, reverse acquired resistance and reduce the frequency of emergence of resistant mutant strains.⁵ According to Stavrī et al.,⁶ there have been no natural EPIs for mycobacteria identified so far.

Every class of the five existing families of efflux pumps is present in *M. tuberculosis*.² Reserpine is an inhibitor of ATP-dependent pumps, verapamil inhibits P-glycoprotein and bacterial efflux pumps in general,² and chlorpromazine affects potassium flux across the membrane in *Staphylococcus aureus* and the yeast *Saccharomyces cerevisiae*.⁷ A feature of all of these compounds is their ability to inhibit potassium transport processes.⁵ These inhibitors were used as reference substances to evaluate the extent of possible efflux inhibition in *Mycobacterium smegmatis mc²155*, which expresses many putative efflux pumps.⁵ In this paper, we present for the first time
plant natural products with inhibiting effects on ethidium bromide (EtBr) efflux of *M. smegmatis* mc²155.

**Materials and methods**

*Chemicals*

EtBr (Sigma-Aldrich, Steinheim, Germany) was dissolved in water. Isoniazid (Sigma-Aldrich) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; Fluka/Sigma-Aldrich) were dissolved in DMSO (Merck Darmstadt, Germany). The following test compounds were dissolved in DMSO: Biochanin A, daidzein and formononetin (Fluka, Sigma-Aldrich). Baicalin 95%, baicalein 98%, resveratrol, chlorpromazine hydrochloride, verapamil hydrochloride 98% and reserpine were purchased from Sigma-Aldrich. Luteolin was acquired from Carl RothKG, Karlsruhe, Germany.

**Bacterial strains and growth conditions**

*M. smegmatis* mc²155 ATCC 70084 (LGC Promochem, Teddington, Middlesex, UK) was used throughout the study. Bacterial cells were grown on Columbia Blood Agar (CBA; Oxoid, Basingstoke, England, UK) supplemented with 7% defibrinated horse blood (Oxoid) at 37°C under aerobic conditions for 2–3 days prior to assays. MIC and modulation assays were performed in cation-adjusted Mueller–Hinton Agar (MHB; Oxoid).

Difco™ Middlebrook 7H9 Broth (Becton, Dickinson and Company, Le Pont de Claix, France) supplemented with 10% BBL™ Middlebrook OADC Enrichment (Becton, Dickinson and Company, Shannon, Ireland) and 0.05% Tween 80 (for molecular biology, Sigma-Aldrich) or 0.4% Difco™ Glycerol (Becton, Dickinson and Company, Sparks, MI, USA) was used for efflux experiments.

**MIC assay and modulation assay**

MICs were determined as described previously.¹⁰ Briefly, a standard MIC determination of serially diluted test compounds in Ca²⁺- and Mg²⁺-adjusted MHB using bacterial inocula with a density of 5 × 10⁸ cfu/mL was conducted. Plates were incubated at 37°C for 72 h. Isoniazid was used as a positive control.

Test compounds were further screened for their synergistic effects with EtBr prior to efflux assays. Compounds were dissolved in DMSO and diluted in MHB at subinhibitory concentrations. The concentration of the modulators remained the same throughout the experiment, whereas the antibiotics were serially diluted for MIC determination with and without modulator, respectively. A ‘modulation factor’ (MF) was used to express the modulating effects of compounds on MIC (EtBr).

\[
MF = \frac{MIC \text{ (antibiotic)}}{MIC \text{ (antibiotic + modulator)}}
\]

The fractional inhibitory concentration index (FICI)¹¹ expressed the effect of the combination of antibacterial agents:

\[
FICI = FIC (A) + FIC (B)
\]

\[
FIC (A) = \frac{MIC (A \text{ in the presence of } B)}{MIC (A \text{ alone})}
\]

\[
FIC (B) = \frac{MIC (B \text{ in the presence of } A)}{MIC (B \text{ alone})}
\]

Synergism, \(FICI \leq 0.5\); antagonism, \(FICI \geq 4.0\); and no interaction, \(FICI > 0.5–4.0\).

**EtBr efflux assay**

This assay was adapted for *M. smegmatis* mc²155 following a method by Kaatz et al.¹² for inhibitors of the proton motive force driven multidrug pump NorA in *S. aureus*.

*M. smegmatis* mc²155 was cultivated on CBA under aerobic conditions at 37°C for 2–3 days, which was then used for inoculating an overnight culture in Tween 80-containing Middlebrook 7H9 broth. This culture was incubated overnight at 37°C, 160 rpm in sterile 50 mL centrifugal tubes and diluted 1:100 in the same medium. Large-scale cultures were grown to mid-exponential phase (OD₆₀₀ ~ 0.8–1.0) at 37°C, 80 rpm for 16–24 h.

Cells were loaded with 100 μM of the proton conductor CCCP and 5 μM EtBr and further incubated for 1 h at 37°C. The inoculum was adjusted to OD₆₀₀ = 0.40 (0.39–0.41) with EtBr- and CCCP-containing Middlebrook 7H9 (with OADC and Tween 80), 4 mL aliquots were spun down with 5000 g for 10 min at 20°C and the pellets were put on ice immediately. Cell pellets were resuspended in 2 mL of glycerol-containing Middlebrook 7H9. EtBr efflux from the cells was monitored at room temperature with a spectrophotofluorimeter (Perkin Elmer LS50B Luminescence Spectrometer) under constant stirring. The excitation and emission wavelengths used were 530 nm (slit width = 5.0 nm) and 600 nm (slit width = 10.0 nm), respectively, and readings were taken every minute for 10 min. The loss of fluorescence indicated efflux activity.

The low background fluorescence of the medium was subtracted from sample and control measurements. Controls without the test compounds were carried out at the beginning and end of the assay, showing that the bacteria did not loose EtBr while stored on ice. Each concentration of the test compounds and the controls was measured at least in duplicate (active compounds at least in triplicate). Mean results were expressed as the percentage reduction of total efflux, which was observed for test strains in the absence of inhibitors for 10 min. Fluorescence levels of all controls and samples had to be within a coefficient of variation of maximal 20%.

**Table 1. MICs and modulation factors of compounds for *M. smegmatis* mc²155**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (mg/L)</th>
<th>Concentration as modulator (mg/L)</th>
<th>Modulation factor (EtBr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalein</td>
<td>64</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Baicalin</td>
<td>256</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>256</td>
<td>10</td>
<td>4–8</td>
</tr>
<tr>
<td>Daidzein</td>
<td>&gt;256</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>Formononetin</td>
<td>256</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>Genistein</td>
<td>256</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Luteolin</td>
<td>32</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Myricetin</td>
<td>32</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>64</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>64</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Reserpine</td>
<td>&gt;128</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Verapamil</td>
<td>&gt;256</td>
<td>20</td>
<td>1–2</td>
</tr>
</tbody>
</table>

MIC of isoniazid = 2 mg/L and MIC of EtBr = 16 mg/L.

Modulation factor = MIC (EtBr)/MIC (EtBr + modulator); n = 4–8.
Results

Minimum inhibitory concentrations
The majority of the compounds exhibited weak antimycobacterial activities (Table 1).

Modulating activities
All compounds were further tested for modulating activities of EtBr at subinhibitory concentrations. Biochanin A was shown to be the best modulator and could decrease the MIC of EtBr 4- to 8-fold at 10 mg/L and 16- to 32-fold at 32 mg/L. The FICI between biochanin A and EtBr showed synergism (FICI = 0.25).11

With the exception of baicalin, formononetin, daidzein and myricetin, all tested compounds could modulate the MIC of EtBr at least to a small extent.

EtBr efflux inhibition experiments
We validated the EtBr efflux assay using reserpine, which demonstrated efflux inhibition of 91.31 ± 9.36% at 160 nM. Each known EPI tested showed efflux inhibition in M. smegmatis mc²155 cells. From all compounds tested, only biochanin A achieved inhibition levels comparable to the standard EPI controls. Luteolin and resveratrol were much less active and myricetin was inactive (Figure 1).

Discussion

The modulation assay with EtBr as an antibiotic seems to be an appropriate pre-screening for flavonoids as EPIs. Therefore, compounds that are able to decrease the MIC of EtBr (MIC = 16 mg/L) in M. smegmatis mc²155 should be further tested in the efflux assay. This can be illustrated by comparison of biochanin A, luteolin and myricetin, which show decreasing modulation factors as well as decreasing efflux-inhibiting activities.

The modulation assay also appears to be suitable for bioassay-guided isolation for mycobacterial EPIs, for example from crude plant extracts. As we did not utilize an overexpressing strain, this might explain the quite high concentrations of EPIs to achieve efflux inhibition.

Luteolin exhibited the same antimycobacterial activity as myricetin, but stronger synergism with EtBr. This showed that the hydroxy group at C-3 as well as the number of hydroxyl groups in ring B of flavones influenced EtBr-modulating but not antimycobacterial activity.

The free hydroxy group in ring B of daidzein slightly increased the modulating activity when compared with formononetin with a para-methoxy group in ring B. In contrast, the para-methoxy group of biochanin A strongly enhanced the modulating activity when compared with its parent compound genistein. Comparison of biochanin A and formononetin illustrated the relevance of a hydroxy group at C-5 for EtBr-modulating activity in general EPIs. Comparing baicalin and its aglycone baicalein showed that glycosylation of the hydroxy group at C-7 of flavones reduced antimycobacterial as well as modulating activities, respectively.

Biochanin A showed comparable efflux inhibitory effects to the reference EPIs. The isoflavone biochanin A and its metabolite genistein are potentiators of the antibacterial activities of norfloxacin and berberine in wild-type S. aureus.14 and the authors assumed an inhibiting effect on MDR pumps. Biochanin A might also reverse MDR by inhibiting the P-glycoprotein function in human breast cancer cells and was shown to increase [³H]daunomycin accumulation much more than the positive control verapamil.15 Combining our results with those of previous literature, biochanin A can override efflux mediated resistance in mammalian as well as bacterial cells.

The experiments of this study were performed on a fast-growing mycobacterial strain, and we emphasize that conclusions on the effects on M. tuberculosis should be drawn with care. However, the results obtained with the isoflavonoid biochanin A should stimulate investigations of this class of compounds as inhibitors of mycobacterial efflux pumps.

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Figure 1. Ethidium efflux inhibition assay from M. smegmatis mc²155 cells. Standard EPIs (broken lines): chlorpromazine, bars; reserpine, filled diamonds; verapamil, filled triangles. Test compounds (continuous lines): biochanin A, filled squares; resveratrol, filled triangles; luteolin, filled circles. Values represent means ± SD, n = 2–6.
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Transparency declarations

None to declare.

References


