Anti-tubercular screening of natural products from Colombian plants: 3-methoxynordomesticine, an inhibitor of MurE ligase of Mycobacterium tuberculosis

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Objectives: New anti-mycobacterial entities with novel mechanisms of action are clinically needed for treating resistant forms of tuberculosis. The purpose of this study was to evaluate anti-tubercular activity and selectivity of seven recently isolated natural products from Colombian plants.

Methods: MICs were determined using a liquid medium growth inhibition assay for Mycobacterium tuberculosis H37Rv and both solid and liquid media growth inhibition assays for Mycobacterium bovis BCG. Escherichia coli growth inhibition and mammalian macrophage cell toxicity were evaluated to establish the degree of selectivity of the natural product against whole cell organisms. Enzymatic inhibition of ATP-dependent MurE ligase from M. tuberculosis was assayed using a colorimetric phosphate detection method. The most active compound, 3-methoxynordomesticine hydrochloride, was further investigated on M. bovis BCG for its inhibition of sigmoidal growth, acid-fast staining and viability counting analysis.

Results: Aporphine alkaloids were found to be potent inhibitors of slow-growing mycobacterial pathogens showing favourable selectivity and cytotoxicity. In terms of their endogenous action, the aporphine alkaloids were found inhibitory to M. tuberculosis ATP-dependent MurE ligase at micromolar concentrations. A significantly low MIC was detected for 3-methoxynordomesticine hydrochloride against both M. bovis BCG and M. tuberculosis H37Rv.

Conclusions: Considering all the data, 3-methoxynordomesticine hydrochloride was found to be a potent anti-tubercular compound with a favourable specificity profile. The alkaloid showed MurE inhibition and is considered an initial hit for exploring related chemical space.

Keywords: TB, aporphine alkaloids, MurE inhibitors

Introduction

Tuberculosis (TB) is an ancient and contagious disease caused by infection with Mycobacterium tuberculosis complex.1,2 It causes characteristic necrotic and caseous lesions, notably in the lungs, but it may also affect the skin, lymph nodes, brain and almost every other organ. More than 9.3 million new cases and 1.8 million deaths were notified globally in 2008 according to the latest WHO report.3 TB was declared to be a global health emergency because of the increase in HIV co-infection and the appearance of multidrug-resistant and extensively drug-resistant strains (MDR- and XDR-TB).4 A TB burden also occurs as a consequence of low compliance due to the long and complex TB regimen and the lack of treatment or adapted treatment derived from the absence of appropriate health programmes.5,6 New anti-mycobacterial entities with novel mechanisms of action are clinically needed for treating resistant forms of TB. A global effort among all sectors of society is required for controlling the burden of TB.7

Natural products are crucial sources of new antimicrobials because of their amazing chemical diversity and their validation...
through centuries of evolution. It is usual for plants (or microorganisms) to fight against environmental infections using their chemical arsenal of secondary metabolites and therefore many types of different structures have been reported to display an antimicrobial function. Colombian Lauraceae, Magnoliaceae and Piperaceae species have been recently studied as sources for bioactive metabolites that might be useful in agricultural and medicinal applications. Ethnopharmacological Ocotea macrophylla Kunth (Lauraceae),12 endangered Dugandiodendron argyrotrichum Lozano (Magnoliaceae),13,14 spicy Piper hispidum Kunth15 and aromatic Piper eriopodon CDC (Piperaceae) were included in this study for evaluating the anti-tubercular activity of some of their constitutive natural products.

The strategy used in this investigation was to screen the purified natural compounds for growth inhibition against two slow-growing mycobacteria (Mycobacterium bovis BCG and M. tuberculosis H37Rv) and against gut bacteria (Escherichia coli) and mammalian macrophages (RAW264.7), in order to gain an idea of their mycobacterial specificity in whole cell experiments. Given the availability of recently characterized ATP-dependent MurE ligase from M. tuberculosis16 of the peptidoglycan biosynthetic pathway, the activity of the enzyme was recorded in the presence of the natural products. The gene encoding for this enzyme occurs as a single copy in the M. tuberculosis genome17 and was found to be essential for bacterial growth and survival.18,19 Furthermore, a detailed study in M. bovis BCG involving the growth curve, acid-fast staining and viability counting was undertaken for the most active compound. To the best of our knowledge, this is the first instance in which a natural product is reported to have inhibitory activity against MurE ligase from any organism.

Materials and methods

Natural products and organisms

Natural products 1 and 2 were previously isolated from O. macrophylla,12 4 and 5 from D. argyrotrichum13,14 and 6 from P. hispidum.15 In this study, compound 3 was obtained in 75% yield by HCl treatment from compound 1 in ethanol and column chromatography on silica gel with CH3Cl2-AcOEt (97:3).16 H and 13C NMR spectroscopy and the two-dimensional techniques of correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) were used to assign unambiguously the signals at δ 10.49 (1H, s) and 9.67 (1H, s) to the diastereoisomeric hydrogens on the positively charged nitrogen atom. The absolute configuration of C-6a was assigned as (R).23 An aliquot of 5 mL of Middlebrook 7H9 (BD Diagnostics) broth supplemented with 10% oleic acid/albumin/dextrose/catalase supplement (OADC; BD Diagnostics) and 0.2% glycerol. The same dilution procedure was performed for M. bovis BCG from a liquid culture having a cell density of 105 cfu/mL. The final cell density added to the microplates was 103 cfu/mL. From a sterile DMSO stock of the compounds at 200 mg/L, a solution in liquid medium was prepared at 512 mg/L (final concentration of DMSO 0.5%). Rifampicin was included as a positive control at 8 mg/L. To a 96-well microplate, 100 μL of liquid medium was added, and then the compounds were serially diluted 2-fold. An aliquot of 100 μL of the inoculum was added and the plates were sealed. Agitation was carried out manually every day, and after 6 days of incubation at 37°C the plates were carefully uncovered and 22 μL of a freshly prepared mixture (10:12) of 5 g/L methyl thiouaryl tetrazolium (MTT) and 20% Tween-80 was added. For M. bovis BCG, 30 μL of freshly prepared aqueous solution containing 0.05% resazurin and 0.1% Tween-80 was added to each well. Results were observed the next day after incubation at 37°C.

Solid plate-based spot culture growth inhibition assay

The spot culture growth inhibition assay was performed as reported elsewhere. An aliquot of 5 mL of Middlebrook 7H10 (BD Diagnostics) supplemented with 0.2% glycerol and 10% OADC was added to a six-well microplate along with 5 μL of natural compounds in a solution of DMSO at a concentration of 50, 25, 10, 5, 1 and 0 g/L. Isoniazid was included at 1, 0.5, 0.2, 0.1, 0.05 and 0 g/L. Afterwards, 5 μL of an appropriately diluted mid-log phase (106 cfu/mL) M. bovis BCG culture was carefully dispensed into the centre of each well. Results were observed after 2 weeks of incubation at 37°C.

Antibacterial assay against E. coli, P. putida and S. aureus

A seed culture of E. coli JM109 was prepared in Luria-Bertani broth and grown overnight at 37°C with shaking at 200 rpm. Then to each tube 5 mL of a 100× diluted culture (106 cfu/mL) and 20 μL of the compounds at 50 g/L stock concentration were added to make a final concentration of 200 mg/L. Controls of isoniazid and kanamycin at the same concentration and two negative controls (with and without DMSO, final concentration 0.1%) were included. The cells were incubated with agitation at
200 rpm and OD<sub>600</sub> was measured each hour up to the stationary phase. The MICs against <i>E. coli</i>, <i>P. putida</i> and <i>S. aureus</i> were determined in duplicate using a microdilution resazurin technique from 512 to 1 mg/L of the natural products following the CLSI (formerly NCCLS) guidelines. The <i>M. tuberculosis</i> protein was used as a positive control for Gram-negative bacteria and norfloxacin for Gram-positive bacteria.

**Mammalian macrophage cytotoxicity assay**

RAW264.7 macrophages were maintained in RPMI medium and passaged twice before the assay. The cells were detached using lidocaine–EDTA and mechanical tapping. Cell viability for macrophages was performed using a trypan blue assay and the cell density was adjusted to 1×10<sup>6</sup> cells/mL with RPMI. To a 96-well microplate, 2 μL aliquots of the 10×g/mL natural product stocks in DMSO were added. Then 200 μL of RPMI was added to the first row and serially diluted 2-fold. Finally, 100 μL of diluted macrophage cells was added. After 48 h of incubation, the cells were washed with PBS twice. Fresh RPMI medium was added and then the plates were revealed with 30 μL of freshly prepared 0.01% resazurin solution. A positive control of digitonin was used in the assay. The plates were incubated overnight and the next day spectrophotometric detection was performed at 590 nm after excitation at 560 nm. The assay was carried out in duplicate on different days. In addition, microscopic observation of treated and untreated cells was performed at ×400 magnification.

**M. tuberculosis MurE activity inhibition assay**

<i>M. tuberculosis</i> MurE activity was assayed as reported previously. The assay mixture contained 25 mM bis-trispropane buffer at pH 8.5, 5 mM MgCl<sub>2</sub>, 100 μM UDP-MurNac-dipeptide (BoCWAU, University of Warwick, UK), 250 μM ATP and 1 mM meso-diaminopimelic acid. The compounds were dissolved in DMSO at concentrations of 25, 8.3, 2.5, 0.83, 0.25 and 0.083 mM and 2 μL aliquots were added to half area 96-well plates. The reaction was initiated by the addition of MurE enzyme at a final concentration of 40 nM to the assay mixture, 48 μL of the mixture was added to each well and the plate was incubated at 37°C for 30 min. Phosphate release was determined in triplicate using the Pi Color-Lock Gold Kit (Innova Biosciences) reagents. Isoniazid was included as negative control at the same concentrations. A control reaction was performed with all the components except the enzyme. Absorbance at 635 nm was measured and percentage inhibition was calculated using a negative control (0% activity) and enzyme reaction (100% activity).

**M. bovis BCG growth curve, acid-fast staining and viability counting**

To roller bottles, 100 mL of Middlebrook 7H9 containing 0.2% glycerol, 0.05% Tween-80 and 10% albumin/dextrose/catalase supplement (ADC; BD Diagnostics) was added. Compound 3 dissolved in DMSO was added to the roller bottles at final concentrations of 5, 2.5, 1 and 0 mg/L. A 1:100 inoculation from a first-passage M. bovis BCG culture in supplemented 7H9 was performed and the bottles were incubated at 37°C, rolling at 2 rpm. OD<sub>600</sub> measurements were taken every 24 h for 2 weeks using an appropriate blank. Serial 10-fold dilutions in Middlebrook 7H10 medium supplemented with 0.2% glycerol and 10% OADC. Counting of single colonies allowed calculation of initial viable bacterial density. To two pairs of glass slides, 50 μL of liquid culture was added and the slides were dried at 110°C for 20 min. The fixed slides were stained with a Tb-color Staining Kit (Merck) using fuchsin, EtOH–HCl and malachite green, and they were observed under the microscope at ×1000 magnification.

**Results**

**Growth inhibition of Mycobacterium spp.**

Natural products 1–7 (Figure 1) were screened for growth inhibition against <i>M. tuberculosis</i> H37Rv in liquid medium and <i>M. bovis</i> BCG in solid and liquid media (Table 1). Interestingly, antitubercular activity was more pronounced for the polar and mildly acidic alkaloid 3 (MIC ≤ 5 mg/L) when compared with more basic compounds 1 (MIC ≥ 25 mg/L) and N-methoxycarbonyl-substituted 2 (MIC ≥ 50 mg/L). The diastereoisomeric erthro and threo mixture of austrobailignan-6 (5) showed inhibition of mycobacterial growth in liquid and solid media and the meso and threo mixture of dihydroguaiareic acid (4) was inactive (Table 1). The diastereoisomeric mixture 4 inhibited <i>M. bovis</i> BCG growth in solid medium at 25 mg/L with significant effects at 10 mg/L. Boldine, a natural aporphine alkaloid structurally related to 3-methoxydomesticine, was shown to be inactive, having an MIC greater than 50 mg/L.

**Antibacterial activity on E. coli, P. putida and S. aureus**

To appraise whether the compounds selectively affected slow-growing mycobacteria in comparison with fast growing bacteria, whole-cell experiments were performed. Comparative time measurement of <i>E. coli</i> growth after exposure to high concentrations of compounds, typically 200 mg/L, was evaluated by measuring OD<sub>600</sub> (Figure 2a). Potent anti-tubercular alkaloids 1–3 showed a similar profile (among the alkaloids 1–3, only 1 is shown in Figure 2a), and weakly affected <i>E. coli</i> growth at concentrations of 25 mg/L. Microscopic observation showed that the diastereoisomers 5, dihydrochalcone 6 and phenol 7 were moderate growth inhibitors of <i>E. coli</i>. The microdilution technique allowed the compounds to be inactive against Gram-negative (<i>E. coli</i> and <i>P. putida</i>) and Gram-positive bacteria (S. aureus) except phenol 7, which significantly inhibited the growth of <i>S. aureus</i> (J. D. Guzman, D. R. Muñoz, W. A. Delgado, L. E. Cuca, S. Gibbons and S. Bhakta, unpublished results).

**Macrophage RAW264.7 cytotoxicity**

Growth inhibitory concentration 50 (IC<sub>50</sub>) was calculated for all of the natural compounds (Table 1). Microscopic slides of RAW264.7 cells at a concentration of 50 mg/L were observed. Viability in the presence of 1–3 demonstrated moderate cytotoxicity. For the alkaloids, the selectivity index (SI = IC<sub>50</sub>/MIC) showed a value ranging from 0.5 to 12, compound 3 being the most specific (SI = 12). Microscopic observation showed that 1 did not affect macrophages at 50 mg/L, although 3 showed some growth inhibition at the same concentration. Moreover, macrophages were strongly susceptible to flavonoid 6 and moderately susceptible to phenol 7 and lignans 4 and 5 (Table 1).

**M. tuberculosis MurE inhibition**

Overexpressed and purified recombinant <i>M. tuberculosis</i> protein MurE was assayed under different concentrations of natural products using colorimetric detection of phosphate, which has been
Figure 1. Chemical structures of 3-methoxynordomesticine (1), N-methoxycarbonyl-3-methoxynordomesticine (2), 3-methoxynordomesticine hydrochloride (3), dihydroquaiaretic acid meso and erythro diastereoisomers (4), austrobailignan-6 threo and erythro diastereoisomers (5), 2',4',6'-trimethoxydihydrochalcone (6), gibbilimbol-B (7) and boldine.

Table 1. MICs, GIC_{50}, SI and MurE IC_{50} of natural compounds 1-7

<table>
<thead>
<tr>
<th>Natural products</th>
<th>M. tuberculosis H_{37}Rv, MTT microtitre assay^21</th>
<th>spot culture growth inhibition assay^21</th>
<th>resazurin microtitre assay^22</th>
<th>GIC_{50} for RAW264.7 (mg/L)</th>
<th>SI^a</th>
<th>IC_{50} for M. tuberculosis MurE (μM) [mg/L]</th>
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<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>25</td>
<td>64</td>
<td>62 ± 5</td>
<td>0.97</td>
<td>67 ± 11 [22.9]</td>
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<td>2</td>
<td>128</td>
<td>50</td>
<td>256</td>
<td>67 ± 7</td>
<td>0.52</td>
<td>75 ± 15 [30.0]</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>5</td>
<td>16</td>
<td>47 ± 6</td>
<td>12</td>
<td>57 ± 14 [19.5]</td>
</tr>
<tr>
<td>4</td>
<td>&gt;128</td>
<td>&gt;50</td>
<td>256</td>
<td>43 ± 13</td>
<td>&lt;0.34</td>
<td>&gt;1000 [&gt;330]</td>
</tr>
<tr>
<td>5</td>
<td>128</td>
<td>50</td>
<td>128</td>
<td>45 ± 6</td>
<td>0.35</td>
<td>286 ± 33 [93.9]</td>
</tr>
<tr>
<td>6</td>
<td>&gt;128</td>
<td>&gt;50</td>
<td>&gt;256</td>
<td>10 ± 8</td>
<td>&lt;0.078</td>
<td>&gt;1000 [&gt;300]</td>
</tr>
<tr>
<td>7</td>
<td>128</td>
<td>25</td>
<td>128</td>
<td>36 ± 8</td>
<td>0.28</td>
<td>184 ± 16 [42.8]</td>
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<tr>
<td>Isoniazid</td>
<td>0.125</td>
<td>0.05</td>
<td>0.0625</td>
<td>&gt;500</td>
<td>&gt;4000</td>
<td>&gt;1000 [&gt;137]</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.25</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&gt;500 [&gt;411]</td>
</tr>
</tbody>
</table>

ND, not determined.

^aThe SI was calculated by dividing the GIC_{50} for RAW264.7 by the MIC for M. tuberculosis H_{37}Rv.
shown to be stoichiometrically coupled to meso-diaminopimelic acid ligation.\textsuperscript{16} A consistent MurE inhibition was observed for aporphine alkaloids 1–3, all having IC\textsubscript{50} values of less than 100 \(\mu\)M (Table 1). Boldine was not inhibitory to MurE even at higher concentrations (IC\textsubscript{50} > 1 mM). Natural products 5 and 7 showed moderate MurE inhibition and compounds 4 and 6 were inactive. Known anti-TB drugs were also tested against MurE inhibition, but none showed activity (isoniazid and rifampicin were included as controls).

**Alteration in mycobacterial growth curve, acid-fast staining and viability counting**

The growth curve of \(M.\ bovis\) BCG in the presence of different concentrations of 3-methoxynordomesticine hydrochloride (3) corroborated the high activity (MIC < 5 mg/L) observed in liquid and solid culture (Figure 2b). After five days of growth (OD ≏ 1.2) cells were acid-fast stained and counted for viability. Not a single acid-fast cell was observable in the 5 mg/L treated sample. Alkaloid 3 decreased by more than 7-log the number of colony forming units (Figure 2c) at 5.0 mg/L, indicating a bactericidal effect.

**Discussion**

The *in vitro* growth of bacteria on solid and in liquid media represents two different physiological states of the microorganism. In solid medium mycobacteria grow more slowly than in liquid medium and the bacilli are restricted to a surface as opposed to a liquid medium, where there is aeration and constant movement. Differences between the activity of the compounds or antibiotics may exist in liquid and solid media. In this work, apolar natural products such as compounds 4, 5 and 7 showed significant differences in their MIC in liquid and solid culture for the same species, \(M.\ bovis\) BCG. Moreover, in liquid medium the MIC was presumably overestimated because of precipitation and hence reduced availability of the compounds in Middlebrook 7H9 and LB broth. We must also consider the longer incubation time for slow-growing mycobacteria, which may increase the amount of precipitation for compounds with low solubility in aqueous medium. On the other hand, the alkaloids 1–3 showed a similar effect in liquid and solid media. There are also reports of interaction of some natural compounds with the MTT viability indicator, especially for flavonoids.\textsuperscript{26} In this work dihydrochalcone 6 reduced the viability indicator MTT in the absence of bacterial cells, introducing some ambiguity to the MIC determination, hence subsequently a solid agar-based screening was appropriately introduced.

The aporphine alkaloids 1–3 were found to be selective inhibitors of slow-growing mycobacteria. Anti-mycobacterial activity has previously been reported for structurally related piperocatechum and cepharadione aporphine alkaloids,\textsuperscript{27} suggesting a promising anti-tubercular hit in related chemical space. Moreover, a recent paper has reported anti-inflammatory activity of alkaloid 1 and, interestingly, also recorded a low antimicrobial effect (MIC > 256 mg/L) against \(E.\ coli\), \(S.\ aureus\) and \(Candida albicans\),\textsuperscript{28} supporting mycobacterial selectivity for this metabolite. Interestingly, the closely related alkaloid boldine was ineffective as a MurE inhibitor as well as not showing any effect on the *in vitro* growth inhibition of slow-growing mycobacteria, indicating that substitution in the aryl rings and/or nitrogen atom is essential for the anti-tubercular activity of aporphines. Dibenzylbutane lignan 5 was a moderate inhibitor of slow-growing mycobacteria of higher concentrations (IC\textsubscript{50} > 1 mM).

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**Figure 2.** Antimicrobial activity of natural products (3–7) against \(E.\ coli\) and effect of 3-methoxynordomesticine hydrochloride (3) on liquid culture of \(M.\ bovis\) BCG. (a) Growth curve of \(E.\ coli\) at 200 mg/mL of the natural compounds. (b) Growth curve of the bacilli under four different concentrations (0, 1.0, 2.5 and 5.0 mg/L) of 3. (c) Bar diagram showing the number of cfu/mL of liquid culture under different concentrations of 3. Error bars show the SD of the mean.
mycobacteria, and related chemical structures have displayed anti-tubercular activity, but are in some way cytotoxic as they also inhibit DNA topoisomerases. All diastereomers of dihydroguaiaretic acid have shown potent antimicrobial activity and this study confirmed this observation. Flavonoid did not show significant inhibition of mycobacteria or E. coli, but was surprisingly toxic to macrophages. Gibbilimbol-B had anti-mycobacterial properties, but lacked selectivity. O-arylated catechol analogues of have been reported as potent inhibitors of the mycolic acid biosynthetic InhA enzyme, a recognized target of isoniazid.

To our understanding, this is the first report of MurE ligase inhibition by natural products. The IC₅₀ of the aporphine alkaloids fell in the micromolar range and these initial hits may be exploited in the future for constructing increased affinity ligands. The fact that the MurE IC₅₀ of the aporphine alkaloids has the same order of magnitude as the Glc₅₀ on macrophages reveals that further structural improvement is needed in order to make the alkaloids less toxic while increasing their affinity for the enzyme. A synthetic route for these alkaloids has already been described and consequently it is possible to generate closely related analogues by varying a single chemical functionality in order to gain structure-activity information. More detailed studies involving fragment screening, inhibition kinetics and structural molecular approaches are envisaged for augmenting the impact of these hits. Additionally, we must consider the analysis of muramidases in treated bacilli at the sub-MIC of the inhibitors for confirming pathway interference as the mode of action of these alkaloids. We believe that the unique upper leaflets (mycolyl-arabinogalactan) covalently bound to the lower polymeric mesh (peptidoglycan) in the asymmetrical model proposed by Minnikin and collaborators are most likely to disappear when lower layers are attacked; thus, inhibitors of a key peptidoglycan biosynthesis step have the potential to be developed into a novel anti-TB chemotheraphy.

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Transparency declarations

None to declare.

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