Interaction of N-methyl-2-alkenyl-4-quinolones with ATP-dependent MurE ligase of Mycobacterium tuberculosis: antibacterial activity, molecular docking and inhibition kinetics

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Objectives: The aim of this study was to comprehensively evaluate the antibacterial activity and MurE inhibition of a set of N-methyl-2-alkenyl-4-quinolones found to inhibit the growth of fast-growing mycobacteria.

Methods: Using the spot culture growth inhibition assay, MICs were determined for Mycobacterium tuberculosis H37Rv, Mycobacterium bovis BCG and Mycobacterium smegmatis mc²155. MICs were determined for Mycobacterium fortuitum, Mycobacterium phlei, methicillin-resistant Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa using microplate dilution assays. Inhibition of M. tuberculosis MurE ligase activity was determined both by colorimetric and HPLC methods. Computational modelling and binding prediction of the quinolones in the MurE structure was performed using Glide. Kinetic experiments were conducted for understanding possible competitive relations of the quinolones with the endogenous substrates of MurE ligase.

Results: The novel synthetic N-methyl-2-alkenyl-4-quinolones were found to be growth inhibitors of M. tuberculosis and rapid-growing mycobacteria as well as methicillin-resistant S. aureus, while showing no inhibition for Escherichia coli and P. aeruginosa. The quinolones were found to be inhibitory to MurE ligase of M. tuberculosis in the micromolar range (IC₅₀ ≏ 40–200 μM) when assayed either spectrophotometrically or by HPLC. Computational docking of the quinolones on the published M. tuberculosis MurE crystal structure suggested that the uracil-recognition site is a probable binding site for the quinolones.

Conclusions: N-methyl-2-alkenyl-4-quinolones are inhibitors of mycobacterial and staphylococcal growth, and show MurE ligase inhibition. Therefore, they are considered as a starting point for the development of increased affinity MurE activity disruptors.

Keywords: 4-quinolones, Mur ligase inhibitors, M. tuberculosis

Introduction

Tuberculosis (TB) is a contagious disease caused by infection with species belonging to the Mycobacterium tuberculosis complex.¹ This slow-growing acid-fast bacterium exerts a tremendous impact on current global health.² Staphylococcus aureus is also a major concern, as this pathogen is the most common cause of bacterial infection worldwide³ and methicillin-resistant S. aureus (MRSA) strains remain difficult to treat,⁴ despite the approval of agents such as linezolid, quinupristin/dalfopristin and daptomycin over the last decade. Infection with drug-resistant M. tuberculosis strains is extremely serious, prolonging...
treatment time, decreasing the probability of cure and increasing the cost of treatment. The current anti-TB chemotherapy must be administered for 6 months for drug-susceptible strains and for ≥2 years for multidrug-resistant (MDR) or extensively drug-resistant (XDR) infections. Outbreaks of drug-resistant pathogens are more and more frequent everywhere, and it would be catastrophic if these pathogens develop total drug resistance. Novel chemical entities are therefore required for treating drug-resistant strains. They must be patent enough to reduce the length of treatment and to prevent the emergence of resistance, but they must also be safer than second-line drugs and not interfere with antiretroviral therapy. Screening for novel mechanisms of action seems a reasonable strategy to develop inhibitors against MDR, XDR and totally drug-resistant strains of *M. tuberculosis*, as the resulting compounds may have a disrupting effect on pathways or enzymes that have never been targeted before.

Natural products are a primordial source of bioactive chemical scaffolds that have been therapeutically exploited for a large number of diseases. Plants and microorganisms have developed many successful secondary metabolites for protection against microbial infection. Antibiotics are, by definition, produced by microorganisms and are one of the most valuable antimicrobial classes, as they gained bacteria-killing competence by targeting essential biochemical pathways through centuries of microbial evolution. β-Lactams, glycopeptides, bacitracin, fosfomycin and cycloserine are all antibiotics targeting the peptidoglycan biosynthetic pathway. Peptidoglycan is an essential bacterial cell wall polymer that is responsible for cell shape and serves as containment for cytoplasmic pressure. Because it is a well-validated pathway, there is growing interest in developing small molecule inhibitors that target novel proteins of this biosynthetic route. Mycobacterial peptidoglycan is the sustaining mesh that supports the myco-arabino-galactan complex and is therefore considered an indispensable building block. Mur ligases are cytoplasmic enzymes that perform the biosynthesis of uridine-diphosphate-N-acetylmuramyl-Δ-Ala-Δ-Glu-m-DAP to form the peptidoglycan pentapeptide chain, and have been shown to act by a similar mechanism to folylpolyglutamate synthetase. The MurE substrate UDP-MurNAc-tripeptide is the product of MurE ligase and can be docked into the published MurE protein X-ray structure (PDB:2wtz) to propose a probable binding site. Docking results and the competition experiments of quinolone 2 with MurE ligands suggest that they bind to a specific hydrophobic pocket close to the uracil-binding site that could be exploited to generate a novel class of antimycobacterials.

**Materials and methods**

**Reagents**

Isoniazid, norfloxacin, kanamycin, resazurin, Tween 80, glycerol, m-DAP, ATP, bis-trispropane, magnesium chloride, Luria–Bertani broth, RPMI-1640, l-glutamine, heat-inactivated fetal calf serum, DMSO and ammonium formate were purchased from Sigma–Aldrich. Middlebrook 7H9, Middlebrook 7H10, and oleic acid, albumin, dextrose and catalase supplement (OADC) were obtained from BD Diagnostics. The MurE substrate UDP-MurNAc-Δ-Ala-Δ-Glu was purchased from the BaCWAN synthetic facility (University of Warwick, UK).

**Synthesis of N-methyl-2-alkenyl-4-quinolones 1–5**

The N-methyl-2-alkenyl-4-quinolones 1–5 were obtained using a synthetic route recently reported. The quinolone alkaloids with a cis-unsaturated aliphatic side chain (1 and 2) were prepared by the reaction of cis-unsaturated methyl ketones with N-methyl isatoic acid anhydride in the presence of lithium disopropylamide (LDA). The trans-α,β-unsaturated methyl ketones were used to prepare alkaloids 3–5 using the same type of condensation with N-methyl isatoic acid anhydride in the presence of LDA. The identity of the quinolone alkaloids and their corresponding intermediates was confirmed by analysis of 1D- and 2D-NMR spectroscopy data. Spectroscopic data of quinolones 1–5 are provided as Supplementary data at JAC Online.

**Bacterial strains and cells**

*M. tuberculosis* H37Rv (ATCC 27294), *M. bovis* BCG (ATCC 35734), *M. smegmatis* mc²155 (ATCC 700884), *M. fortuitum* (ATCC 6841), *M. phlei* (ATCC 11758), *Escherichia coli* JM109 (ATCC 53323), *Pseudomonas aeruginosa* (ATCC 25668) and murine RAW264.7 macrophages (ATCC TIB71) were used in this study. EMRSA-15 and -16 were gifts from Dr Paul Stapleton (School of Pharmacy, University of London, UK). Competent *E. coli* BL21(DE3)pLysS cells (New England Biolabs, UK) were used for overproducing MurE ligase of *M. tuberculosis* H37Rv.

**Drug susceptibility**

The spot culture susceptibility assay for *M. tuberculosis* H37Rv, *M. bovis* BCG and *M. smegmatis* mc²155 species was performed as described previously. Briefly, Middlebrook 7H9 mycobacterial cultures were serially diluted to 10⁻⁶ cfu/mL. A 5 μL aliquot of the diluted culture (≤500 viable cells) was spotted onto 5 mL of solidified Middlebrook 7H10 agar medium, supplemented with 10% (v/v) OADC in a six-well plate containing various concentrations of compounds 1–5. A negative control containing only DMSO was included in each plate. A six-well plate containing various concentrations of isoniazid was also used as a positive control. Following incubation at 37°C for 2 weeks for slow growers and 3 days for *M. tuberculosis*. 

Inhibitors of MurE ligase of *Mycobacterium tuberculosis* shown to be effective inhibitors of rapid-growing resistant (XDR) infections. Outbreaks of drug-resistant pathogens of *M. tuberculosis* have been computationally modelled and docked into the published *M. tuberculosis* MurE protein X-ray structure (PDB:2wtz) to propose a probable binding site. The docking results and the competition experiments of quinolone 2 with MurE ligands suggest that they bind to a specific hydrophobic pocket close to the uracil-binding site that could be exploited to generate a novel class of antimycobacterials.
smegmatis, the MIC was determined as the concentration at which there was no visible mycobacterial growth. Microdilution-based methods using Mueller-Hinton broth for S. aureus, and Luria-Bertani broth for E. coli and P. aeruginosa were employed for the MIC determination of the quinolones. Kanamycin and norfloxacin were used as positive controls. Susceptibilities of M. fortuitum and M. phlei were assessed as reported previously in a microdilution assay in cation-adjusted Mueller-Hinton broth using isoniazid as a positive control.

Cytotoxicity towards RAW264.7 macrophages

RAW264.7 macrophages (National Collection of Type Cultures) were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum, in a humidified incubator containing 5% CO₂, at 37 °C, and passed twice before the assay. The cell suspension was adjusted to 5 × 10⁵ cells/mL, and the assay was performed in 96-well cell culture flat-bottom plates (Costar 3596; VWR) in triplicate. Firstly, 2 μL of the 10 μg/mL stock solution of compounds 1–5 was added to 200 μL of RPMI-1640 medium in the first row and then 2-fold serially diluted. In each well, 100 μL of diluted macrophage cells was added. After 48 h of incubation, the monocytes were washed twice with PBS and fresh RPMI-1640 medium was added. The plates were then revealed with 30 μL of a freshly prepared and filter-sterilized aqueous 0.01% resazurin solution, and incubated overnight at 37 °C. The following day, fluorescence was measured at 590 nm with excitation at 560 nm using a Fluostar Optima microplate reader (BMG LABTECH).

Mureligase inhibition assay

The Mure protein of M. tuberculosis was overexpressed in E. coli BL21(DE3)pLysS and purified as previously reported. The phosphate colorimetric detection method was performed for the preliminary screen of the small molecules. Additionally, M. tuberculosis Mure inhibition was assayed using HPLC analysis. A solution containing 25 mM bis-trispropane buffer (pH 8.5), 5 mM MgCl₂, 100 mM UDP-MurNAc-di-peptide, 250 μM ATP and 1 mM m-DAP was prepared in water as the enzyme–substrate mixture. Quinolones 1–5 were dissolved in DMSO at a concentration of 25, 8.3, 2.5, 0.83 and 0.25 mM, and 2 μL was dispensed into 0.5 mL Eppendorfs. Mure enzyme was added to the enzyme–substrate mixture at a final concentration of ~40 mM, and 48 μL of the mixture was rapidly added to each Eppendorf and incubated at 37 °C for 30 min. The reaction was stopped by denaturing the protein at 100 °C using a block heater for 10 min. The content was centrifuged at 10,000 rpm for 30 s and then transferred to 200 μL glass inserts (Supelco) fitted to HPLC vials that were analysed by the phosphate detection colorimetric method (Figure 1a) were significantly active in susceptibility testing on rapid-growing mycobacteria. They also showed growth inhibition of slow-growing mycobacteria (M. tuberculosis H₃⁷Rv and M. bovis BCG), having an MIC value between 5 and 25 mg/L (Table 1). The quinolones were more active against rapid-growing species, showing an MIC value between 0.5 and 10 mg/L. The growth of the highly problematic EMRSA strains was also notably inhibited, revealing MIC values in the range of 0.5–4 mg/L. Moreover, the quinolones did not show any significant inhibition at 50 mg/L for E. coli, behaving similarly to the control, isoniazid. The MIC values of 1–5 for E. coli were >1000 mg/L and for P. aeruginosa they were >256 mg/L. These compounds were moderately cytotoxic towards macrophage RAW264.7 cells, having a 50% growth inhibition concentration (GIC₅₀) of between 24 and 112 mg/L (Table 1). The selectivity index (SI), defined as the GIC₅₀ value divided by the MIC, varied between 1.0 and 5.6 for M. tuberculosis, but was much higher for S. aureus (SI: 9.75–112).

M. tuberculosis MurE ligase inhibition

Mure ligase activity in the presence of compounds was assayed by HPLC quantification of the product (UDP-MurNAc–L-Ala–μ-DAP) formed after 30 min and by the phosphate colorimetric detection method. Clearly, all of quinolones 1–5 showed inhibition of MurE (Figures 1b and S1 (Figure S1 is available as Supplementary Online)), displaying an IC₅₀ value of 95–207 μM when determined by HPLC and of 36–72 μM when analysed by the phosphate detection colorimetric method (Table 1). Quinolone 2 was the most active MurE inhibitor, with an IC₅₀ value of 95 μM, as can be inferred from the chromatograms at different concentrations of the inhibitor (Figure 1b). The difference between the MurE inhibition values determined...
Docking of the quinolones in the MurE structure

The MurE substrate UDP-MurNAc-L-Ala-D-Glu used as a self-dock test was effectively docked in the same orientation as the published MurE-substrate crystal structure (PDB:2wtz), displaying a GlideScore of $-9.92 \text{kcal/mol}$. The root mean square distance of the heavy atoms of the UDP-MurNAc-L-Ala-D-Glu substrate in the docked and crystal structures was 3.09 Å, indicating a good preparation of the protein and adequate docking parameters. Our results suggested that all of the quinolones interacted in a similar fashion to a pocket located near the uracil recognition site (Figure 2a) of the Rossmann fold in domain 1 of MurE. According to the calculated hydrophilicity surface of the protein (Figure 2b), the quinolone was attracted to the lipophilic patches on the protein surface. The GlideScore for quinolones 1–5 was in the range of $-2.46$ to $-4.51 \text{kcal/mol}$, with quinolone 4 having the highest score in absolute value. This range indicated a rather weak binding, as can be observed in the orientations with a single hydrogen bond participating in the interaction (via the hydroxyl group of Thr-176 to the tertiary nitrogen atom of the quinolone in Figure 2c).

Kinetic competition between the quinolones and MurE substrates

The velocity of formation of the MurE product in the presence of quinolone 2 displayed a dependence on the concentration of the UDP-MurNAc-dipeptide substrate. At a high concentration of UDP-MurNAc-dipeptide (300 and 100 µM), the velocity decreased with an increase in the concentration of the quinolone (Figure 3a). For these two concentrations, the fitted lines converge to a point of intersection, therefore indicating competitive inhibition between the UDP-MurNAc-dipeptide and the quinolone. However, at a low concentration of UDP-MurNAc-dipeptide (30, 10 and 3 µM), the velocity of MurE product formation was independent of the concentration of the quinolone. For the two other substrates, namely ATP (Figure 3b) and m-DAP (Figure 3c), the change in the velocity was less drastic when varying the concentration of the quinolone, and the curves had the same tendency without converging to a point of intersection, indicating uncompetitive inhibition.

Discussion

Initially isolated as the active antimycobacterial entities from the fruits of the traditional Chinese medicinal tree Evodia rutaecarpa (Rutaceae), the N-methyl-2-alkenyl-4-quinolone chemotype was further exploited by synthetic methods in order to explore chemical variation and improve activity. A group of these chemical entities, which showed the highest activity on rapidly growing mycobacteria, were selected for a comprehensive biological evaluation. We found that these compounds were growth inhibitors of mycobacterial species and the highly problematic EMRSA strains, being also inhibitors of the MurE ligase of M. tuberculosis. The MIC indicated that these compounds are notable antibacterials, particularly against EMRSA-15 and -16, which are regularly encountered in UK hospitals. The macrophage SI in relation to the H37Rv strain for the quinolones was considerably low (SI<10); however, the SI in relation to EMRSA-15 and -16 was much higher (SI range 9.75–112), indicating a promising selectivity. Moreover, the compounds did not show inhibition of E. coli growth up to a high dose and they are probably innocuous to bacterial gut flora. It was also noted that the cytotoxicity was reduced when the linear alkenyl chain was extended, suggesting an interesting relation for differential selectivity.

The 4-quinolone nucleus is a specific class of compound that has attracted and continues to attract significant interest from the pharmaceutical industry, principally because of the impact of the fluoroquinolones, which inhibit both DNA gyrase and topoisomerase IV, and the anticancer 2-phenyl-4-quinolones targeting tubulin. In comparison to the fluoroquinolones, the compounds of the present study lack a carboxyl group at position C-3, which is considered to be essential for DNA gyrase inhibition, and therefore a further mechanism of action must be assumed. Moreover, this class of chemicals may also be
exploited for the discovery and development of bacterial Mur ligase inhibitors. Using both colorimetric and HPLC methods, it was found that the compounds reproducibly inhibited in vitro the ligase activity of MurE from M. tuberculosis. Comparing 1 and 2, a slight influence on MurE inhibition was observed for the position of the double bond in the alkenyl chain. Furthermore, the percentage of inhibition when determined using the phosphate colorimetric method was slightly higher than when compared with the HPLC method, probably because of a small decoupling between phosphatase and ligase activities.

For the computational prediction of the binding of potential ligands to a protein, it is advisable to use a 3D structural model of atomic resolution, typically below 2 Å. However, in the absence of structures of MurE from M. tuberculosis below 2 Å resolution, we used the available published model at 3 Å as a preliminary basis for assessing probable binding sites. Interestingly, quinolones 1–5 were predicted to bind to a hydrophobic pocket located near the uridine recognition site in domain 1 of the enzyme. The GlideScore was low (~4 kcal/mol) in comparison with general reported values for inhibitors (~10 kcal/mol), however, considering the weak interactions between the quinolones and the protein, it is not surprising to observe low GlideScore values. This also indicates that there is space for improvement in the search for chemical and steric complementarities, and the possibility of using this lipophilic cavity for rational drug design. We hypothesize that the aliphatic lipophilic chain of 1–5 interacts with the buried hydrophobic residues of MurE ligase, probably inducing a change in the conformation that prevents binding to UDP-MurNAc-dipeptide. In order to gain further evidence of this possibility, a kinetic competition experiment showed that at a low concentration of the dipeptide, the velocity of the reaction was unaltered by the concentration of the inhibitor. At low concentration of the dipeptide substrate, the majority of the enzyme was free and, 

### Table 1. MICs for different species of bacteria, GIC50 and SI for macrophage cells and MurE IC50 of the synthetic quinolones 1–5

| Compound | MIC in mg/L (µM) | Murine macrophages | M. tuberculosis | M. smegmatis | S. aureus | GIC50 (mg/L) | SI | IC50 (µM) |
|----------|------------------|---------------------|----------------|--------------|-----------|--------------|----|-----------|
|          | M. tuberculosis | H37Rv | M. bovis BCG | mc²155 | M. fortuitum | M. phlei | EMRSA-15 | EMRSA-16 |
| 1        | 25 (70.7)        | 10 (28.3) | 10 (28.3) | 0.5 (1.41) | 0.5 (1.4) | 1 (2.8) | 0.5 (1.4) | 24 ± 9 |
| 2        | 10 (28.3)        | 10 (28.3) | 10 (28.3) | 1 (2.83) | 1 (2.8) | 1 (2.8) | 0.5 (1.4) | 39 ± 12 |
| 3        | 25 (84.0)        | 25 (84.0) | 5 (16.8) | 2 (6.72) | 1 (3.4) | 4 (13.4) | 2 (6.72) | 39 ± 11 |
| 4        | 10 (30.7)        | 5 (15.3) | 5 (15.3) | 1 (3.06) | 0.5 (1.5) | 2 (6.12) | 2 (6.12) | 40 ± 7 |
| 5        | 20 (58.9)        | 10 (29.4) | 10 (29.4) | 1 (2.94) | 1 (2.9) | 2 (5.8) | 1 (2.9) | 112 ± 10 |
| Isoniazid | 0.1 (0.73) | 0.1 (0.73) | 5 (18.5) | 1 (3.65) | 0 (0) | ND | 0 (0) | >500 |
|          | S. aureus        | phosphate-based method | HPLC method |
|          |          |          |          |          |          |          |          |          |
|          |          |          |          |          |          |          |          |          |

ND, not determined.
The MIC of norfloxacin was 0.5 and 256 mg/L for EMRSA-15 and -16, respectively. The SI was calculated by dividing the GIC50 by the MIC for M. tuberculosis H37Rv. IC50 and GIC50 values are shown ±SD.

Figure 2. Lowest GlideScore docking pose of quinolone 4 interacting with MurE (PDB:2wt2) of M. tuberculosis. 17 (a) Presumed binding pocket of the quinolones (in yellow) near the uracil recognition site of UDP-MurNAc-dipeptide (in magenta). (b) Protein surface showing the high hydrophobicity (in red) of the quinolone binding pocket calculated using UCSF Chimera software. 34 (c) MurE residues that may interact with the quinolones: Thr-176, Ala-168, Gln-45, His-66 and Thr-61. This figure appears in colour in the online version of JAC, and in black and white in the print version of JAC.
therefore, according to our results, the conformation of the free MurE enzyme has low interaction with the quinolones. However, when the concentration of the dipeptide substrate was high, most of the enzyme formed the enzyme–substrate complex, which, in agreement with the induced-fit theory, has a different protein conformation. Our results suggested that this induced-fit enzyme–substrate conformation of MurE interacted with the quinolones and when this conformation occurred, inhibition of ligase activity took place. This novel type of MurE inhibitor can be included as a starting point in virtual and fragment screening MurE projects that may help in the detection of structure–activity relationships. Structural modifications of a particularly interesting MurE inhibitor pharmacophore can potentially lead to potent and selective antibacterials in the near future.

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Transparency declarations

None to declare.

Supplementary data

The spectroscopic data of the quinolones 1–5 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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