Lipiarmycin targets RNA polymerase and has good activity against multidrug-resistant strains of *Mycobacterium tuberculosis*

Mekonnen Kurabachew¹, Stephen H. J. Lu¹, Philipp Krastel², Esther K. Schmitt², Bangalore L. Suresh¹, Anne Goh¹, John E. Knox¹, Ngai Ling Ma¹, Jan Jiricek¹, David Beer¹, Michael Cynamon³, Frank Petersen², Veronique Dartois¹, Thomas Keller¹, Thomas Dick¹ and Vasan K. Sambandamurthy¹*¹

¹Novartis Institute for Tropical Diseases, 10 Biopolis Road, #05-01 Chromos, Singapore 138670; ²Natural Products Unit, Novartis Institute for Biomedical Research, CH-4002 Basel, Switzerland; ³Veterans Affairs Medical Center, 800 Irving Avenue, Syracuse, NY, USA

Received 22 January 2008; returned 30 April 2008; revised 22 May 2008; accepted 2 June 2008

**Objectives**: The aim of this study was to determine the *in vitro* activity of lipiarmycin against drug-resistant strains of *Mycobacterium tuberculosis* (MTB) and to establish the resistance mechanism of MTB against lipiarmycin using genetic approaches.

**Methods**: MIC values were measured against a panel of drug-resistant strains of MTB using the broth microdilution method. Spontaneous lipiarmycin-resistant mutants of MTB were tested for cross-resistance to standard anti-TB drugs, and their *rpoB* and *rpoC* genes were sequenced to identify mutations.

**Results**: Lipiarmycin exhibited excellent inhibitory activity against multidrug-resistant strains of MTB with MIC values of <0.1 mg/L. Sequence analysis of the *rpoB* and *rpoC* genes from spontaneous lipiarmycin-resistant mutants of MTB revealed that missense mutations in these genes caused resistance to lipiarmycin. Although both lipiarmycin and rifampicin are known to inhibit the bacterial RNA polymerase, the sites of mutation in the *rpoB* gene were found to be different in MTB strains resistant to these inhibitors. Whereas all six rifampicin-resistant MTB strains tested had mutation in the 81 bp hotspot region of the *rpoB* gene spanning codons 507–533, 16 of 18 lipiarmycin-resistant strains exhibited mutation between codons 977 and 1150. The remaining two lipiarmycin-resistant strains had mutation in the *rpoC* gene.

**Conclusions**: Lipiarmycin has excellent bactericidal activity against MTB and lacks cross-resistance to standard anti-TB drugs. Furthermore, rifampicin-resistant strains remained fully susceptible to lipiarmycin, and none of the lipiarmycin-resistant MTB strains became resistant to rifampicin, highlighting the lack of cross-resistance.

**Keywords**: antimycobacterial activity, resistance mechanism, transcription

**Introduction**

Tuberculosis (TB) remains a disease of major public health concern globally, and it is estimated that about a third of the world’s population is infected with *Mycobacterium tuberculosis* (MTB). Approximately 9 million new cases of TB and an estimated 2 million deaths occur each year, with sub-Saharan Africa and Southeast Asia having the highest burden of disease.¹ The high rate of drug-resistant MTB strains in some countries, especially in the former Soviet Union, is also a matter of great concern.² The increasing prevalence of MTB strains resistant to the frontline drugs, rifampicin and isoniazid, considerably limits therapeutic options and highlights the urgent need to develop new molecules with activity against these drug-resistant strains.³

RNA polymerase (RNAP), a target of several natural antibiotics, is a vital enzyme in bacterial cells and plays a key role in transcription.⁴,⁵ Rifampicin, one of the most potent RNAP inhibitors, is an effective anti-TB agent. The ability of
rifampicin to easily diffuse into tissues, living cells and bacteria has made it a highly effective drug against intracellular pathogens such as MTB.9 The use of rifampicin has considerably shortened the duration of chemotherapy required for the successful treatment of drug-susceptible TB in humans.7 However, due to the emergence of MTB strains resistant to rifampicin, there is an urgent need to identify transcription inhibitors that lack cross-resistance to rifampicin.

Several RNAP inhibitors have been isolated from natural sources.8,9 Natural products are widely recognized in the pharmaceutical industry for their extensive structural diversity as well as for their wide range of pharmacological activities and have served as a major source of drugs for several decades.10,11

In the course of a natural product-screening programme for new anti-TB agents, we have identified a known natural product, lipaemyacin A3 (LIP), also known as tiacumin B or OPT-80, as a hit.12,13 Lipaemyacin is a macro-cyclic antibiotic displaying moderate activity against Gram-positive bacteria, such as staphylococci and enterococci, but excellent activity against clostridia.13 OPT-80 is currently undergoing Phase III clinical studies for use in Clostridium difficile-associated diarrhoea, a serious health problem in patients on broad-spectrum antibiotic treatment.14

Lipaemyacin is a transcription inhibitor and is shown to inhibit the holoenzyme better than the core enzyme.15 Moreover, lipaemyacin inhibits early transcription, but has no effect on middle or late transcription.16 In the present study, we show that lipaemyacin has excellent activity against multidrug-resistant strains of MTB and exhibits no cross-resistance to rifampicin. In addition, we provide data for the genetic basis of resistance of MTB to lipaemyacin.

Materials and methods

Bacterial strains, growth condition and plasmids

M. tuberculosis H37Rv (ATCC 27294), Mycobacterium bovis BCG (ATCC 35734) and Mycobacterium smegmatis (mc²155) were cultured in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% (v/v) ADS or grown on Middlebrook 7H10 (Difco) agar plates supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% (v/v) OADC enrichment (BBL). Escherichia coli strains were cultured in Luria–Bertani (LB) broth (Becton–Dickinson) and on LB agar plates. When required, plates were supplemented with kanamycin, 50 mg/L for E. coli and 25 mg/L for mycobacteria. pCR®-XL-TOPO (Invitrogen) and the mycobacterial-E. coli shuttle plasmid pMV261-kan17 were used for cloning purposes.

Isolation of genomic DNA, PCR amplification and sequencing

Genomic DNA from mycobacterial isolates was extracted for molecular analysis, as described previously.18 The amplification reaction mixture (25 μL) consisted of ~100 ng of purified genomic DNA, 0.5 μM each of upstream and downstream rpoB or rpoC gene primer set and 12.5 μL of Py Turbo Hotstart DNA polymerase (Stratagene). The samples were amplified by PCR (Biometra, T3000 Thermocycler, Germany) with 2 min of enzyme activation at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 2 min, and a final extension of 72 °C for 10 min. The PCR products were gel-purified (Qiagen) and sequenced using the ABI PRISM 3100 sequencer.

Primers

All rpoB and rpoC primers (Table 1) were designed based on the rpoB and rpoC gene sequences from MTB H37Rv. To amplify the full-length rpoB and rpoC genes, primer sets rpoB.PPF1 and rpoB.PPR1, and rpoC.PPF1 and rpoC.PPR1 were used, respectively. The primer set rpoB.PPF2 and rpoB.PPR2 was used for the amplification of a selected 520 bp region of the rpoB gene, spanning codons 977–1150. This includes codons 1048 and 1117, the most prevalent mutation sites associated with lipaemyacin resistance in this study. The primer set RRDR.F1 and RRDR.R1 was used to amplify a 350 bp fragment that includes codons 507–533, a hotspot region known to mediate resistance to rifampicin.19

Cloning of rpoB gene and complementation studies

The rpoB gene was PCR-amplified using primers rpoB.PPF1 and rpoB.PPR1 (Table 1). PCR products were ligated to the TOPO®-XL vector (Invitrogen) and sequenced. The TOPO-XL-rpoB plasmid was digested with EcoRI and Ncol, and the resulting rpoB DNA fragment was ligated with EcoRI-digested and shrimp alkaline phosphatase-treated pMV261-kan. The pMV261-rpoB plasmid was used to transform E. coli, which were grown overnight at 37 °C on LB agar supplemented with kanamycin. Twelve colonies were randomly selected and grown overnight at 37 °C in 5 mL of LB broth supplemented with kanamycin prior to plasmid purification (Qiagen). The purified plasmid was digested with BgIII to check for the orientation of the gene with respect to the hsp65 promoter. Plasmids with correct orientation were electroporated onto BCG, MTB or M. smegmatis, and transformants were selected on 7H10 agar plates supplemented with kanamycin (25 mg/L) and lipaemyacin (1 or 5 mg/L). To demonstrate that the resistance to lipaemyacin can be transferred, the mutant pMV261-rpoB plasmid was electroporated into wild-type BCG, MTB or M. smegmatis.

Isolation of lipaemyacin A3

Lipaemyacin A3 (Figure 1) was isolated from an actinomycetes strain, Catellatospora sp. Bp3323-81, taken from the Novartis strain collection. A 90 L scale stirred tank fermentor containing 50 L of production medium (20 g/L glycerol, 25 g/L potato starch, 5 g/L pharma media, 15 g/L corn steep powder, 5 g CaCO₃ and 3 g/L yeast extract) was inoculated with 5% of a seed culture and stirred for 6 days at 28 °C. After harvesting, the culture broth was extracted overnight with 100 L of ethyl acetate followed by phase separation and evaporation of the organic layer. The resulting extract (54.7 g) was dissolved in methanol/H₂O (9:1) and extracted three times with an equal volume of cyclohexane to eliminate the fatty acids from the extract. The remaining methanol/H₂O phase was partly evaporated and extracted with ethyl acetate. The ethyl acetate phase was evaporated, yielding ~1.6 g of defatted extract. For purification of lipaemyacin A3, the extract was applied to a normal-phase column containing silica gel K60, and the compounds were eluted with a ternary gradient using cyclohexane, ethyl acetate and methanol as solvents. Fractions containing lipaemyacin were further purified using reversed-phase chromatography (Sunfire RP18 column) with a gradient of 10% acetonitrile up to 60% acetonitrile (solvents containing 0.1% formic acid). Fractions containing lipaemyacin A3 were pooled and evaporated to dryness, yielding ~1.8 g of pure lipaemyacin A3. The structure was assigned through comparison of the analytical data (IR, NMR and MS) with the data published in the literature.20

Kurabachew et al.
Antimycobacterial activity of lipiarmycin A3

The MIC<sub>50</sub> in this study is defined as the lowest concentration of compound that inhibited 50% growth of the wild-type strain. The MIC<sub>50</sub> values of lipiarmycin and four TB drugs, namely rifampicin, isoniazid, streptomycin and moxifloxacin, were determined by the broth microdilution method using a 96-well plate as recommended by the CLSI (formerly the NCCLS). The test inoculum was prepared by adjusting the turbidity of a logarithmic phase BCG or MTB broth culture in 7H9 medium to an optical density at 600 nm (OD<sub>600</sub>) of 0.04. Subsequently, 0.1 mL of cell suspension was added into each well of a 96-well plate containing an equal volume of drug-containing 7H9 broth, thus bringing the final inoculum density to ~1 × 10<sup>5</sup> cfu/mL. The MIC was read after 5 days of incubation at 37°C. After determining the MIC<sub>50</sub> value, 50 μL of culture from the wells corresponding to MIC, 2<sup>1/2</sup> MIC and 4<sup>1/2</sup> MIC values was plated from the assay plate at different dilutions onto 7H10 agar plates to determine the minimum bactericidal concentration (MBC<sub>90</sub>) values. Plates were incubated at 37°C for 3–4 weeks before determining the cfu. The MBC<sub>90</sub> is defined as the minimum concentration of compound that kills 90% of the wild-type strain.

Frequency of spontaneous lipiarmycin-resistant mutants in mycobacteria

Briefly, wild-type MTB, BCG or <i>M. smegmatis</i> cultures were grown in 7H9 broth to an OD<sub>600</sub> of 0.4–0.5, and 10<sup>10</sup> cfu/mL was evenly spread onto drug-free 7H10 agar plates or onto 7H10 plates containing different concentrations of lipiarmycin (5 × and 10 × MIC). Plates were incubated at 37°C for 4–6 weeks, and the colonies were counted. The frequency at which spontaneous drug-resistant mutants arose in a given culture was calculated as the ratio of the average number of colonies that grew on drug-containing plates to those growing on drug-free plates. In a similar manner, spontaneous rifampicin-resistant mutants of MTB and BCG were isolated at 10 × MIC (0.05 mg/L).

Structural modelling of RpoB protein

A homology model of the MTB RpoB protein was constructed using the Schrödinger software (Prime, version 1.2, Schrödinger, LLC, New York, NY, 2005). A BLAST search was performed to find the homologue using the P0A680 MTB sequence. Structural discontinuities in 1IW7 (insertions of 13 residues and deletions) were omitted, as they were not in regions of interest. In addition, the model was only built up to the outermost aligned residue. Side chains and the loop were optimized using the default settings. The Q1048H mutant homology model was constructed in the manner discussed above.
Results

Antimycobacterial activity of lipiarmycin

Lipiarmycin was evaluated for its inhibitory and bactericidal activity against MTB (H37Rv), BCG and M. smegmatis. Comparison of the MIC₅₀ and MBC₉₀ of lipiarmycin with standard frontline TB drugs (Table 2) clearly showed that the inhibitory and bactericidal activity of lipiarmycin against MTB is better than streptomycin and comparable to moxifloxacin. Similar results were obtained for BCG and M. smegmatis (data not shown). However, lipiarmycin displayed a higher MIC and MBC than isoniazid and rifampicin. Interestingly, lipiarmycin also showed excellent activity against several single-drug- and multidrug-resistant clinical isolates of MTB (Table 3).

Isolation of lipiarmycin-resistant mutants and determination of cross-resistance

Lipiarmycin-resistant mutants were isolated as spontaneous variants of MTB that were able to grow on 7H10 plates containing 10 × MIC (0.8 mg/L) of lipiarmycin. At this concentration, the frequency of spontaneous resistant colonies was <10⁻⁸. The mutation frequency observed here is equivalent to the frequency reported for rifampicin. Repeated MIC determination using the microplate dilution method showed that most, but not all, of the colonies that appeared were stably resistant to lipiarmycin. The acquisition of lipiarmycin resistance did not alter the susceptibility of these mutant strains to rifampicin or to other standard TB drugs used in this study (Table 4). In addition, a number of spontaneous rifampicin-resistant mutants of MTB were also picked at random, and their MICs of rifampicin were determined. This resulted in the identification of mutants for which the MICs of lipiarmycin were in the range of 0.13–0.85 mg/L, an approximate 20- to 140-fold increase in MIC compared with the MIC for the wild-type H37Rv strain (data not shown).

Sequence analyses of lipiarmycin-resistant strains

Sequence analyses of the rpoB and rpoC genes from 18 different lipiarmycin-resistant MTB strains revealed a single point mutation in the rpoB gene for 16 strains and a point mutation in the rpoC gene for 2 strains (Table 4). In all lipiarmycin-resistant MTB mutants, a single nucleotide substitution resulted in a missense mutation. In M. bovis BCG, all the six lipiarmycin-resistant mutants displayed mutation in the rpoB gene. A guanine (G) to cytosine (C) change at nucleotide position 3350 resulting in a valine to leucine amino acid change was the most dominant mutation, as four of six mutants showed a change at this position. The remaining two isolates also had mutations in the rpoB gene, one at nucleotide position 3142 (C to A change) and the other at nucleotide position 3239 (C to A change). In both cases, the change resulted in a missense mutation, glutamine to lysine and glutamic acid to lysine, respectively (data not shown). Interestingly, the mutation that occurred at nucleotide position 3239 was unique to BCG strains and was not seen in MTB mutants resistant to lipiarmycin. Sequencing of an 81 bp region of the rpoB gene associated with rifampicin resistance for six randomly chosen rifampicin-resistant MTB strains revealed a point mutation in the hotspot region in all of the six strains (data not shown).

To confirm the association between resistance to lipiarmycin and mutation in the rpoB gene, complementation studies were performed. A PCR-amplified rpoB gene from a lipiarmycin-resistant mutant was cloned into pMV261-kan and then transformed into wild-type MTB. The MIC results showed that the wild-type MTB strain complemented with the mutant lipiarmycin-resistant rpoB gene displayed an increased level of resistance only to lipiarmycin (a 15-fold increase in MIC₅₀ value), but remained susceptible to rifampicin and other standard TB drugs. Similarly, wild-type MTB strains complemented with the mutant rifampicin-resistant rpoB gene remained susceptible to lipiarmycin while acquiring resistance to rifampicin (data not shown). To demonstrate further this genetic association in fast-growing mycobacteria, wild-type M. smegmatis (mc²155) strain was transformed with pMV261-kan containing rpoB gene from several lipiarmycin-resistant MTB mutants. All the transformants displayed MIC₅₀ values of >20 mg/L, which is approximately a 25-fold increase in the MIC₅₀ value for the wild-type M. smegmatis mc²155 strain (0.78 mg/L) (data not shown).

MIC results for the various lipiarmycin-resistant strains analysed in this study showed that, with a few exceptions, specific

### Table 2. Inhibitory and bactericidal activity of lipiarmycin and other standard anti-TB drugs against M. tuberculosis

<table>
<thead>
<tr>
<th>Compound/drug</th>
<th>MIC₅₀ (mg/L)</th>
<th>MBC₉₀ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipiarmycin</td>
<td>0.085</td>
<td>0.328</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.005</td>
<td>0.016</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.034</td>
<td>0.054</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.335</td>
<td>2.273</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.044</td>
<td>0.313</td>
</tr>
</tbody>
</table>

### Table 3. MIC values of lipiarmycin against various drug-resistant strains of M. tuberculosis

<table>
<thead>
<tr>
<th>Strain</th>
<th>LIP (mg/L)</th>
<th>INH (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv (ATCC 27294)</td>
<td>0.015</td>
<td>0.045</td>
</tr>
<tr>
<td>Beijing MDR</td>
<td>0.004</td>
<td>2.0</td>
</tr>
<tr>
<td>Beijing W MDR</td>
<td>0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>Beijing F29 MDR 1</td>
<td>0.045</td>
<td>0.25</td>
</tr>
<tr>
<td>Beijing F29 MDR 2</td>
<td>0.06</td>
<td>0.37</td>
</tr>
<tr>
<td>INH r SDR</td>
<td>0.011</td>
<td>4.0</td>
</tr>
<tr>
<td>STR r SDR</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>Rif r SDR</td>
<td>0.022</td>
<td>0.03</td>
</tr>
<tr>
<td>Clinical MDR 1</td>
<td>0.015</td>
<td>2.0</td>
</tr>
<tr>
<td>Clinical MDR 2</td>
<td>0.06</td>
<td>4.0</td>
</tr>
<tr>
<td>Clinical MDR 3</td>
<td>&lt;0.008</td>
<td>2.0</td>
</tr>
<tr>
<td>Clinical MDR 4</td>
<td>0.011</td>
<td>2.0</td>
</tr>
<tr>
<td>Clinical MDR 5</td>
<td>0.008</td>
<td>1.25</td>
</tr>
<tr>
<td>Clinical MDR 6</td>
<td>0.09</td>
<td>1.5</td>
</tr>
<tr>
<td>Clinical MDR 7</td>
<td>0.011</td>
<td>2.0</td>
</tr>
<tr>
<td>Clinical MDR 8</td>
<td>0.015</td>
<td>3.0</td>
</tr>
</tbody>
</table>

MDR, multidrug resistance; SDR, single-drug resistance; INH, isoniazid; STR, streptomycin; Rif, rifampicin; LIP, lipiarmycin.
All MIC determinations were done twice and the average values are presented.
mutations within the \textit{rpoB} gene were associated with different levels of resistance to lipiarmycin. All of the five Q1048H mutants displayed high-level resistance (10.7–18.9 mg/L) to lipiarmycin compared with all six Q1048K/E mutants that showed only intermediate to low-level resistance (4.0–8.9 mg/L). Similarly, the V1117G mutants displayed high-level resistance compared with the V1117L mutants (Table 4).

Since the crystal structure of the MTB RNAP has not been determined, a threedimensional model for RpoB protein of MTB was constructed based on the known crystal structure of \textit{T. thermophilus} RpoB protein. The RNAP of \textit{T. thermophilus} [PDB code: 1IW7 (chain C/M)] was found to have high homology (49\%) at the amino acid level. Interestingly, there was a high sequence homology between them in the regions where mutations in the \textit{rpoB} gene occurred in lipiarmycin-resistant strains. In particular, regions around the Q1048 mutation (Figure 2a) and the V1117 mutation (Figure 2b) were highly conserved.

In the homology model, both the backbone and side chain of Q1048 (in a loop) are hydrogen bonded to the side chain of R1093 in an adjacent helix. In the Q1048K/E mutants, the neutral wild-type amino acid (Q) is changed to a charged amino acid (H, K and E). The change in the character of the side chain from neutral to charged due to a mutation perturbs the interaction between residues 1048 and 1093, leading to the movement of

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{LIP\textsuperscript{R} mutant no.} & \textbf{SNPs} & \textbf{AA} & \textbf{SNPs} & \textbf{AA} & \textbf{LIP} & \textbf{RIF} & \textbf{INH} & \textbf{STP} & \textbf{MOX} \\
\hline
M1 & G3144T & Q1048H & — & — & 10.73 & 0.016 & 0.021 & 0.25 & 0.09 \\
M2 & — & — & C977G & P326R & 20.33 & 0.008 & 0.014 & 0.12 & 0.07 \\
M3 & T3350G & V1117G & — & — & 12.8 & 0.008 & 0.021 & 0.16 & 0.08 \\
M4 & — & — & G1235A & R412Q & 7.49 & 0.025 & 0.014 & 0.23 & 0.08 \\
M5 & G3144T & Q1048H & — & — & 14.63 & 0.025 & 0.021 & 0.22 & 0.08 \\
M6 & T3350G & V1117G & — & — & 18.65 & 0.016 & 0.014 & 0.23 & 0.08 \\
M7 & T3350G & V1117G & — & — & 17.21 & 0.016 & 0.016 & 0.28 & 0.11 \\
M8 & C3142A & Q1048K & — & — & 4.04 & 0.016 & 0.031 & 0.20 & 0.08 \\
M9 & G3144T & Q1048H & — & — & 18.91 & 0.025 & 0.039 & 0.19 & 0.09 \\
M10 & C3142A & Q1048K & — & — & 5.67 & 0.008 & 0.025 & 0.16 & 0.08 \\
M11 & G3144T & Q1048H & — & — & 11.8 & 0.008 & 0.018 & 0.16 & 0.08 \\
M12 & C3142G & Q1048E & — & — & 6.61 & 0.016 & 0.039 & 0.16 & 0.05 \\
M13 & C3142G & Q1048E & — & — & 8.18 & 0.008 & 0.039 & 0.16 & 0.06 \\
M14 & G3349C & V1117L & — & — & 1.72 & 0.074 & 0.023 & 0.19 & 0.06 \\
M15 & G3349C & V1117L & — & — & 2.32 & 0.008 & 0.034 & 0.22 & 0.07 \\
M16 & C3142A & Q1048K & — & — & 4.02 & 0.008 & 0.021 & 0.07 & 0.06 \\
M17 & G3144T & Q1048H & — & — & 13.86 & 0.025 & 0.032 & 0.17 & 0.08 \\
M18 & C3142A & Q1048K & — & — & 8.93 & 0.016 & 0.026 & 0.19 & 0.07 \\
H37Rv & — & — & — & — & 0.08 & 0.008 & 0.026 & 0.20 & 0.07 \\
\hline
\end{tabular}
\caption{Mutations identified in \textit{rpoB} and \textit{rpoC} genes of lipiarmycin-resistant mutants of \textit{M. tuberculosis} H37Rv and their MICs of standard TB drugs.}
\end{table}

\textbf{Homology model of the MTB RpoB protein}

Since the crystal structure of the MTB RNAP has not been determined, a three-dimensional model for RpoB protein of MTB was constructed based on the known crystal structure of \textit{T. thermophilus} RpoB protein. The RNAP of \textit{T. thermophilus} [PDB code: 1IW7 (chain C/M)] was found to have high homology (49\%) at the amino acid level. Interestingly, there was a high sequence homology between them in the regions where mutations in the \textit{rpoB} gene occurred in lipiarmycin-resistant strains. In particular, regions around the Q1048 mutation (Figure 2a) and the V1117 mutation (Figure 2b) were highly conserved.

In the homology model, both the backbone and side chain of Q1048 (in a loop) are hydrogen bonded to the side chain of R1093 in an adjacent helix. In the Q1048K/E mutants, the neutral wild-type amino acid (Q) is changed to a charged amino acid (H, K and E). The change in the character of the side chain from neutral to charged due to a mutation perturbs the interaction between residues 1048 and 1093, leading to the movement of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Sequence alignment of the P0A680 RpoB protein and chain M of 1IW7 showing the high sequence homology around the Q1048 mutation (a) and the V1117 mutation (b) with arrows pointing to the mutated residues. P0A680 represents the MTB RpoB sequence, 1IW7_M_ssa is the secondary structure of the template protein (blue arrows represent \(\beta\) sheets, red rods represent helices and a single line represents no secondary structure) and 1IW7_M is the sequence from \textit{T. thermophilus} RpoB. A colour version of this figure is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).}
\end{figure}
the loop (Figure 3). This conformational change may affect the lipiarmycin binding site and result in the disruption of the molecular recognition, thereby resulting in resistance. However, as lipiarmycin is a large molecule with many potential hydrogen donating/accepting groups, it is difficult to speculate how the molecule would bind to the MTB RpoB. In contrast, the V1117G/L mutations are located on the outside of a helix not interacting with RpoB itself and thus cannot be explained by the homology model. It is possible though that this terminal helix interacts with one of the other RNAP subunits giving rise to the observed resistance to lipiarmycin.

**Discussion**

RNAP is an attractive drug target because RNA chain elongation is vital for bacterial growth. Rifampicin, which inhibits bacterial RNAP, has been used clinically to treat TB patients for decades. However, owing to the emergence of MTB strains that are resistant to this important drug, there is a need for new and improved drugs against MTB. Lipiarmycin, which works on the same target as rifampicin but without showing any cross-resistance to rifampicin and other frontline TB drugs, could serve as a good starting point in the identification of novel transcription inhibitors.

This study demonstrates that lipiarmycin has potent antimycobacterial activity and its inhibitory and bactericidal activity is better than streptomycin and comparable to moxifloxacin (Table 2). The antimycobacterial spectrum of lipiarmycin is further evidenced by its excellent activity against a panel of clinical multidrug-resistant strains of MTB, including some Beijing strains (Table 3). Interestingly, spontaneous mutants that are resistant to a high concentration of lipiarmycin arise at a low frequency (~1 in $10^8$ cells/mL).

Previous studies have shown that rifampicin-resistant strains had mutation in the 81 bp hotspot region of rpoB, stretching from codons 507 to 533. In the present study, mutations linked to lipiarmycin resistance were found near the C-terminal region of the RpoB protein spanning codons 977–1150. Interestingly, rifampicin-resistant strains remained susceptible to lipiarmycin, and none of the 18 lipiarmycin-resistant MTB strains showed an increase in the MIC of rifampicin or the other three TB drugs, namely isoniazid, streptomycin and moxifloxacin (Table 4). This clearly indicates that there is no cross-resistance between lipiarmycin and rifampicin.

In summary, lipiarmycin has excellent activity against multidrug-resistant strains of MTB, has a low mutation frequency and shows no cross-resistance to rifampicin. However, understanding of the potential clinical role of lipiarmycin in the treatment of TB infection requires further studies, which include pharmacokinetic–pharmacodynamic and experimental animal studies.

**Acknowledgements**

We thank Bill Jacobs for providing pMV261-kan, which was used for the complementation studies.

**Funding**

This work was supported by Novartis Institute for Tropical Diseases, Singapore.

**Transparency declarations**


**Supplementary data**

Colour versions of Figures 2 and 3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**

Antimycobacterial activity of lipiarmycin