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Identification of novel inhibitors of phospho-MurNAc-pentapeptide translocase MraY from library screening: Isoquinoline alkaloid michellamine B and xanthene dye phloxine B

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ABSTRACT

The National Cancer Institute (NCI) Diversity Set was screened for potential inhibitors of phospho-MurNAc-pentapeptide translocase MraY from *Escherichia coli* using a primary fluorescence enhancement assay, followed by a secondary radiochemical assay. One new MraY inhibitor was identified from this screen, a naphthylisoquinoline alkaloid michellamine B, which inhibited *E. coli* MraY (IC_{50} 456 μM) and *Bacillus subtilis* MraY (IC_{50} 386 μM), and which showed antimicrobial activity against *B. subtilis* (MIC 16 μg/mL). Following an earlier report of halogenated fluoresceins identified from a combined MraY/MurG screen, three halogenated fluoresceins were tested as inhibitors of *E. coli* MraY and *E. coli* MurG, and phloxine B was identified as an inhibitor of *E. coli* MraY (IC_{50} 32 μM). Molecular docking of inhibitor structures against the structure of *Aquifex aeolicus* MraY indicates that phloxine B appears to bind to the Mg^{2+} cofactor in the enzyme active site, while michellamine B binds to a hydrophobic groove formed between transmembrane helices 5 and 9.

1. Introduction

The biosynthesis of the peptidoglycan layer of bacterial cell walls is a well established target for antibacterial action, with the beta-lactam and glycopeptide antibiotics targeting the penicillin-binding proteins which catalyse the final steps of the biosynthetic pathway, and fosfomycin and D-cycloserine inhibiting enolpyruvyl transferase MurA and D-alanine/D-alanine ligase at the start of the pathway.[1] In principle all the enzymes of the peptidoglycan pathway are good antibacterial targets, and the enzymes MurA-F catalysing the cytoplasmic steps have been subject to high-throughput screening programmes since the mid-1990s, but relatively few hits have emerged,[2] and very few of those hits show antimicrobial activity.[3]

Enzymes of the lipid-linked cycle of peptidoglycan biosynthesis are targets for a number of natural product agents,[4] but there are very few reports of high-throughput screening studies. The enzymes phosphoMurNAc-pentapeptide translocase MraY and glycosyltransferase MurG catalyse the formation of lipid intermediates 1 and 2 (see Fig. 1), for which there are only two reports of high-throughput screening studies. For MurG, a screen of commercially available compound libraries using a fluorescence binding assay yielded a number of heterocyclic derivatives, for which no antimicrobial activity was reported.[5] A combined MraY/MurG screen using a scintillation proximity assay by Bristol-Myers Squibb led to the identification of four structures with IC_{50} <20 μM, including two halogenated fluorescein compounds,[6] however, it is not known which of the two enzymes are targeted by these compounds.

We have reported a continuous fluorescence enhancement assay for translocase MraY, which we have used to study the inhibition of MraY by nucleoside natural product antimicrobial agents mureidomycin A, liposidomycin B, and tunicamycin.[7,8] Further nucleoside natural product families, namely the pacidamycins,[9] caprazamycins,[10] muraymycins[11] and capuramycins[12] have also been reported as MraY inhibitors, though each are relatively high molecular weight compounds with high polarity, and so far it has proved difficult to identify low molecular weight analogues that retain high activity and antimicrobial activity.[13] Since the fluorescence assay is amenable to high-throughput format screening, we have undertaken the screening of the National Cancer Institute Diversity Set for novel MraY inhibitors, and further structure–activity analysis of halogenated fluoresceins implicated in the Bristol–Myers Squibb study, and we report the identification of two new inhibitor structures.

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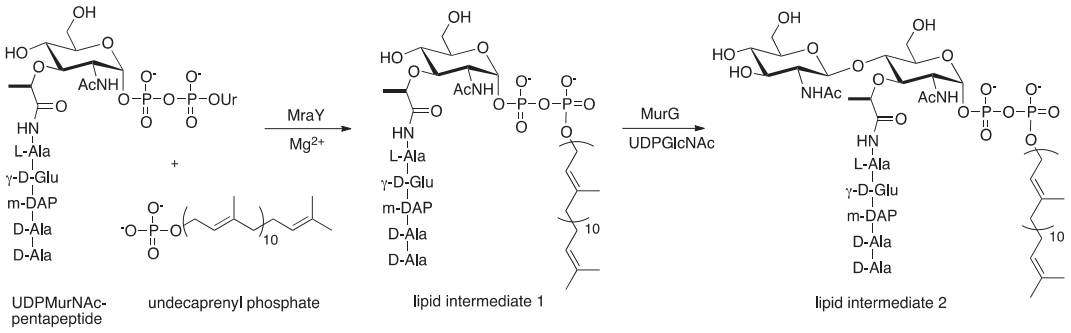

Figure 1. Reactions catalysed by translocase MraY and glycosyltransferase MurG.

2. Results

2.1. Screening of NCI diversity set against *Escherichia coli* MraY

The fluorescence enhancement MraY assay was carried out on 96-well microtitre plates using overexpressed *E. coli* MraY activity, with the following assay conditions: 10 μM *N*-dansyl-UDPMurNAc-pentapeptide, 15 μM heptaprenyl phosphate, 40 μg membranes containing overexpressed *E. coli* MraY, in 72 mM Tris buffer pH 7.5, with excitation at 340 nm and detection at 530 nm, giving fluorescence enhancement of 5000–6000 FAU over a 20 min assay (see Supporting information S1). 25 μM tunicamycin was used as an inhibitor control, giving >95% inhibition of fluorescence enhancement.

The NCI Diversity Set (1717 compounds) was then screened at 100 μM concentration under these conditions. 1340 compounds showed no observable inhibition, 243 compounds showed background fluorescence that interfered with these assay conditions, and 134 compounds showed reduced fluorescence over a 20 min assay. These 134 compounds were then examined in a continuous fluorescence cuvette-based assay at 60 μM concentration (see Supporting information S2). 83 compounds showed an immediate drop in fluorescence, followed by time-dependent increase in fluorescence (low fluorescence with activity, LFA), while 51 compounds showed an immediate drop in fluorescence, followed by no subsequent change in fluorescence (low fluorescence, LF). However, testing of the 83 LFA and 51 LF compounds with a secondary radiochemical MraY assay with [^{14}C-D-Ala]-UDPMurNAc-pentapeptide (see Section 4) unfortunately revealed that none of these compounds caused inhibition of MraY at 100 μM concentration, hence these compounds were causing fluorescence quenching in the original screen.

The radiochemical assay was then used to screen 21 high fluorescence compounds and 11 low fluorescence compounds from the natural products collection in the NCI Diversity Set (these 32 compounds were from the group of 243 showing background fluorescence, but also members of the 120 natural products in the Diversity Set). In this set of compounds, one test compound showed approx. 20% inhibition of MraY activity at 100 μM concentration, and re-testing of this compound at 500 μM concentration showed approx 60% inhibition. This compound was michellamine B, a naphthylisoquinoline alkaloid isolated from *Ancistrocladus korupensis*[14] (Fig. 2).

Testing of michellamine B at a range of concentrations against *E. coli* MraY using the radiochemical assay gave an IC_{50} value of 456 μM (see Supporting information S3). This compound was also assayed against recombinant MraY enzymes from *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, and showed slightly higher inhibitory activity against *B. subtilis* MraY (IC_{50} 386 μM, see Supporting information S4), but no inhibition of *S. aureus* MraY (see Table 1).

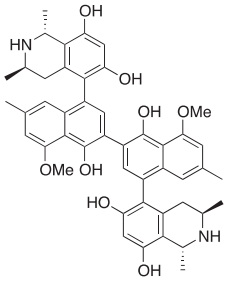

Figure 2. Structure of michellamine B.

Samples of michellamine B were also tested for antimicrobial activity, using a liquid culture microtitre plate assay. Inhibition of bacterial growth was observed for *Bacillus subtilis* (MIC 16 μg/mL), but no growth inhibition was observed against *Escherichia coli*, *Pseudomonas putida* or *Micrococcus flavus*.

2.2. Assay of halogenated fluoresceins against *E. coli* MraY and *E. coli* MurG

In order to investigate whether the halogenated fluorescein derivatives reported by Zawadzke et al. (compounds **3** and **4** in Fig. 3) inhibited MraY or MurG,[6] 2,7-dichlorofluorescein **1** and 2,7-dibromofluorescein **2** were prepared using the method of Lyttle et al.,[16] and the commercially available phloxine B was also tested for activity. Assay against *E. coli* MraY using the radiochemical assay revealed that fluoresceins **1** and **2** were inactive as inhibitors, but inhibition was observed using phloxine B.

Assay of phloxine B at a range of inhibitor concentrations gave an IC_{50} value of 32 μM against *E. coli* MraY (see Fig. 4), and weaker IC_{50} values of 128-254 μM against MraY enzymes from other bacteria (see Table 1). These compounds were also assayed against recombinant *E. coli* MurG, using a radiochemical assay previously reported. [17] No MurG inhibition was observed at up to 500 μg/mL concentration, whereas >80% inhibition was observed using 50 μg/mL ramoplanin, which inhibits MurG via complexation of the lipid I substrate.

2.3. Docking of michellamine B and phloxine B to structure of *Aquifex aeolicus* MraY

The crystal structure of *Aquifex aeolicus* MraY has recently been reported,[18] which allowed us to dock the structures of michellamine B and phloxine B against the reported structure. After minimisation of the MraY enzyme and ligand structures, molecular docking was first performed with Auto Dock vina software, which searches for the best binding sites among all the probable cavities in the protein structure, with a rigid binding site, but flexible ligand.[19] Four potential binding sites were observed: the active

Table 1
Activity of michellamine B and phloxine B against recombinant MraY enzymes

Recombinant MraY enzyme	MraY activity (fluorescence units min^{-1} mg $protein^{-1}$)	Michellamine B IC_{50} (μM) or % inhibition @ 500 μM	Phloxine B IC_{50} (μM)
E. coli	2.27	456 ± 55	32 ± 1.2
B. subtilis	2.71	386 ± 60	165 ± 32
P. aeruginosa	4.91	38%	128 ± 25
S. aureus	2.98	0%	254 ± 33
M. flavus[a]	0.71	14%	195 ± 28

[a] Using *Micrococcus flavus* membranes containing naturally enhanced levels of MraY and MurG.

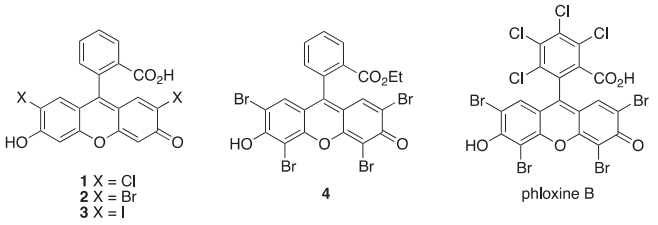

Figure 3. Structures of halogenated fluorescein ligands tested in this study (**1**, **2**, phloxine B), and the compounds reported by Zawadzke et al (**3**, **4**).[6]

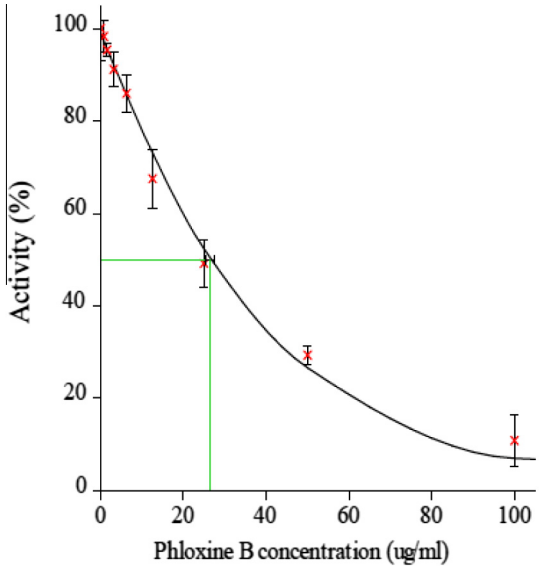

Figure 4. Inhibition of *E. coli* MraY by phloxine B. Assay conditions described in Section 4.

site, in the vicinity of Asp-117, Asp-118, Asp-265 and the Mg^{2+} cofactor;[18,20] a hydrophobic groove formed between transmembrane helices 5 and 9; and putative 'gate' and 'space' sites formed at the interface between the two protein sub-units, as shown in Fig. 5A. Phloxine B was found to bind favourably either at the active site (affinity −6.9 kcal/mol) or the inter-subunit space (affinity −6.9–7.6 kcal/mol), whereas michellamine B was found to bind preferentially to the hydrophobic groove (affinity −8.5–9.0 kcal/mol), and the natural substrate UDPMurNAc-pentapeptide was found to bind to the active site (affinity −5.2 kcal/mol).

In order to find the optimum binding conformation, GOLD software was used to study molecular docking of each ligand to each binding site determined by Autodock Vina, with both binding site and ligand flexible.[21] Scoring function Chem Score Fitness was used to rank different binding conformations, and Chem Score DG to calculate binding affinity.[21] For phloxine B, the protonated form was found to bind to the active site (Fitness score 21.5); the optimum binding conformation (Chem Score DG-28.9, see Supporting information S6) is shown in Figure 5B. The carboxylic acid of phloxine

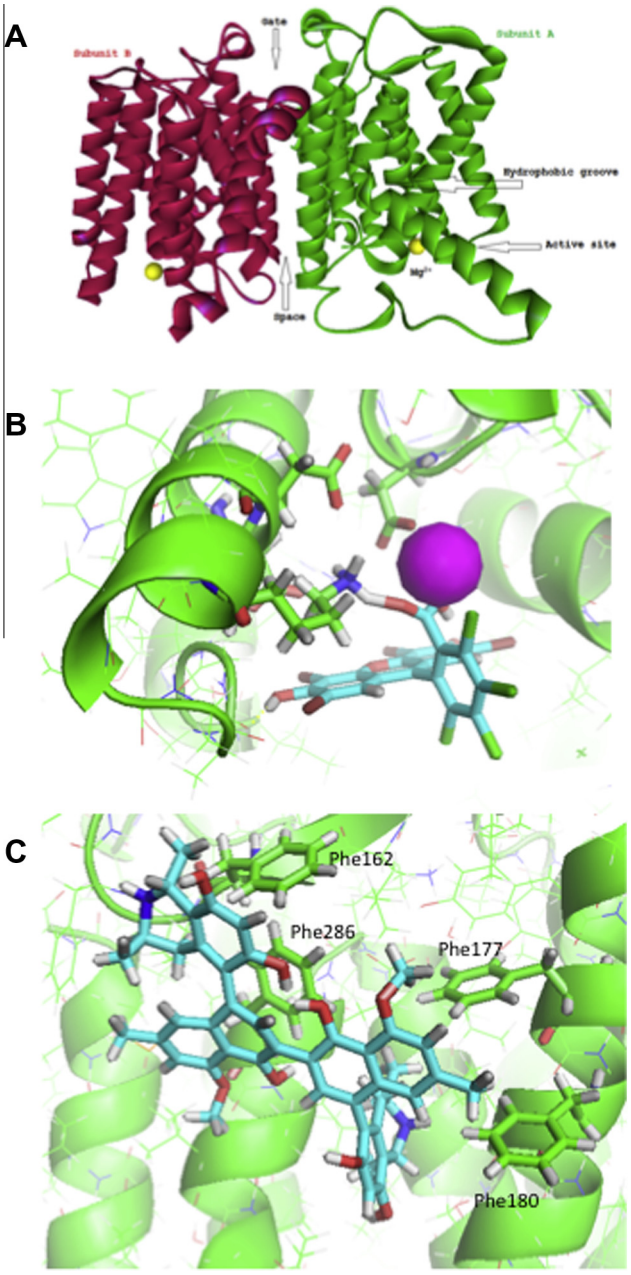

Figure 5. Docking of ligands to *A. aeolicus* MraY structure. (A) Binding sites observed in MraY protein structure; (B) binding of phloxine B to MraY active site, near Mg^{2+} cofactor and Lys-121; (C) binding of michellamine B to hydrophobic groove, near Phe-286 on helix 9, Phe-180 and Phe-177 on helix 5, and Phe-162.

B binds to the Mg^{2+} cofactor and Lys-121, while the two aromatic rings form favourable π–cation (with Lys-121 and Mg^{2+} cofactor) and hydrophobic contacts with the active site.

Michellamine B showed preferential binding to the hydrophobic groove identified in Figure 5A. Several possible binding conformations were found (see Supporting information S5), in which there are close contacts with the sidechains of four phenylalanine residues: Phe-286 on helix 9, Phe-180 and Phe-177 on helix 5, and Phe-162. The four aromatic ring systems of michellamine B are able to form favourable π–π interactions with two, three or four of these Phe residues, as shown in Figure 5C.

3. Discussion

Screening of the NCI Diversity Set (1717 compounds) against *E. coli* translocase MraY has revealed only one new inhibitor, michellamine B, a naphthylisoquinoline alkaloid isolated from *Ancistrocladus korupensis*.[14] Michellamine B has anti-HIV antiviral activity,[22] and has also been identified as an inhibitor of human 12-hLO lipoxygenase from library screening.[23] Docking of michellamine B to the structure of *A. aeolicus* MraY indicates that it appears to bind to a hydrophobic groove formed between helices 5 and 9, forming interactions with four phenylalanine residues. One of these residues, Phe-288 (Phe-286 in *A. aeolicus* MraY), is part of an interaction site with the antibacterial lysis protein E from bacteriophage φX174, that is proposed to be a protein-protein interaction site for MraY.[15] Of the two phenylalanine residues on helix 5, Phe-180 (in *A. aeolicus* MraY sequence) is conserved in an amino acid sequence alignment, whereas Phe-177 is not (see Supporting information S7). These four phenylalanine residues form a hydrophobic binding site, and the binding of michellamine B to this site suggests that it could be targeted by other small molecule ligands. We note that the *S. aureus* MraY is not inhibited by michellamine B, this might be due to the presence of a polar Gln residue two residues after the equivalent to Phe-180, which would probably alter the structure of helix 5 in this enzyme.

Testing of three halogenated fluoresceins has identified phloxine B as an inhibitor of MraY, and not MurG, and that MraY inhibitory activity appears to be highly dependent on the halogenation of the carbon skeleton. Although our halogenated fluorescein derivatives are not identical to those reported by Zawadzke et al.,[6] it seems likely that their observed effects were due to inhibition of MraY rather than MurG, and the IC_{50} value measured here for phloxine B (32 μM) is comparable to the reported values for diiodofluorescein **3** (16 μg/mL) and tetra-bromo derivative **4** (7 μg/mL) in a combined MraY/MurG assay.[6] It should be noted that our study used membranes containing overexpressed MraY, in which the measured IC_{50} may differ from assays using detergent-solubilised MraY,[7,8] however the study by Zawadzke et al. also used membranes containing overexpressed MraY.[6] Inhibition of peptidoglycan synthesis via MraY inhibition may therefore be responsible for the antimicrobial activity of phloxine B against *Staphylococcus aureus*, which was also reported to be highly dependent on the degree of halogenation.[24] Docking of phloxine B to the *A. aeolicus* MraY structure indicates that it appears to bind to the MraY active site, via the Mg^{2+} cofactor.

This work provides some further potential ligands for translocase MraY. It is hoped that understanding of the molecular basis for inhibition of MraY by these compounds may lead to the identification of further small molecule inhibitors for this antibacterial target enzyme.

4. Experimental section

4.1. Materials

The NCI diversity set III, natural products set II, and a sample of michellamine B diacetate (MW 877) were supplied by the Developmental Therapeutics Program of the National Cancer Institute (Rockville, MD, USA). 2,7-Dichlorofluorescein (**1**) and 2,7-dibromofluorescein (**2**) were prepared by reaction of 4-chlororesorcinol (Sigma–Aldrich) or 4-bromoresorcinol[25] with phthalic anhydride, using methanesulfonic acid as catalyst, following the method of Lyttle et al.[16] UDPMurNAc-pentapeptide and UDPMurNAc-tripeptide were obtained from the BaCWAN Synthesis facility (School of Life Sciences, University of Warwick). *N*-Dansyl-UDPMurNAc-pentapeptide was prepared by dansylation of UDPMurNAc-pentapeptide, after desalting via Sephadex G25 gel filtration and lyophilisation, using the method of Brandish et al.[7] Chemicals and biochemical were purchased from Sigma–Aldrich, undecaprenyl phosphate and heptaprenyl phosphate were purchased from Larodan Fine Chemicals (Malmö, Sweden).

4.2. Screening of NCI diversity set via microtitre plate assay

Assays were carried out in a 100 μl total volume, containing 85 μl master mix (containing 11.2 μM dansyl-labelled UDPMurNAc-pentapeptide, 17 μg/mL heptaprenyl phosphate in 85 mM Tris buffer pH 7.5, 21.25 mM $MgCl_2$), 5 μl test compound solution at 100 μM concentration final concentration (from 2 mM stock in 200 mM Tris at pH 7.5, 50 mM $MgCl_2$) and 10 μl *E. coli* MraY membranes, containing 40 μg protein. 25 μg/mL tunicamycin was used as a positive inhibitor control; negative controls were run containing no inhibitor, or no membranes. Assays were carried out in duplicate. Assays were performed at room temperature in a Tecan Genios Plate-Reader. Fluorescence measurements were taken before the start of the reaction, then over 20 min at 5 min intervals, and once again at 30 min, monitoring at excitation 340 nm, emission 535 nm.

4.3. Continuous fluorescence MraY assays

Continuous fluorescence assays of MraY were carried out as described by Brandish et al.,[7] using over-expressed *E. coli* MraY. The hits from the microtitre fluorescence MraY assay were tested at 60 μM concentration in a total volume of 170 μl containing 15 μM dansyl-labelled UDPMurNAc-pentapeptide, 35 μg/mL heptaprenyl phosphate, 83 mM Tris pH 7.5, 21 mM $MgCl_2$, 6 % glycerol and 0.15% Triton X-100 by the continuous fluorescence MraY assay. The reaction was started by the addition of 60 μg MraY membranes. 14.7 μg/mL tunicamycin was used as a positive control. The continuous fluorescence assays were performed at 25 °C in a PerkinElmer fluorimeter. The MraY reaction was monitored at 340 nm for excitation and at 530 nm for emission.

4.4. Preparation of [^{14}C-D-Ala]-UDPMurNAc-pentapeptide

The synthesis incubation (total volume 750 μl) containing UDP-MurNAc-tripeptide (1 μmole), [^{14}C]-L-alanine (3 μmol, specific activity 10 μCi/μmol), purified *E. coli* alanine racemase (200 μg), purified *E. coli* D-alanine-D-alanine ligase (DdlB, 70 μg), purified *Pseudomonas aeruginosa* MurF (240 μg), 100 μl ATP (25 mM, 19 μmole), pyridoxal phosphate (0.5 mM) in 250 mM Tris buffer pH 8.0 was incubated for 16 h at 20 °C. The enzymes were removed by ultrafiltration with a Centricon membrane (cut off 10,000 Da). The filtrate was purified by gel filtration (Sephadex G 25, 30 × 1 cm) eluting with water, collecting 4 mL fractions. The highest molecular weight fractions with absorbance maxima at 210 and 260 nm were collected and counted in a scintillation counter, and highest activity fractions (1,000–2,000 cpm/μl) were pooled, and stored for assays.

4.5. Radiochemical assay for MraY

Assays were carried out as described by A. Lloyd et al. [20] A solution of [^{14}C]-UDPMurNAc-pentapeptide (1–4 nCi/assay) was

incubated for 30 min at 35 °C with 27 μg/mL undecaprenyl or heptaprenyl phosphate and 30–50 μg overexpressed MraY membranes in a buffer containing 100 mM Tris pH 7.5, 17.5 mM $MgCl_2$, 4.0% (vol/vol) glycerol, 2.3 % (vol/vol) DMSO, 0.1% Triton X-100, in a total volume of 100 μl. The mixture was vortexed and left for 30 min at 35 °C. The reaction was stopped by the addition of 6 M pyridinium acetate pH 4.2 (100 μl), and an additional 200 μl of 1-butanol was added. The mixture was vortexed and centrifuged at 13,000×*g*. A 200 μl volume of the upper butanol layer was pipetted, and placed in ~5 mL ScintiSafe scintillation liquid and quantified by liquid scintillation counting by a PerkinElmer scintillation counter (dpm). Control assays lacking MraY membranes gave an 'extraction control' background, which was subtracted to give the final measurement. Assays were carried out in duplicate. Further controls included a no inhibitor negative control, and a positive inhibitor control tunicamycin (25 μM). IC_{50} was measured from a plot of enzyme activity vs. inhibitor concentration where the negative controls lacking inhibitor was taken as 100 % activity, and error calculated by a curve fitting program in GenStat.

4.6. Radiochemical assay for MurG

Assays were carried out as described in Trunkfield et al.[17] Lipid I was generated from UDPMurNAc-L-Ala-γ-D-Glu-*m*-Dap-D-Ala-D-Ala and undecaprenyl phosphate by overexpressed *E. coli* MraY membranes and then converted in the presence of [^{3}H]-UDPGlcNAc and *E. coli* MurG to give radiolabelled lipid II. Assays (total volume 100 μl) contained 100 μM UDPMurNAc-L-Ala-γ-D-Glu-*m*-Dap-D-Ala-D-Ala, 23.3 μg/mL undecaprenyl phosphate, 6.6 μM [^{3}H]-UDPGlcNAc (2.53 nCi/assay), 70 mM Tris pH 7.5, 17.5 mM $MgCl_2$, 4.0% glycerol (vol/vol), 2.3% (vol/vol) DMSO, 0.1% Triton X-100 to which was added 60 μg *E. coli* membranes containing overexpressed *E. coli* MraY, and 60 μg *E. coli* MurG solution. The mixture was incubated at 35 °C for 30 min and reactions were stopped by the addition of 100 μl 6 M pyridinium acetate, pH 4.2. The lipid products were extracted into 200 μl 1-butanol and quantified by liquid scintillation counting. Assays were carried out in duplicates in the presence of inhibitor at final concentrations of 165 μg/mL dichlorofluorescein and 325 μg/mL dibromofluorescein. Inhibition by 70 μM ramoplanin was used as a positive control. Mixtures for control experiments lacked *E. coli* MraY membranes and *E. coli* MurG.

4.7. MIC determination

Antibacterial activity was measured against *E. coli* DH5α, *B. subtilis* (W23) and *P. putida* (ATCC 33015). Overnight cultures were diluted 100-fold into 3 × 10 mL Luria-Bertani medium, and incubated at 37 °C with shaking at 180 rpm until OD_{600} was 0.6. The culture was diluted 100 fold and 95 μl of this diluted culture was placed in 96-well plates. To each well, 5 μl inhibitor solution was added, to a total volume of 100 μl. Michellamine B was tested at 125, 62.5, 31.3, 15.6, 7.81, 3.91 and 1.95 μg/mL final concentrations. Samples were carried out in triplicates. Control wells contained 100 μl LB The plates were incubated and shaken at 37 °C and bacterial growth was monitored at 595 nm.

4.8. Computational methods

Molecular docking was used to predict the binding sites and binding affinities for *A. aeolicus* MraY (PDB code 4J72). Pdb files for ligands were obtained by drawing them by in ChemDraw Pro 13.0 software. After minimisation of MraY enzyme and ligand structures, molecular docking was performed with two different softwares.

Autodock Vina software (http://vina.scripps.edu/index.html) was used for searching of the best binding sites among all the probable cavities in MraY.[19] The receptor and ligands coordinate files were converted into PDBQT format using MGLTools (version 1.5.6). All parameters were kept at their default values. A grid box was prepared to cover the full length of subunit A of MraY, to allow the ligand molecule to explore all the possible binding sites of enzyme.[26]

GOLD (Genetic Optimisation for Ligand Docking) was then used to study molecular docking of each ligand into the each binding site found by Autodock Vina. The scoring functions method of the docking program evaluates the accuracy of the docking procedure. Based on the fitness function scores and ligand binding position, the best-docked poses for each ligand are selected. GOLD provides two scoring functions, GoldScore and ChemScore.[26,27] ChemScore function ranks the ligands by the energy levels. In this study the ligands were docked into MraY enzyme using the scoring function of ChemScore. The ChemScore function estimates the free energy of binding of the ligand to a receptor as follows:

$$\Delta G_{\text{bind}} = \Delta G_0 + \Delta G_{\text{hbond}} S_{\text{hbond}} + \Delta G_{\text{metal}} S_{\text{metal}} + \Delta G_{\text{lipo}} S_{\text{lipo}} + \Delta G_{\text{rot}} S_{\text{rot}} \quad (1)$$

S_{hbond}, S_{metal} and S_{lipo} are the scores for hydrogen bonding, acceptor-metal and lipophilic interactions, respectively. The score of S_{rot} represents the loss of conformational entropy of the ligand upon binding to the protein. The final ChemScore value is obtained by adding a clash penalty and internal torsion terms, which operate against the establishment of close contacts in the docking and poor internal conformations. Covalent and constraint scores may also be included as follows:

$$\text{ChemScore} = \Delta G_{\text{bind}} + \Delta E_{\text{clash}} + \Delta E_{\text{internal}} \quad (2)$$

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.07.035.

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