Electronic Supplementary Information

New Application of Tiplaxtinin as an Effective FtsZ-targeting Chemotype for the Antimicrobial Study

Supplementary Materials and Methods

1. Materials
Tiplaxtinin was purchased from Sigma-Aldrich as catalogue #PZ0295. *B. subtilis*, *S. aureus* USA300 #417 and *S. aureus* USA300 #2690 tested in this assay were already available in our in-house collection, other strains were purchased from American Type Culture Collection (ATCC, USA).

2. Cell division inhibitory screening assay
Cell division inhibitory activity of the tested compounds was performed as described previously\(^1\). Overnight *B. subtilis* culture was grown in starvation medium supplemented with 1% hydrolyzed casein and then diluted in starvation medium supplemented with 3% hydrolyzed casein and grown at 37 °C. The culture was diluted to \(A_{600}\) of ~0.06, and 10 \(\mu\)L aliquots were added to transparent 96-well microtiter plates containing dilutions of the screening compounds in 100 \(\mu\)L volumes of medium. After incubation for approximately 5 h at 37 °C, 10 \(\mu\)L culture samples were transferred to poly-L-lysine-coated slides for microscopy. Cell morphology was observed by phase-contrast light microscopy.

3. Antimicrobial susceptibility assay
Antimicrobial susceptibility tests were conducted in 96-well microplates using the broth microdilution procedure described in the Clinical and Laboratory Standards Institute (CLSI) guidelines\(^2\). Cation-adjusted Mueller Hinton broth for all the *S. aureus* strains, or brain heart infusion broth for antibiotic-susceptible *E. faecium* strain ATCC 49624 and vancomycin-resistant *E. faecium* strain ATCC 700221, or Mueller Hinton broth for the other strains were used in the assays. After incubation for 18 h at 37 °C, the absorbance at 600 nm (\(A_{600}\)) was recorded using a microplate reader (Bio-Rad laboratory Ltd., UK) and the percentage of bacterial cell inhibition with respect to vehicles (1% DMSO) was calculated. The MIC was defined as the lowest compound concentration at which the growth of bacteria was inhibited by \(\geq 90\%\). Three independent assays were performed for each test.

4. Bacterial survival assays
A growing culture of *S. aureus* ATCC 29213 or *B. subtilis* 168 were diluted to approximately \(10^5\) CFU.mL\(^{-1}\) in volumes of Cation-adjusted Mueller Hinton broth or Mueller Hinton broth respectively, containing various concentrations of tiplaxtinin. Cultures were incubated at 37 °C, shaking. At the appropriate time intervals, 100 \(\mu\)L samples were removed for serial dilution in 900 \(\mu\)L volumes of Cation-adjusted Mueller
Hinton broth or Mueller Hinton broth, and 100 μL volumes from three dilutions were spread on to MH agar. Cell counts (CFU.ml⁻¹) were enumerated after incubating the plates at 37 °C for 18 hours.

5. Monitoring FtsZ assembly with light scattering
_S. aureus_ FtsZ was cloned, overexpressed, and purified as described previously[^3]. The light scattering assay was performed using a protocol adapted from the literature[^4]. The polymerization of recombinant _SaFtsZ_ was measured using 90° light scattering in a thermostatically (37 °C) controlled fluorescence spectrometer (Agilent Cary Eclipse). Both excitation and emission wavelengths were set to 600 nm with a slit width of 2.5 nm. FtsZ (6 μM) in 20 mM of Tris buffer (pH 7.4, containing 0.01% Triton X-100 to avoid compound aggregation) was placed in a 10 x 2 mm (excitation path) cell, the reaction was started by consecutive additions of 20 mM KCl, 5 mM MgCl₂, 1mM GTP and different concentrations of the test compound. 1% DMSO and 20 μg/mL methicillin were tested as vehicle and negative in this assay.

6. Transmission electron microscopy (TEM)
_S. aureus_ FtsZ (7.5 μM) was incubated in the absence and in the presence of 4 μg/mL of tiplaxtinin in 20 mM of Tris buffer (pH 7.4, containing 0.01% Triton X-100 to avoid compound aggregation) at 25 °C. After 10 min, 5 mM MgCl₂, 20 mM KCl, and 1 mM GTP were added to the reaction mixtures and incubated at 37 °C for 15 min. Then, 10 μL of the sample mixtures were placed on a glow-discharged Formvar carbon-coated copper grid (400 mesh) for 10 min. The grids were subsequently subjected to negative staining using 10 μL of 0.5% phosphotungstic acid (PTA) for 30 s, air-dried and digital images of the specimen were observed with a transmission electron microscope (JEOL model JEM 2010) operated at 200 kV and equipped with a Gatan MSC 794 CCD camera.

7. GTPase activity assay
The Effect of tiplaxtinin on the GTPase activity of recombinant _SaFtsZ_ was measured in 96-well microplates using a CytoPhos phosphate assay Biochem Kit (Cytoskeleton, USA) according to an optimized protocol and the manufacturer’s instructions[^3]. FtsZ (6 μM) was preincubated with vehicle (1% DMSO) or different concentrations of each test compound in 20 mM Tris buffer (pH 7.4, containing 0.01% Triton X-100 to avoid compound aggregation) for 10 min at 25 °C. Then 5 mM of MgCl₂ and 200 mM of KCl were added. Reactions were started with the addition of 500 mM GTP and incubated at 37°C. After 30 min, the reactions were quenched by adding 100 mL of Cytophos reagent for 10 min. Inorganic phosphate was quantified by measuring the absorbance at 650 nm with a microplate reader (Bio-Rad laboratory Ltd., UK).

8. Z-ring visualization in _B. subtilis_ cells
A culture of _B. subtilis_ containing the IPTG-inducible plasmid for the overexpression
of FtsZ fused with green fluorescence protein (GFP) was grown in LB medium supplemented with 30 μg/mL of chloramphenicol. After overnight incubation, a sample of the culture was diluted to 1% in the LB medium containing 2 μg/mL of tiplaxtinin and 40 μM of IPTG. After 4 h incubation at 37 °C, the B. subtilis cells were harvested, then resuspended in PBS buffer containing 0.25% of agarose. 10 μL of sample mixture were added to a pretreated microscopic slide with 0.1% (w/v) poly-L-lysine and visualized using a fluorescence microscope with a standard FITC filter set.

9. Molecular docking simulation of tiplaxtinin
The molecular modeling were performed using Discovery Studio 2016. The X-ray crystal structure of FtsZ in complex with a cell division inhibitor (PC190723) and GDP was downloaded from the PDB database (PDB entry: 4DXD; resolution: 2.0 Å). Water molecules and co-crystal ligands were removed from the structure and the protein was prepared for docking using an automated procedure of Discovery Studio. The structures of tiplaxtinin were sketched in 2D and converted into 3D using the Discovery Studio small molecule tools. Automated docking studies were carried out using Discovery Studio as implemented through the graphical user interface DS-CDocker protocol. The top-scoring poses were visually inspected.

References