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New application of tiplaxtinin as an effective FtsZ-targeting chemotype for an antimicrobial study†‡

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The filamenting temperature-sensitive mutant Z (FtsZ) protein is generally recognized as a promising anti-microbial drug target. In the present study, a small organic molecule (tiplaxtinin) was identified for the first time as an excellent cell division inhibitor by using a cell-based screening approach from a library with 250 compounds. Tiplaxtinin possesses potent antibacterial activity against Gram-positive pathogens. Both *in vitro* and *in vivo* results reveal that the compound is able to disrupt dynamic assembly of FtsZ and Z-ring formation effectively through the mechanism of stimulating FtsZ polymerization and impairing GTPase activity.

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Introduction

Bacterial pathogens cause a wide range of severe illnesses such as infections of the skin and soft tissues, septicaemia, pneumonia, and meningitis. Over the past few decades, the worldwide use of antibiotics has greatly improved public health, however, their efficacy has been found to decrease rapidly due to overuse/abuse or misuse that stimulates bacteria to develop resistance.^{1,2} Treatment of antibiotic-resistant infections has therefore become more and more difficult. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) are typical examples of bacteria showing resistance to widely prescribed antibiotics.^{3,4} The emergence of drug-resistant bacteria causes serious difficulties in the therapy of common bacterial infections. Thus, there is an urgent need for new antibacterial agents with innovative mechanisms of action.⁵

Bacterial divisomes having a complex set of biochemical machinery that contains many proteins are believed to be a new area for antibiotic research currently. In particular, filamenting temperature-sensitive mutant Z (FtsZ) has been

recognized as a very essential protein in the process of bacterial cell division.^{6,7} In the bacterial cell division process, FtsZ monomers self-assemble into a Z-ring and a highly dynamic cytoskeleton scaffold is generated at the site of septum formation.^{8,9} The mechanism that regulates the assembly and organization of FtsZ into a ring-like structure involves GTP binding and hydrolysis, which are modulated by the interaction of the N-terminal nucleotide binding domain of one FtsZ monomer with the C-terminal GTPase-activating domain (T7-loop) on the adjacent FtsZ monomer.^{8,9} Subsequently, FtsZ recruits other proteins to form a cell-division complex known as the divisome. Once the divisome is fully assembled, bacterial cell division is achieved by coordinated constriction and splitting of the daughter cells.^{10,11} In addition to its critical role in bacterial cytokinesis, FtsZ is a highly conserved

Fig. 1 Chemical structures of tiplaxtinin and some reported FtsZ inhibitors.

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protein in prokaryotes and is absent in eukaryotic cells,¹² thereby making it a promising antibacterial drug target.

Nowadays, some FtsZ inhibitors, as shown in Fig. 1, are known to inhibit bacterial cell division effectively.^{13–20} These molecules are able to impair bacterial growth through disrupting the dynamic assembly and/or GTP hydrolysis of FtsZ. Among these reported compounds, PC190723 is the most studied inhibitor of FtsZ to date and shows powerful antibacterial activity against most *Staphylococci* including MRSA, but exerts little effect on Gram-negative strains.^{16,21,22} However, PC190723 is poorly soluble for formulation and has limitations for clinical use.²¹ In other words, no FtsZ inhibitor has reached clinical trials yet. Expanding the existing chemical diversity with new chemotypes targeting bacterial cell division should be a better choice for further development. For this purpose, we identified tiplaxtinin (2-(1-benzyl-5-(4-(trifluoromethoxy)phenyl)-1*H*-indol-3-yl)-2-oxoacetic acid) as a potent cell division inhibitor (Fig. 1) by cell-based screening.

Interestingly, tiplaxtinin is reported as an orally efficacious plasminogen activator inhibitor-1 (PAI-1) inhibitor. In a rat carotid thrombosis model, tiplaxtinin (1 mg kg⁻¹, p.o.) increases the occlusion time and prevents carotid blood flow reduction.²³ In mice models bearing cervical xenografts, tiplaxtinin was found to markedly inhibit the growth of tumors. In addition, no toxicity or weight loss is noted in the mice model treated with 20 mg kg⁻¹ of tiplaxtinin *via* oral gavage.²⁴ Therefore it is a very promising drug candidate for further development. In addition, the experimental results obtained from the present study evidently suggest that the compound can act as an effective potent inhibitor of FtsZ and exhibits a cell division inhibition phenomenon with promising antibacterial activity against Gram-positive strains.

Results and discussion

FtsZ targeting compounds can disrupt the cell division function of the FtsZ protein, and then lead rod-shaped bacteria into cell elongation. We therefore screened *B. subtilis* 168 in the presence of a small compound library including 150 general compounds, 100 natural products and their derivatives which are either commercially available or synthesized by our research group. The screen²⁵ generated one potential hit (tiplaxtinin). The compound is an inhibitor of plasminogen activator inhibitor-1 which was reported to prevent carotid blood flow reduction in the rat model of carotid thrombosis.²³ As shown in Fig. 2, tiplaxtinin can significantly increase the cell length of *B. subtilis* at a concentration of 2 µg mL⁻¹, through a mechanism of antibacterial-induced cell filamentation. We further tested the antibacterial activity of the compound against an extended panel of clinically relevant bacterial strains, including antibiotic-resistant strains. The results show that tiplaxtinin has potent antibacterial activity against Gram-positive bacteria including *S. aureus*, *B. subtilis*, *E. faecium*, *E. faecalis*, and *S. epidermidis*, with the minimum inhibitory concentration (MIC) in the range of 2 to 4 µg mL⁻¹

Fig. 2 Inhibitory effect of tiplaxtinin on *B. subtilis*. Cells of *B. subtilis* 168 were grown in the absence (A) and presence of 2 µg mL⁻¹ of tiplaxtinin (B). Scale bar = 15 µm.

(Table 1). The potency of the compound was more than 100 times greater than that of methicillin against most of the MRSA strains. In addition, the compound also strongly inhibited the growth of a vancomycin-resistant *E. faecium* (VREF) with a MIC value of 4 µg mL⁻¹. However, tiplaxtinin cannot inhibit the growth of Gram-negative strains, such as *E. coli*, *P. aeruginosa* and *K. pneumoniae*, even at a concentration of 48 µg mL⁻¹. This antibacterial profile is similar to those of reported FtsZ inhibitors such as benzamide derivatives and 9-phenoxyalkylberberine derivatives.^{19,26}

In bacterial survival assays, time-kill curve determinations were performed to investigate whether tiplaxtinin is bactericidal or not. The concentration used, either equivalent to or greater than the MIC, caused a significant reduction in viable *S. aureus* ATCC 29213 or *B. subtilis* 168 cell numbers of more than three logarithms within 24 hours (Fig. 3), which was consistent with a bactericidal mode of action.²⁷

Because similar cell filamentation phenomena can also be found with FtsZ inhibitors from different chemotypes, such as 9-phenoxyalkylberberine derivatives, quinoline derivatives

Table 1 Minimum inhibitory concentrations of tiplaxtinin against a panel of bacterial strains

Organism	MIC (µg mL ⁻¹)	
	Tiplaxtinin	Methicillin
<i>B. subtilis</i> 168	2	<1
<i>S. aureus</i> ATCC 29213	2	<1
<i>S. aureus</i> ATCC 29247	2	6
<i>S. aureus</i> ATCC 33591 ^a	4	1024
<i>S. aureus</i> ATCC 33592 ^a	4	512
<i>S. aureus</i> ATCC 43300 ^a	4	512
<i>S. aureus</i> ATCC BAA-41 ^a	4	1024
<i>S. aureus</i> ATCC BAA-1717 ^a	4	512
<i>S. aureus</i> ATCC BAA-1720 ^a	4	1024
<i>S. aureus</i> ATCC BAA-1747 ^a	4	256
<i>S. aureus</i> USA300 #417 ^a	2	512
<i>S. aureus</i> USA300 #2690 ^a	2	256
<i>S. epidermidis</i> ATCC 12228	2	0.75
<i>E. faecalis</i> ATCC 29212	4	0.75
<i>E. faecium</i> ATCC 49624	4	1.5
<i>E. faecium</i> ATCC 700221 ^b	4	1.5
<i>E. coli</i> ATCC 25922	>48	3
<i>P. aeruginosa</i> ATCC BAA-2108	>48	>256
<i>K. pneumoniae</i> ATCC BAA-1144	>48	>256

^a These strains are MRSA. ^b A vancomycin-resistant strain; the MIC of vancomycin is higher than 96 µg mL⁻¹.

Fig. 3 Bacterial survival curve of tiplaxtinin. At time zero, samples of a growing culture of *S. aureus* ATCC 29213 (A) or *B. subtilis* 168 (B) were incubated with concentrations of tiplaxtinin equivalent to 1× (red), 2× (green), and 4× (blue) the MIC. A vehicle (1% DMSO; black) was included. Samples were removed at the time intervals indicated for the determination of viable cell counts.

and benzamide derivatives,^{19,20,28} further investigation of the antibacterial activity and the cell elongation effect of tiplaxtinin with FtsZ was conducted. To study the interaction between FtsZ and tiplaxtinin, *S. aureus* FtsZ proteins were cloned, overexpressed, and purified, and some biochemical assays were employed. As the first step in validating FtsZ as an antibacterial target of tiplaxtinin, we assessed the impact of tiplaxtinin on the polymerization dynamics of FtsZ using 90° light scattering in a thermostatically controlled fluorescence spectrometer in which changes in FtsZ polymerization are reflected by corresponding changes in absorbance at 600 nm.²⁹ Fig. 4A shows the time-dependent polymerization profiles of FtsZ in the absence and presence of tiplaxtinin in the concentration range from 2 to 8 μg mL⁻¹. The results show that tiplaxtinin is able to enhance FtsZ polymerization in a concentration-dependent manner, which is similar to the reported FtsZ-targeting compounds.^{30–33} Methicillin was used

Fig. 4 *In vitro* effects of tiplaxtinin on FtsZ. (A) Time-dependent polymerization profiles of *S. aureus* FtsZ in the absence and presence of tiplaxtinin in the concentration range from 2 to 8 μg mL⁻¹. (B) Inhibition of GTPase activity of FtsZ by tiplaxtinin. (C) and (D) Electron micrographs of FtsZ polymers in the absence (C) and in the presence (D) of 4 μg mL⁻¹ of tiplaxtinin. Scale bar = 500 nm.

in this assay as a non-FtsZ-targeting control antibiotic and it does not influence FtsZ polymerization. The impact of tiplaxtinin on FtsZ polymer formation was further visualized by using transmission electron microscopy (TEM). It was found that the size of the FtsZ polymers and the bundling of the FtsZ protofilaments were dramatically increased at the concentration of 4 μg mL⁻¹ (Fig. 4C and D). Moreover, the effect of tiplaxtinin on the GTPase activity of FtsZ was studied. The results show that tiplaxtinin decreases the rate of GTP hydrolysis of FtsZ in a concentration-dependent manner (Fig. 4B). The result indicates that tiplaxtinin inhibits the rate of GTP hydrolysis of FtsZ, which may be due to the disruption of the assembly of FtsZ.

Apart from the *in vitro* study, the on-target effect of tiplaxtinin in live bacteria is critical. A green fluorescent protein (GFP)–FtsZ constructed in *B. subtilis* 168 was used to monitor the effect of tiplaxtinin on the formation of FtsZ Z-rings.²⁵ We treated the bacteria with DMSO (control) or tiplaxtinin, and then examined the bacteria using fluorescence microscopy. In the absence of the compound, fluorescent foci corresponding to the Z-rings are localized at the midcell or the septum (Fig. 5A). On the other hand, FtsZ was found to be distributed as discrete and punctate foci throughout the elongated cell in the bacteria that were treated with tiplaxtinin, indicating the misformation of the Z-ring (Fig. 5B). As demonstrated, tiplaxtinin can also disrupt the polymerization and GTP hydrolysis of FtsZ; therefore, it is likely that the discrete and punctate fluorescent foci distributed throughout the bacteria treated with tiplaxtinin reflect multiple non-functional FtsZ polymeric structures distinct from Z-rings. This finding is in accord with the reported results using other chemotypes as FtsZ inhibitors.¹⁴

Molecular modelling was also used to predict the potential binding site for this class of compounds in the FtsZ protein. A 2.01 Å crystal structure of the *S. aureus* FtsZ apo-form (4DXD)³⁴ was used for the docking study. The highest docking score positioned the binding site of the ligands near the T7-loop and H7-helix. As the binding site is a relatively narrow cleft which is delimited by the H7-helix, the T7-loop, and a four-stranded β-sheet, FtsZ inhibitors therefore require some degree of planarity in order to fit. The docking results showed that a large number of favorable hydrophobic interactions were predicted to be established between tiplaxtinin

Fig. 5 Effect of tiplaxtinin on the Z-ring formation in *B. subtilis*. Bacteria were grown in the absence (A) and presence of 2 μg mL⁻¹ of tiplaxtinin (B). Scale bar = 10 μm.

Fig. 6 Predicted binding modes of tiplaxtinin bound to FtsZ. (A) Tiplaxtinin bound to the C-terminal interdomain cleft of FtsZ (PDB: 4DXD); (B) predicted interactions between tiplaxtinin and the amino acids of FtsZ.

and the side chains of Val189, Gln192, Gly193, Asp199, Leu200, Leu209, Met226, Gly227, Ile228, Thr256 and Val297. In addition, the trifluoromethoxy group of tiplaxtinin was predicted to participate in one conventional hydrogen bond with the side chain of Gly227 and three carbon-hydrogen bonds with the backbones of Gly193 and Gly227. The carbonyl group was supposed to form a hydrogen bond with Thr256. Furthermore, the trifluoromethoxy group could also interact with Val189, Gln192, Gly193, and Met226 through halogen bonds (Fig. 6).

Conclusions

In conclusion, we used a cell-based screening approach to identify the low toxic compound tiplaxtinin that is able to disrupt the dynamics of FtsZ. The compound was found to effectively enhance FtsZ protein assembly, disrupt GTP hydrolysis of FtsZ, block bacterial cytokinesis, and eventually impair bacterial cell division. Furthermore, tiplaxtinin displays potent antibacterial activity against Gram-positive pathogenic bacteria including drug-resistant strains: MRSA and VREF. These merit characteristics of the compound plus its low tox-

icity to animal models make it an attractive lead compound for further structure modification and assessment of efficacy in appropriate *in vivo* models of infection.

Conflicts of interest

The authors declare no competing interests.

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