International Journal of Antimicrobial Agents 49 (2017) 355–363

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Lipoteichoic acid synthesis inhibition in combination with antibiotics abrogates growth of multidrug-resistant *Enterococcus faecium*

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ARTICLE INFO

Article history:
Received 1 September 2016
Accepted 3 December 2016

Keywords:
Lipoteichoic acid
Enterococcus faecium
Antibiotic resistance
Biofilms
Compound 1771
LtaS

ABSTRACT

Enterococcus faecium is a multidrug-resistant (MDR) nosocomial pathogen causing significant morbidity in debilitated patients. New antimicrobials are needed to treat antibiotic-resistant *E. faecium* infections in hospitalised patients. *E. faecium* incorporates lipoteichoic acid (LTA) (1,3-polyglycerol-phosphate linked to glycolipid) in its cell wall. The small-molecule inhibitor 1771 [2-oxo-2-(5-phenyl-1,3,4-oxadiazol-2-ylamino)ethyl 2-naphtho[2,1-*b*]furan-1-ylacetate] specifically blocks the activity of *Staphylococcus aureus* LtaS synthase, which polymerises 1,3-glycerolphosphate into LTA polymers. Here we characterised the effects of the small-molecule inhibitor 1771 on the growth of *E. faecium* isolates, alone (28 strains) or in combination with the antibiotics vancomycin, daptomycin, ampicillin, gentamicin or linezolid (15 strains), and on biofilm formation (16 strains). Inhibition of LTA synthesis at the surface of the cell by compound 1771 in combination with current antibiotic therapy abrogates enterococcal growth *in vitro* but does not affect mature *E. faecium* biofilms. Targeting LTA synthesis may provide new possibilities to treat MDR *E. faecium* infections.

1. Introduction

Antibiotic-resistant bacteria have emerged as an important health concern worldwide, with increasing rates of hospital-associated infections by a group of drug-resistant pathogenic bacteria, affecting patient morbidity and mortality [1]. This group of pathogens has been coined the 'ESKAPE pathogens', for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. Currently, the ESKAPE pathogens cause the majority of hospital infections and effectively 'escape' the effects of antibacterial drugs [2]. *E. faecium* has, when it acquired vancomycin-resistance, emerged as a significant resistance problem in hospitals for which the costs of hospital care are increasing [3]. *E. faecium* infections are often difficult to treat because of its intrinsic and acquired resistance against a plethora of antibiotics, including ampicillin and vancomycin as well as the relatively new 'last-resort' antibiotics such as daptomycin and linezolid [4,5]. Novel antimicrobial agents are needed to counter the continuous evolution of drug resistance among bacteria causing infections in humans. There are some novel promising antimicrobial compound classes in pre-clinical stage targeting Gram-positive bacteria, including *E. faecium*. These include teixobactin targeting lipid II and lipid III [6], lipopeptides derived from nisin targeting lipid II [7], sortase transpeptidase inhibitors targeting sortase [8–10], alanine racemase enzyme inhibitors [11], and lipoteichoic acid (LTA) synthesis inhibitors that target LtaS [12]. The targets of these novel compounds and modes of action make them very promising for antimicrobial drug development and future treatment of bacterial infections.

LTA is an important cell wall component of Gram-positive bacteria, including enterococci, comprising a polymer of 1,3-polyglycerol-phosphate tethered to a glycolipid moiety Glc_2-DAG (β-gentiobiosyldiacylglycerol[glucosyl-(1→6)-glucosyl-(1→3)-diacylglycerol]), which provides for non-covalent anchoring in the cell membrane [13,14]. In *S. aureus*, synthesis of Glc_2-DAG is catalysed by PgcA, GtaB and YpfP, followed by translocation of the glycolipid across the membrane by LtaA [15–17]. The LTA synthase enzyme, LtaS, catalyses the polymerisation of polyglycerol phosphate from phosphatidylglycerol and its transfer to Glc_2-DAG [18]. LtaS is a transmembrane protein with a large extracellular C-terminal sulphatase domain implicated in this process [19]. Mutations in the *ltaS* gene of *S. aureus*, *Listeria monocytogenes*, *Bacillus subtilis* and *Bacillus anthracis* revealed an altered cell wall morphology and affected cell division and growth, suggesting that the LtaS enzyme is a suitable target for the development of novel antimicrobials [19–23]. Indeed, Richter et al used a library of 167,405 compounds to identify a small-molecule inhibitor termed compound

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http://dx.doi.org/10.1016/j.ijantimicag.2016.12.002
0924-8579/

1771 [2-oxo-2-(5-phenyl-1,3,4-oxadiazol-2-ylamino)ethyl 2-naphtho[2,1-*b*]furan-1-ylacetate] that inhibits LTA synthesis in *S. aureus* [12]. This small-molecule inhibitor probably binds the active site of LtaS to prevent the cleavage of phosphatidylglycerol and thus inhibits LTA synthesis, thereby decreasing cell viability significantly. Relatively little is known about LTA synthesis and LTA structure in *E. faecium*. However, *ltaS* homologues are present in *E. faecium* genomes, suggesting a comparable LTA biogenesis pathway [12]. This is supported by the fact that compound 1771 also has an inhibitory effect on the growth of *E. faecium* TX0016 [12].

To gain an insight in LTA synthesis inhibition by small-molecule inhibitor 1771 in multidrug-resistant (MDR) *E. faecium*, in this study the susceptibility of a panel of *E. faecium* strains to compound 1771, alone and in combination with either ampicillin, vancomycin, daptomycin, linezolid or gentamicin, was tested and the impact of this compound on enterococcal LTA synthesis was determined.

2. Materials and methods

2.1. Bacterial strains

A total of 28 *E. faecium* isolates collected from 11 different countries worldwide, representing clinical and hospital outbreak isolates from hospitalised patients ($n = 20$) and non-hospital-associated isolates ($n = 8$), were used in this study (Table 1). The set of strains included isolates that were resistant to vancomycin, ampicillin, gentamicin, linezolid and daptomycin. All bacterial strains were grown aerobically at 37 °C on trypticase soy agar II plates supplemented with 5% sheep blood (Becton Dickinson, Breda, The Netherlands) from glycerol stocks from –80 °C and in tryptic soy broth (TSB) (Oxoid B.V., Landsmeer, The Netherlands) supplemented with 1% glucose (TSBg) and 1% dimethyl sulfoxide (DMSO) (Merck, Amsterdam, The Netherlands).

2.2. In silico *analysis of* E. faecium *LtaS enzymes*

A total of 423 draft assemblies of *E. faecium* genomes publicly available at the PATRIC database (https://www.patricbrc.org; interrogated 28 May 2016) were searched for proteins annotated as 'LtaS' or 'lipoteichoic acid synthase' using the protein family sorting tool [24]. The multiple LtaS synthase type IIb and type IIc proteins and alignments were examined visually. The TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict transmembrane helices in LtaS proteins. The *E. faecium* E745 genome was sequenced previously by our group (van Schaik et al, genome to be deposited under project ID PRJNA295268). The *E. faecium* E745 LtaS type IIb amino acid sequence was submitted to the Phyre 2.0 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) to identify structural homologues [25]. The structure of LtaS of *E. faecium*, termed $LtaS_{fm}$, was analysed using the PyMOL Molecular Graphics System v.1.8 (Schrödinger, LLC, New York, NY, USA).

2.3. Small-molecule inhibitor 1771

Compound 1771 [2-oxo-2-(5-phenyl-1,3,4-oxadiazol-2-ylamino)ethyl 2-naphtho[2,1-*b*]furan-1-ylacetate] was purchased from Enamine Ltd. (Kiev, Ukraine). A 200 mM stock concentration of compound 1771 was prepared by dissolving in 100% DMSO at 50 °C.

2.4. Determination of growth curves

A Bioscreen C instrument (Oy Growth Curves AB, Helsinki, Finland) was used to monitor the effects of antibiotics, compound 1771 or a combination of different antibiotics plus compound 1771 on the growth of resistant *E. faecium* strains. A final concentration of 20 μM of compound 1771 was used alone or in combination with

Table 1
Enterococcus faecium strains used in this study.[a]

Strain	Epidemiology	Isolation source	Country	Year	MIC (μg/mL)					
					Vancomycin	Ampicillin	Gentamicin	Daptomycin	Linezolid	Compound 1771
E155	Hospital-associated	Faeces	USA	1995	1024	128	≥1024	n.d.	n.d.	8
E300	Hospital-associated	Urine	USA	1994	1024	256	16	n.d.	n.d.	8
E317	Hospital-associated	Urine	USA	n.d.	1024	256	16	n.d.	n.d.	16
E333	Hospital-associated	Blood	ISR	1997	1024	64	≥1024	n.d.	n.d.	8
E336	Hospital-associated	Urine	ITA	n.d.	≤8	128	32	n.d.	n.d.	8
E745	Hospital-associated	Faeces	NL	2000	512	64	16	n.d.	n.d.	8
E980	Community-associated	Faeces	NL	1998	≤8	≤4	32	n.d.	n.d.	8
E1007	Community-associated	Faeces	NL	1998	≤8	≤4	32	n.d.	n.d.	16
E1050	Community-associated	Faeces	NL	1998	≤8	128	16	n.d.	n.d.	16
E1162	Hospital-associated	Blood	FR	1997	16	128	32	n.d.	n.d.	8
E1172	Hospital-associated	Urine	POL	1998	≤8	128	≥1024	n.d.	n.d.	4
E1393	Hospital-associated	n.d.	GBR	2000	512	128	≥1024	n.d.	n.d.	32
E1527	Hospital-associated	n.d.	AUT	n.d.	≤8	512	≥1024	n.d.	≥32	32
E1531	Hospitalised patient	n.d.	GBR	n.d.	512	32	≤8	n.d.	≥32	4
E1590	Community-associated	Faeces	IRL	2001	≤8	≤4	≤8	n.d.	n.d.	≤0.5
E1604	Food	Cheese	NOR	1956	128	32	≥1024	n.d.	n.d.	16
E1613	Food	Fish burger	NOR	1964	≤8	≤4	1024	n.d.	n.d.	32
E1630	Environmental	Water	NL	1981	≤8	≤4	1024	n.d.	n.d.	≤0.5
E1861	Community-associated	Faeces	ESP	2001	≤8	≤4	1024	n.d.	n.d.	64
E1972	Hospital-associated	Blood	GER	2000	≤8	≤4	1024	n.d.	n.d.	16
E2039	Hospital-associated	Catheter	GER	2000	≤8	≤4	1024	n.d.	n.d.	32
E2297	Hospital-associated	Urine	USA	2001	512	512	≥1024	n.d.	n.d.	8
E2560	Hospital-associated	Blood	NL	2006	1024	256	≥1024	n.d.	n.d.	16
E7127	Hospital-associated	Wound	USA	2004	1024	512	1024	n.d.	≥32	16
E7128	Hospital-associated	Blood	ESP	2001	1024	128	1024	6	n.d.	16
E7129	Hospital-associated	Blood	ESP	2008	1024	128	1024	n.d.	n.d.	8
E7130	Hospital-associated	Blood	ESP	2011	1024	256	≥1024	12	n.d.	16
E7150	Hospital-associated	n.d.	GBR	n.d.	1024	128	≥1024	8	n.d.	16

MIC, minimum inhibitory concentration; n.d., not determined; ISR, Israel; ITA, Italy; NL, The Netherlands; FR, France; POL, Poland; GBR, Great Britain; AUT, Austria; IRL, Ireland; NOR, Norway; ESP, Spain; GER, Germany.

[a] Interpretive standards for *Enterococcus* spp. according to Clinical and Laboratory Standards Institute (CLSI) document M100-S26, Table 2D: ampicillin resistance, ≥16 μg/mL; vancomycin resistance, ≥32 μg/mL; linezolid resistance, ≥8 μg/mL; and daptomycin susceptible, ≤4 μg/mL; and according to CLSI document M100-S26, Table 3I: high-level gentamicin resistance, ≥500 μg/mL.