Journal of Medicinal Chemistry

Article

Design and syntheses of highly potent teixobactin analogues against Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant Enterococci (VRE) in vitro and in vivo

Anish Parmar, Rajamani Lakshminarayanan, Abhishek Iyer, Venkatesh Mayandi, Eunice Tze Leng Goh, Daniel G. Lloyd, Madhavi Latha S. Chalasani, Navin Kumar Verma, Stephen H Prior, Roger W Beuerman, Annemieke Madder, Edward J. Taylor, and Ishwar Singh

Just Accepted

ACS Publications

Design and syntheses of highly potent teixobactin analogues against *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci (VRE) *in vitro* and *in vivo*

Anish Parmar,[a]† Rajamani Lakshminarayanan,[b]† Abhishek Iyer,[a][c] Venkatesh Mayandi,[b] Eunice Tze Leng Goh,[b] Daniel G. Lloyd,[d], Madhavi Latha S. Chalasani,[e] Navin K. Verma,[e][b] Stephen H. Prior, [f] Roger W. Beuerman,[b] Annemieke Madder,[c] Edward J. Taylor,[d] and Ishwar Singh* [a]

[a] School of Pharmacy, Joseph Banks Laboratories, University of Lincoln, Green Lane, Lincoln LN6 7DL, United Kingdom.

[b] Singapore Eye Research Institute, The Academia, Discovery Tower Level 6, 20 College Road Singapore 169857

[c] Organic and Biomimetic Chemistry Research Group, Department of Organic and Macromolecular Chemistry, Ghent University, Krijgslaan 281 (S4), B-9000 Ghent, Belgium

[d] School of Life Sciences, Joseph Banks Laboratories, University of Lincoln, Green Lane, Lincoln LN6 7DL, United Kingdom.

[e] Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 636921

[f] School of Chemistry, Joseph Banks Laboratories, University of Lincoln, Green Lane, Lincoln LN6 7DL, United Kingdom.

Abstract: The cyclic depsipeptide, teixobactin kills a number of Gram positive bacteria including Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* without detectable resistance. To date, teixobactin is the only molecule in its class which has shown *in vivo* antibacterial efficacy. In this work, we designed and synthesized 10 new *in vivo* ready teixobactin analogues. These analogues showed highly potent antibacterial activity against *Staphylococcus aureus*, MRSA, and vancomycin-resistant Enterococci (VRE) *in vitro*. One analogue, D-Arg$_4$-Leu$_{10}$-teixobactin **2** was found to be non-cytotoxic *in vitro* and *in vivo*. Moreover, topical instillation of peptide **2** in a mice model of *S. aureus* keratitis decreased the bacterial bioburden (>99.0% reduction) and corneal edema significantly when compared to untreated mice cornea. Collectively, our results have established the high therapeutic potential of a teixobactin analogue in attenuating bacterial infections and associated severities *in vivo*.

Introduction

The increasing bacterial resistance against currently used antibiotics and lack of new antibiotics to combat antimicrobial resistance (AMR) are major challenges to global health and wealth. These major challenges are estimated to cause 10 million deaths every year and $100 trillion in lost productivity to the global economy by 2050.[1] Therefore, there is a continual need to develop new antibacterial compounds. The recently discovered natural product, teixobactin has shown remarkable activity against a broad range of Gram positive bacteria, including resistant bacterial strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus spp.* (vancomycin-resistant enterococci, VRE) and
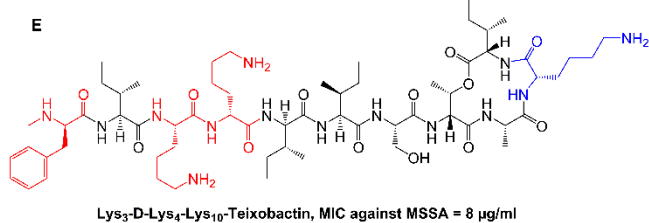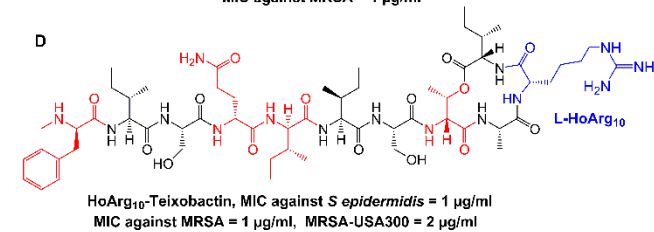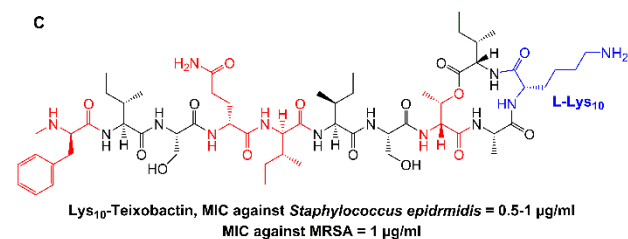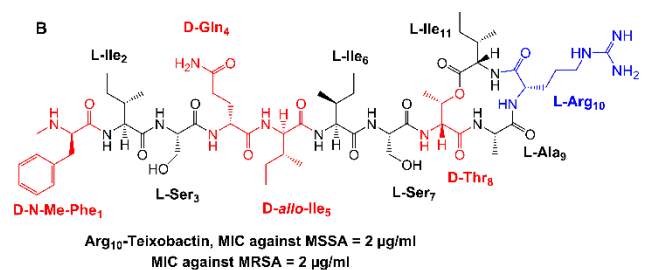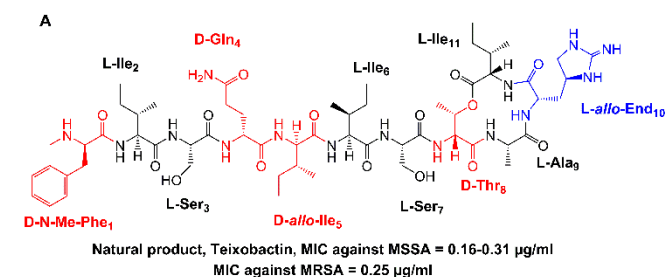
Mycobacterium tuberculosis.[2] Teixobactin is a nonribosomal undecapeptide. It contains four

D amino acids namely *N*-Me-D-Phe$_1$, D-Gln$_4$, D-*allo*-Ile$_5$ and D-Thr$_8$ and the rare L-allo-

enduracididine amino acid (Figure 1A, marked in red and blue respectively). Teixobactin kills

bacteria without detectable resistance and bacteria are less likely to develop resistance

because it operates by at least two unique modes of action. Notably, teixobactin binds to the

highly conserved pyrophosphate motifs of multiple bacterial cell wall substrates such as lipid

II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid).[2]

The total syntheses of teixobactin[3-4] and its analogues[5-7] and their biological activities have

been published in the past year. We[6] and others[5, 7] have reported the synthesis of Arg$_{10}$-

teixobactin by replacing the synthetically challenging enduracididine amino acid at position

10 with arginine. We have reported the first structure activity relationships (SAR) of Arg$_{10}$-

teixobactin and established the importance D amino acids for antibacterial activity.[4, 6-8] In

previous work, we also elucidated the 3D molecular structures of teixobactin analogues. The

disordered structure of teixobactin analogues was found to be vital for their biological activity,

D-Gln$_4$ being essential and D-*allo*-Ile$_5$ being important to maintain the disordered structure.

However, the replacement of D-Gln4 and D-*allo*-Ile$_5$ with L counterparts provided a more

ordered structure of teixobactin.[9]

The minimum pharmacophore of teixobactin was reported by Nowick et al.[7] A lysine scan of

Arg$_{10}$-teixobactin was reported by the Albericio group.[8] Replacement of any of the four

isoleucine residues with lysine led to a complete loss of activity. The replacement of Ser$_3$,

Gln$_4$ and Ala$_9$ by lysine was tolerated well and biological activity was maintained. The replacement of cationic residues such as arginine or lysine at position 10 with histidine led to inferior biological activity.[10]

Figure 1. Teixobactin and its analogues containing cationic and hydrophobic amino acids. Cationic analogues A[2-4], B[5-6, 11], C[7, 11], D[11-12], E[13] and hydrophobic analogues F[14], G[14], H[14], I[14], J[14] (D amino acids highlighted in red and the position 10 amino acids are highlighted in blue).

The L-*allo*-enduracididine was reported to be important for high antibacterial potency of teixobactin.[3] However, it is also a key bottleneck in the production and development of

teixobactin analogues due to various synthetic challenges.[15] We have reported the design and synthesis of potent teixobactin analogues against MRSA through the isosteric replacement of L-*allo*-enduracididine.[11] Recently, Brimble and coworkers reported the synthesis of teixobactin analogues through replacement of L-*allo*-enduracididine with amino acid isosteres and evaluated biological activity against MRSA and VRE.[12]

To expedite access to highly potent teixobactin analogues, we recently reported a new design by replacing the synthetically challenging enduracididine with commercially available hydrophobic residues such as leucine and isoleucine.[14] Leu_{10}- teixobactin and Ile_{10}-teixobactin showed identical activity to teixobactin against MRSA *in vitro*. However, increased hydrophobicity may have an adverse influence on the *in vivo* capacity to be further developed as therapeutic drugs. Teixobactin and key teixobactin analogues and their antibacterial activities are summarised in figure 1.

Teixobactin has shown antibacterial efficacy *in vivo* in three mouse models of infection. Although these results are encouraging, a significant amount of work remains in the development of teixobactin as a therapeutic antibiotic for human use.[15] The translation of molecules from a discovery phase to useful therapeutic antibiotics is prone to high failure due to numerous challenges, such as balancing high efficacy *in vivo* against a broad spectrum of pathogens with minimal liabilities against human targets and the balancing of hydrophobicity

with hydrophilicity to address water solubility issues.[16] There is a pressing need for highly

potent analogues of teixobactin to address common drug development challenges. To date,

there have been no *in vivo* evaluation studies of teixobactin analogues.

To address such teixobactin development challenges, we report herein the design and

synthesis of 10 highly potent teixobactin analogues (Figure 2) and their antibacterial

evaluations aganist *S. aureus*, MRSA, VRE and *in vivo* evaluation of one analogue in a mice

model of *S. aureus* keratitis. This work lays the foundation for the development of *in vivo*

ready teixobactin analogues.
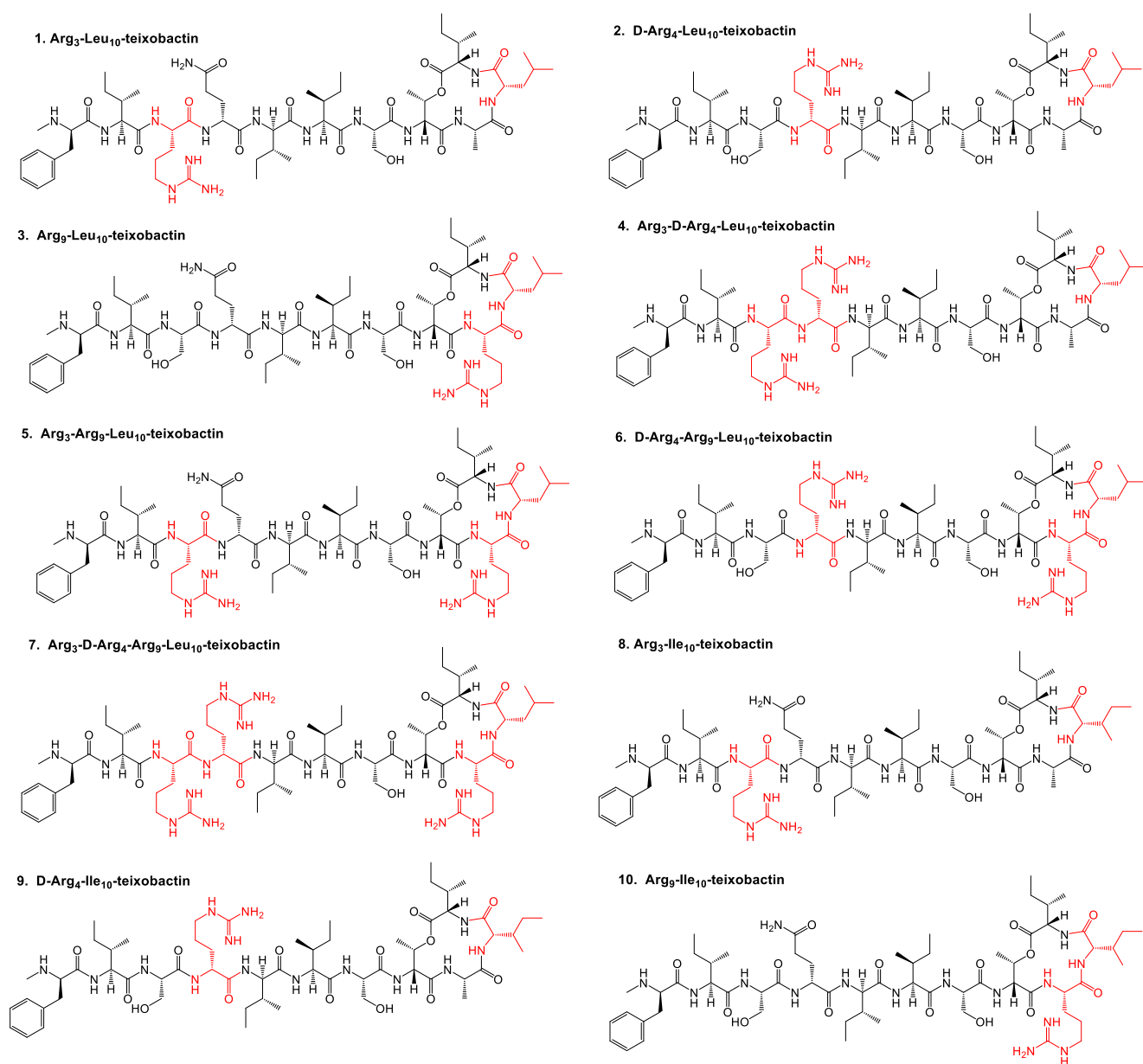
Figure 2. Structure of teixobactin analogues **1-10**.

Results and discussion

Design and synthesis:

To date, teixobactin is the only molecule in its class which has shown *in vivo* antibacterial efficacy. To realise the therapeutic potential of molecules based on the teixobactin scaffold, there is a pressing need for *in vivo* ready, simplified teixobactin analogues with ease of access to address the current challenges associated due to the lengthy and daunting total synthesis of teixobactins.

In this work, to address such teixobactin development challenges, we speculated that replacement of Ser_3, $D\text{-}Gln_4$ and Ala_9 of Leu_{10}-teixobactin and Ile_{10}-teixobactin with cationic arginine would mimic a suitable balance of hyrophobicity and hydrophilicity similar to natural teixobactin. We thus replaced the Ser_3, $D\text{-}Gln_4$ and Ala_9 of Leu_{10}-teixobactin and Ile_{10}-teixobactin with arginine in a systematic fashion (**1**-**10**, Figure 2). In this way, we realized an optimal balance between hyrophobicity and hydrophilicity. Six of these analogues (**1**-**3**, **8**-**10**, Figure 2) have a hyrophobic-hydrophilic profile (two positive charges at physiological pH) similar to natural teixobactin. Three analogues (**4**-**6**, Figure 2) feature three positive charges and one analogue (**7**, Figure 2) bears four positive charges. In total, we synthesised 10 new and highly potent teixobactin analogues (Figure 2, **1**-**10**) in a similar fashion to our recently reported highly efficient strategy (scheme 1 and experimental section).[14]
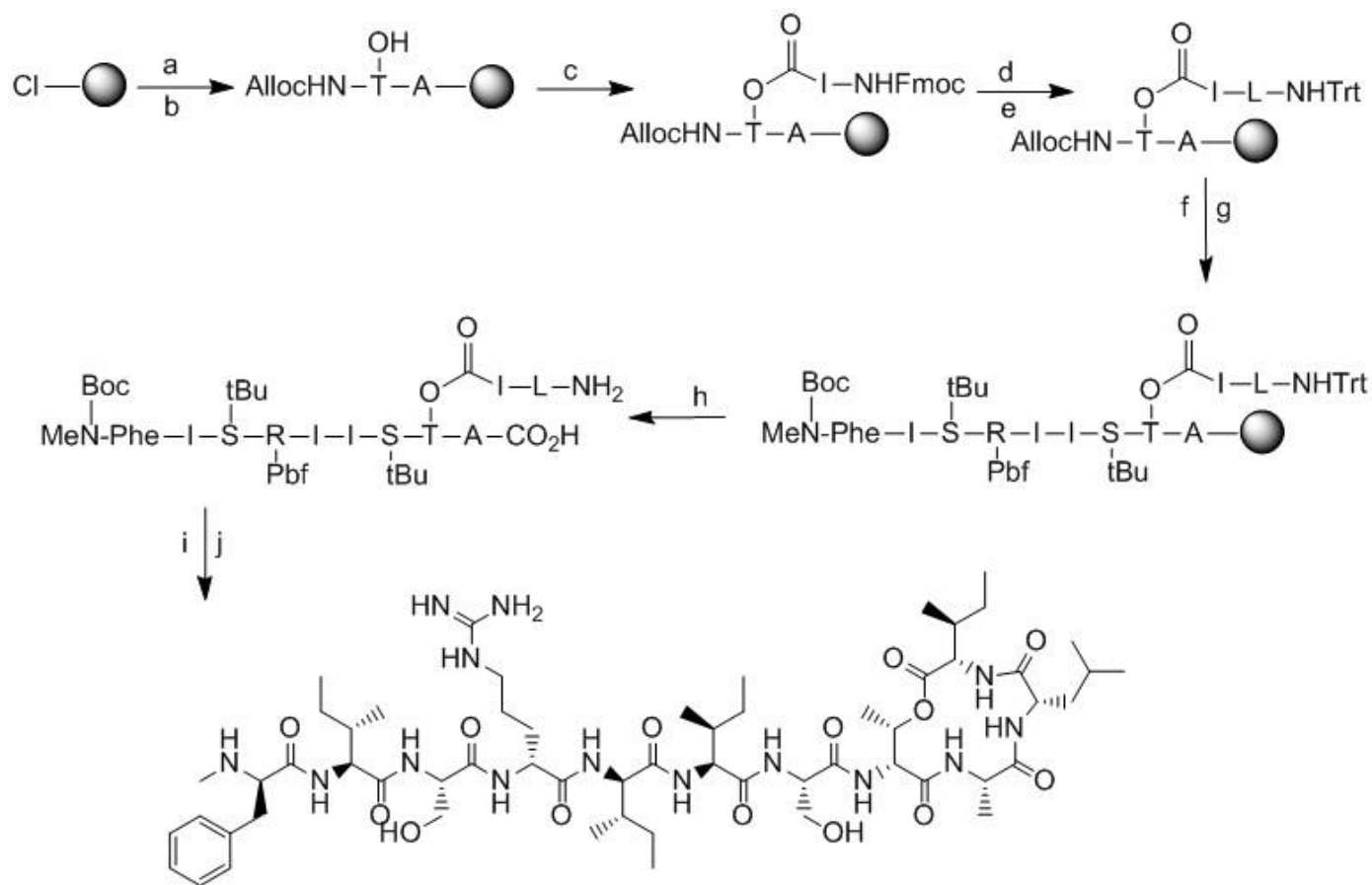
Scheme 1. Synthesis of D-Arg$_4$-Leu$_{10}$-teixobactin starting from 2-chlorotritylchloride resin: a. 4 eq. Fmoc-Ala-OH/8 eq. DIPEA in DCM, 3h.   b. 20% piperidine in DMF followed by 3 eq. AllocHN-D-Thr-OH, 3 eq. HATU/6 eq. DIPEA, 1.5h   c. 10 eq. Fmoc-Ile-OH, 10 eq. DIC, 5 mol% DMAP in DCM, 2h followed by capping with Ac$_2$O/DIPEA 10% in DMF, 20% piperidine in DMF   d. 4 eq. Fmoc-Leu-OH, 4 eq. HATU/8 eq. DIPEA in DMF, 1h followed by 20% piperidine in DMF   e. 10 eq. Trt-Cl, 15% Et$_3$N in DCM, 1h. f. 0.2 eq. [Pd(PPh$_3$)$_4$]^0 + 24 eq. PhSiH$_3$ in dry DCM, 1 x 20 min, 1 x 45 min.   g. 4 eq. Fmoc/Boc-AA(PG)-OH (AA = amino acid, PG = protecting group), 4 eq. DIC/Oxyma (µwave, 10 min) followed by 20% piperidine in DMF (3 min, 10 min).   h. TFA:TIS:DCM = 2:5:93, 1h. i. 1 eq. HATU/10 eq. DIPEA in DMF, 30 min.   j. TFA:TIS:H2O = 95:2.5:2.5, 1h.

Table 1. List of teixobactin analogues (**1-12**). MIC: Minimum Inhibitory Concentration. *MRSA ATCC 33591 used.

Compound	Name	MIC* (μg/mL)
1	Arg_3-Leu_{10}-texiobactin	0.125
2	D-Arg_4-Leu_{10}-texiobactin	0.125
3	Arg_9-Leu_{10}-texiobactin	0.125
4	Arg_3-D-Arg_4-Leu_{10}-teixobactin	0.25
5	Arg_3-Arg_9-Leu_{10}-teixobactin	1
6	D-Arg_4-Arg_9-Leu_{10}-teixobactin	1
7	Arg_3-D-Arg_4-Arg_9-Leu_{10}-teixobactin	1
8	Arg_3-Ile_{10}-texiobactin	0.25
9	D-Arg_4-Ile_{10}-texiobactin	0.125
10	Arg_9-Leu_{10}-texiobactin	0.25
11	Leu_{10}-teixobactin	0.25
12	Teixobactin	0.25

In vitro **antibacterial studies:**

The antimicrobial potency of teixobactin analogues **1-10** was assessed against MRSA ATCC 33591. The Leu_{10}-teixobactin and natural teixobactin were included as benchmarks for activity. The six analogues **1**-**3**, **8**-**10** with two cationic charges have hydrophobic-hydrophilic balances similar to natural teixobactin (two cationic charges). These analogues showed comparable potency (MIC 0.125 - 0.25μg/ ml) to natural teixobactin (MIC 0.25μg/ ml, Table 1). The three analogues **4-6** each possess three cationic charges. Interestingly, analogue **4** showed comparable antimicrobial activity (MIC 0.25μg/ ml) to natural teixobactin. However, analogues **5** and **6** showed 4 times reduced antibacterial activity (MIC 1μg/ ml) than natural

teixobactin or Leu$_{10}$-teixobactin. The analogue **7** with four cationic charges also showed reduced antibacterial activity (MIC 1µg/ ml).

The teixobactin analogues **1-10** were further assessed against a panel of antibiotic-resistant and antibiotic susceptible Gram-positive pathogens and comparator antibiotics, daptomycin (Figure 3). The MIC results indicate that the synthetic analogues are potent against the various strains tested, but their MIC distribution differs significantly. Interestingly, we observed a wider distribution of MIC values as the overall net charge of the peptide was increased (Table 1 and 2).

Notably, the MIC values for *Staphylococcus* were not altered whereas a significant increase in *Enterococcus* was observed with four cationic charges (**7**, MIC 2-8µg/ ml). Similar trends have been reported for teixobactin analogues, whereby increases in positive charges give increases in MICs against *Staphylococcus* aureus ATCC 29213.[13] Herein, for example, Lys$_3$-D-Lys$_4$-Lys$_{10}$-teixobactin (four cationic charges, Figure1E) has a reported MIC of 8µg/ ml against *Staphylococcus* aureus ATCC 29213;[13] whereas, we observed an MIC of 1µg/ ml (8 times improvement) for Arg$_3$-D-Arg$_4$-Arg$_9$-Leu$_{10}$-teixobactin **7** (four cationic charges, Figure2) against the same bacterial strain.

The inclusion of 3 arginines in the above case likely perturbs the amphiphilic character of the teixobactin, resulting in a decrease in activity. The six analogues **1-3**, **8-10** with two cationic charges showed comparable antibacterial potency to Leu$_{10}$-teixobactin. Importantly, the

hydrophobic-hydrophilic balance of these analogues was similar to natural teixobactin (two cationic charges). The analogues **4**-**6** with three cationic charges also showed comparable antibacterial potency to Leu$_{10}$-teixobactin. All synthesized analogues showed good potency against a broad panel of bacteria. The nine analogues **1**-**6**, and **8**-**10** showed drug like profiles such as high antibacterial potency with optimal balance of hydrophobicity and hydrophilicity. We have further determined the minimum bactericidal concentrations (MBC) of teixobactin analogues against *S. aureus*/MRSA strains (Table S3). Compound **2** displayed highly potent bactericidal properties, as its MBC values did not increase above 4 times the MIC against the tested strains. Compound **2** was found inactive against *Pseudomonas aeruginosa* (Gram negative bacteria, table S2). In view of narrow MIC distribution values and bactericidal properties, we focused our attention on compound **2** and further investigated its biological properties.
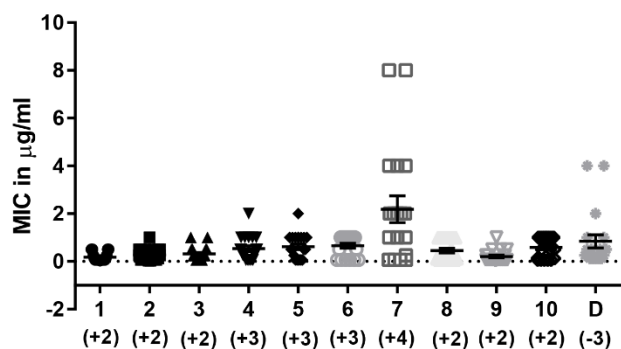
Figure 3 MIC distribution of various analogues of teixobactin (**1**-**10**) against 19 different Gram-positive pathogens (Table S2). The teixobactin analogues, daptomycin (labelled as **D**) was used as the comparator drug. Note the increase in MIC distribution as the overall net charge on the teixobactin analogues was increased. The number in parenthesis indicates the overall net charge of the peptides.

Table 2. MIC values of compounds **1**-**10** against a broad panel of bacteria. *Enterococcus faecalis*, VRE 1001-

1002, 1004, 1008 are clinical isolates. MRSA 42412, MRSA 21455 and MRSA 1003 are clinical isolates.

	Compound ➜ No.	1	2	3	4	5	6	7	8	9	10	11
	Strain ⬇											
1.	*Staphylococcus saprophyticus* ATCC BAA 750	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	-
2.	*Staphylococcus saprophyticus* ATCC 15305	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.25	<0.0625	<0.0625	<0.0625	-
3.	*Staphylococcus saprophyticus* ATCC 49453	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	-
4.	*Staphylococcus saprophyticus* ATCC 49907	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	-
5.	VRE 1001	0.25	0.5	0.5	1	0.5	1	2	1	0.5	1	-
6.	VRE 1002	0.5	1	1	1	1	1	8	1	1	1	-
7.	VRE 1004	<0.0625	0.25	0.25	0.5	0.5	1	4	1	0.5	1	-
8.	VRE 1008	0.125	0.5	0.25	0.5	0.5	1	8	1	0.5	1	-
9.	VRE ATCC 700802	0.5	0.5	0.5	2	1	1	4	1	0.25	1	0.25
10.	VRE ATCC 29212	0.5	0.5	1	1	1	1	4	1	0.25	1	0.25
11.	MRSA ATCC 700699	0.5	0.25	0.5	0.5	1	1	2	1	0.25	1	0.25
12.	MRSA 42412	<0.0625	0.0313	<0.0625	0.25	0.25	1	2	0.125	<0.0625	0.125	<0.0625
13.	MRSA 21455	0.03125	0.0313	0.25	0.5	1	1	2	0.25	0.03125	0.5	<0.0625
14.	MRSA 1003	<0.0625	0.5	0.25	1	2	0.5	2	0.125	<0.0625	0.5	-
15.	SA29213	0.25	<0.0625	0.5	0.25	1	1	1	0.5	0.0625	1	-
16.	SA4299	0.125	-	0.25	0.25	0.5	0.5	1	0.125	<0.0625	1	-
17.	SE12228	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	-
18.	*Bacillus Cereus* ATCC 11788	<0.0625	0.5	0.25	1	1	1	1	0.125	<0.0625	0.5	-
19.	*Bacillus Subtilis* ATCC 6633	<0.0625	0.125	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.125	-

Resistance studies and time dependent killing of bacteria using teixobactin analogue 2:

D-Arg$_4$-Leu$_{10}$-teixobactin (**2**) was evaluated for single step resistance in *S. aureus* ATCC 29213 and MRSA ATCC 33591. We were unable to obtain mutants of *S. aureus* ATCC 29213 or MRSA ATCC 33591 resistant to teixobactin analogue **2** (5x, 10x, 20x MIC). The calculated frequency of resistance to teixobactin analogue **2** was found to be <10^{-10} (SI page S17) which is comparable to teixobactin.[2] A lack of resistance in preliminary studies against **2** is promising in the development of drug like molecules against resistant bacteria.

Time-kill kinetics studies of D-Arg$_4$-Leu$_{10}$-teixobactin **2** against *S. aureus* ATCC 29213 was investigated to ascertain if the chemical modifications retained the bactericidal properties. The exposure of bacterial inoculum to 0.5 µg/ml or 1 µg/ml of compound **2** resulted in $\geq$ 2 log10 decrease in bacterial viability at 8 h (Figure S24), which is comparable with previous reports of teixobactin analogues and teixobactin. [2, 14]

In vitro **cytotoxicity studies:**

It was important to evaluate the cytotoxicity of compound **2** on mammalian cells prior to *in vivo* studies. We determined the cytotoxicity of **2** in human lung epithelial cell line A549 and primary dermal fibroblasts (hDFs). Both of these cell culture models are already established for evaluation of cytotoxicity of antimicrobial peptides.[17-18] An MTS assay indicated that both mammalian cell-types exposed to various concentrations of the peptide retained significant metabolic activity ($\geq$ 80% viability, Figure 4 a,b), even at a concentration that was ~900 times (250 µg/ml) higher than the average MIC (0.27 µg/ml) values, indicating excellent cell

selectivity of the teixobactin analogues. High content images indicated the absence of any cytoskeletal and nuclear disruption upon exposure of both epithelial and fibroblasts cells to compound **2** (Figure 4 c,d), establishing its non-cytotoxic properties. The morphology of mammalian cells exposed to **2** appeared similar to the untreated cells. However, exposure of cells to an antineoplastic agent (nocodazole, used as a control) resulted in substantial loss of adhered cells, confirming its cytotoxicity.
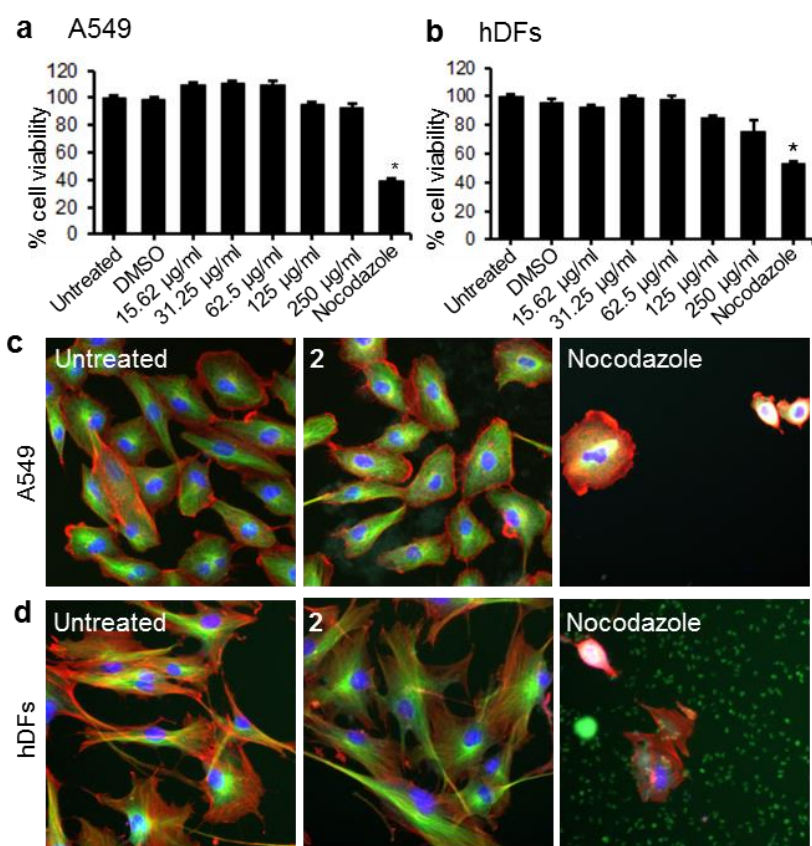

Figure 4. Cytotoxicity evaluation of **2** in A549 lung epithelial cell line and human primary dermal fibroblasts (hDFs). Both A549 cells (a) and hDFs (b) were treated with increasing concentrations of 2 (ranging from 15.62 μg/ml to 250 μg/ml) for 24 h. The stock solution of **2** (500 μg/ml) was prepared fresh by directly dissolving **2** in cell culture medium and used. Cells were treated with dimethyl sulfoxide (DMSO, 0.1% *v/v*)

or nocodazole (5 µg/ml dissolved in DMSO) as controls. At the end of the treatment period, metabolic

activities of cells were quantified by MTS-based cell viability assay. Data represents mean ± SEM of three

independent triplicate experiments, *p>0.05. After 24 h treatment with **2**, A549 cells (c) and hDFs (d) were

fixed, fluorescently stained with rhodamine-phalloidin (red), alexa fluor 488 conjugated anti-α-tubulin

(green) and Hoechst 33342 (blue) and imaged using IN Cell Analyzer 2200 automated microscope.

Representative images of cells treated with 2 (62.5 µg/ml for 24 h) or nocodazole (10 µg/ml, toxicity control)

are shown.

In vivo toxicity studies:

We examined the *in vivo* toxicity of **2** in a rabbit corneal damage model. A 50 µl of 0.3%

(w/v) solution was applied topically (4 times/day) to the circularly debrided cornea and re-

epithelialization was monitored by fluorescein staining. Vehicle alone served as control.

Figure 5 shows the decrease in fluorescein staining with time for both control wounds and

wounds treated with **2**. There was no significant difference in wound closure between PBS-

treated wounds or wounds treated with **2** (Figure S25). The lack of any delay in the re-

epithelialization and wound closure for the injured cornea treated with **2** suggests good

biocompatibility of the peptide.
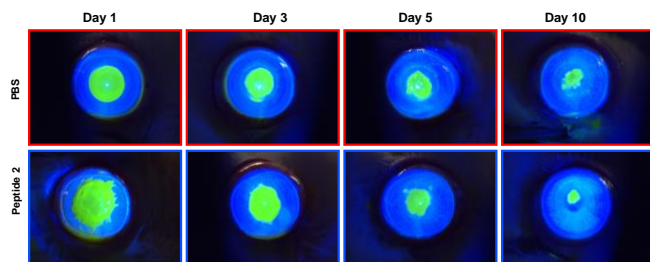
Figure 5. Representative slit lamp fluorescence images showing the time-dependent changes in wound closure of the cornea after application of PBS (2 eyes) or 0.3% peptide **2** (4 eyes). The wounded cornea was stained fluorescein to observe epithelial defects and imaged by slit lamp biomicroscopy.

## *In vivo* antibacterial efficacy of D-Arg$_4$-Leu$_{10}$-teixobactin in bacterial keratitis model:

We examined the *in vivo* efficacy of peptide **2** in the mice-eye model of *S.aureus* keratitis. *S. aureus* is one of the major etiological agents for bacterial keratitis and the toxic secretions produced by this microorganism have been implicated in corneal melt, leading to significant morbidity and vision loss.[19-20] Scarified cornea of the mice were infected with *S. aureus* ATCC 29213 inoculum (15 µl of 6×10^6 CFU/ml). At 6 h post infections (p.i.), the infected cornea were treated with vehicle (PBS), peptide **2** (0.3% w/v in PBS) and moxifloxacin (0.3%). A total of 8 doses were applied and the progression of the infection was monitored by slit lamp examination, anterior segment optical coherent tomography (AS-OCT) and microbiological enumeration of the bacterial bioburden. Mice cornea treated with PBS had severe clinical presentation indicated by chemosis, significant presence of hypopyon like materials and corneal infiltrates (Figure 6).
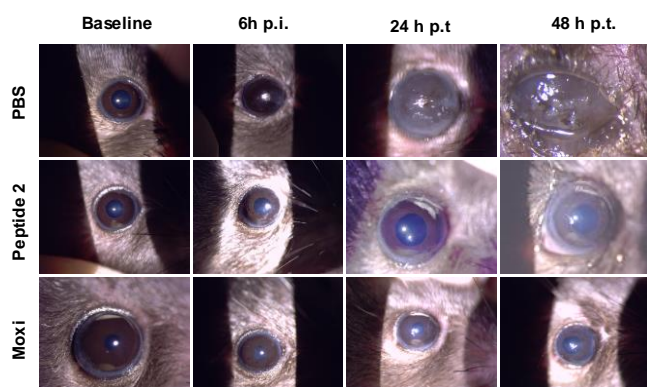
Figure 6 Slit lamp examination of mice infected with *S. aureus* ATCC 29213 strains. After scratching the corneal epithelium with scalpel blade, the scarified cornea was infected with a bacterial inoculum of 6 x 10^6 CFU/ml (15 µl/cornea). At 6 h post infections, the infected cornea were treated with 15 µl of PBS, peptide **2** (0.3% w/v in PBS) and moxifloxacin (0.3% w/v in PBS). Note the significant presence of corneal haze and mucopurulent discharge in PBS treated cornea whereas peptide **2** or moxifloxacin treated cornea remained clear and no signs of corneal defects.

Notably, infected cornea treated with peptide **2** or a fluoroquinalone antibiotic, had similar clinical appearance presentation, as indicated by lack of any conjunctival chemosis and corneal infiltrates. These results indicate that peptide **2** halted the progression of *S. aureus* infections and the activity was comparable to moxifloxacin. To determine the effect of treatments on tissue severity, we determined the corneal thickness from various groups (Figure 7a, Figure S26). The baseline corneal thickness of mice (93.8±2.9 µm) decreased moderately (79.0±3.4 µm) after de-epithelialization followed by *S. aureus* infection (6h p.i.). Treatment of the infected cornea with vehicle alone (PBS) resulted in substantial increase in corneal thickness after 24 h (151.7±12.7 µm) and 48 h (186.2±17.5 µm), indicating corneal edema after infection. Infected cornea treated with peptide **2** had a mean corneal thickness of

92.3±12.5 µm and 121.7±3.2 µm after 24 h and 48 h post treatment (p.t.), respectively. For

the moxifloxacin-treated cornea the mean corneal thickness was 124.2±9.4 µm after 24 h p.t.

and 140.3±10.3 µm after 48 h p.t. These results suggested that peptide **2** treatment resulted in

significant decrease in corneal edema after *S. aureus* infections when compared PBS treated

or moxifloxacin-treated groups.
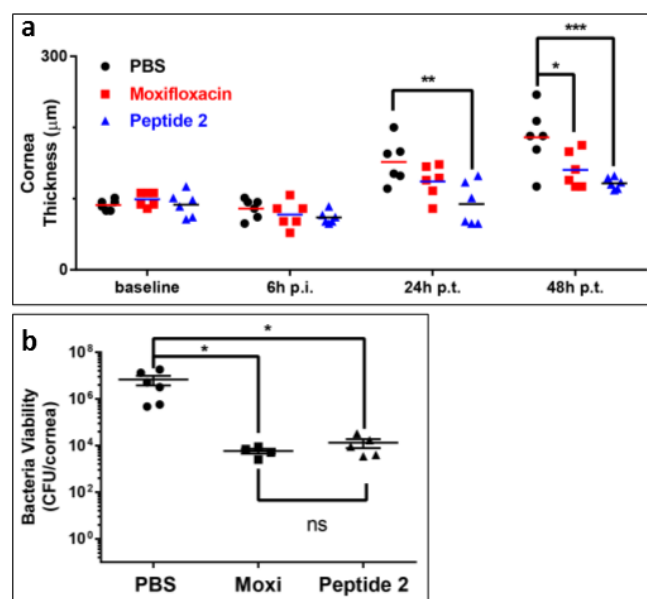
Figure 7. a) Changes in corneal thickness (CT) of mice before and after infections and treatment with various groups. Note that the CT values for peptide **2** treated cornea approached the baseline values after 48 h p.t., which was absent in the case of PBS-/Moxifloxacin-treated corneas. Note that a significant decrease in corneal edema was observed for infected cornea treated with peptide **2** compared to untreated cornea (p, 0.01 two-way ANOVA) as early after 3 doses which decreased further after 8 doses (p, 0.001). The results indicated a marked decrease in the severity (due to infections) after treatment with **2** when compared to standard antibiotic treatment. b) Bacterial bioburden in the infected corneal after 48 h treatment with various groups. Values represent colony counts from individual cornea and bars represent mean CFU/tissue ± standard errors of the mean.

Bacterial enumeration of the corneal tissues harvested after 8 dosages confirmed the *in vivo* efficacy of peptide **2** (Figure 7b). All the infected cornea that received PBS treatment contained significant presence of bacteria, varying from $4.7 \times 10^5 - 1.3 \times 10^7$ CFU/tissue. The mean $\log_{10}$ CFU/tissue $\pm$ standard error of the mean for PBS treated cornea was 6.51 ± 0.27. Five out of six cornea treated with peptide **2** had detectable bacterial colonies. The mean $\log_{10}$ CFU/tissue for peptide **2** treated cornea was 3.97 ± 0.19. Four infected corneas treated with moxifloxacin contained detectable bacterial colonies with a mean $\log_{10}$ CFU/tissue of 3.7 ± 0.24 was observed. These results confirmed that peptide **2** had a similar antibacterial effect as an established antibiotic in decreasing the bacterial bioburden, thus demonstrating its potential as a safe therapeutic for topical applications.

Conclusion

In conclusion, we have designed and synthesized 10 novel analogues of teixobactin through the selective replacement of Ser_3, $D\text{-}Gln_4$ and Ala_9 residues by D/L arginines in Leu_{10}-teixobactin and Ile_{10}-teixobacin. We have successfully achieved a fine balance of hyrophobicity and hydrophilicity while maintaining high antibacterial potency both *in vitro* and *in vivo*. Importantly, most of these teixobactin analogues showed highly potent antibacterial activity against *S. aureus*, MRSA, and VRE comparable to Leu_{10}-teixobactin and Ile_{10}-teixobactin. The MIC values on a broad panel of Gram-positive bacteria indicate a

direct correlation between overall net charge and a narrow distribution of MIC values; for example, a wider distribution of MIC results as the overall net charge of the peptide increases.

The teixobactin-based peptide analogue **2** was found non-cytotoxic both *in vitro* and *in vivo*. In a mice model of infectious keratitis, the topical instillation of **2** resulted in >99.0% reduction in bacterial bioburden and the efficacy was comparable to moxifloxacin. Notably, *S. aureus* is one of the major etiological agents for bacterial keratitis and has been implicated in corneal melt, leading to significant morbidity and vision loss[15,16]. Furthermore in our keratitis mice models, the synthetic teixobactin-analogue **2** decreased corneal edema (severity) significantly when compared to untreated cornea or moxifloxacin treated cornea. To the best of our knowledge, this work is the first *in vivo* demonstration of the excellent therapeutic potential of a teixobactin analogue in attenuating bacterial infections and associated severity. We believe this work represents a significant advancement in the development of *in vivo* ready simplified teixobactin analogues. Thus, the design of safe and highly potent synthetic peptide analogues of teixobactin presented here will enable the development of new drug like analogues against antibiotic-resistant bacterial strains. The findings presented in this work have broad implications and are expected to facilitate the development of peptide based therapies to combat the serious global challenges posed by AMR.

Experimental

Synthesis of D-Arg$_4$-Leu$_{10}$-teixobactin (2)

D-Arg$_4$-Leu$_{10}$-teixobactin (**2**) was synthesised as described in **scheme 1** using our previously

reported procedure.[14] (step a) Commercially available 2-chlorotrityl chloride resin

(manufacturer's loading = 1.2 mmol/g, 170 mg resin) was swelled in DCM in a reactor. To

this resin was added 4 eq. Fmoc-Ala-OH/8 eq. DIPEA in DCM and the reactor was shaken

for 3h. The loading determined by UV absorption of the piperidine-dibenzofulvene adduct

was calculated to be 0.6 mmol/g, (170mg resin, 0.102 mmol). Any unreacted resin was capped

with MeOH:DIPEA:DCM = 1:2:7 by shaking for 1h. (step b) The Fmoc protecting group was

deprotected using 20% piperdine in DMF by shaking for 3 min, followed by draining and

shaking again with 20% piperidine in DMF for 10 min. AllocHN-D-Thr-OH was then coupled

to the resin by adding 3 eq. of the AA, 3 eq. HATU and 6 eq. DIPEA in DMF and shaking

for 1.5h at room temperature. (step c) Esterification was performed using 10 eq. of Fmoc-Ile-

OH, 10 eq. DIC and 5 mol% DMAP in DCM and shaking the reaction for 2h. This was

followed by capping the unreacted alcohol using 10% Ac$_2$O/DIPEA in DMF shaking for 30

min and Fmoc was removed using protocol described earlier in step (b). (step d) Fmoc-Leu-

OH was coupled using 4 eq. of AA, 4 eq. HATU and 8 eq. DIPEA in DMF and shaking for

1h followed by Fmoc deprotection using 20% piperidine in DMF as described earlier. (step

e) The N terminus of Leu was protected using 10 eq. Trt-Cl and 15% Et$_3$N in DCM and

shaking for 1h. The protection was verified by the Ninhydrin colour test. (step f) The Alloc

protecting group of D-Thr was removed using 0.2 eq. [Pd(PPh^3)]^0 and 24 eq. PhSiH$_3$ in dry

DCM under argon for 20 min. This procedure was repeated again increasing the time to 45 min and the resin was washed thoroughly with DCM and DMF to remove any Pd stuck to the resin. (step g) All amino acids were coupled using 4 eq. Amino Acid, 4 eq. DIC/Oxyma using a microwave peptide synthesizer. Coupling time was 10 min. Deprotection cycles were performed as described earlier. (step h) The peptide was cleaved from the resin without cleaving off the protecting groups of the amino acid side chains using TFA:TIS:DCM = 2:5:93 and shaking for 1h. (step i) The solvent was evaporated and the peptide was redissolved in DMF to which 1 eq. HATU and 10 eq. DIPEA were added and the reaction was stirred for 30 min to perform the cyclization. (step j) The side-chain protecting groups were then cleaved off using TFA:TIS:H_2O = 95:2.5:2.5 by stirring for 1h. The peptide was precipitated using cold Et_2O (-20°C) and centrifuging at 7000 rpm to obtain a white solid. This solid was further purified by RP-HPLC using the protocols described in supporting information SII.

All teixobactin analogues were synthesised by using the method described above. The overall yields after HPLC purifications were typically in the range of 13-22%. All teixobactin analogues **1**-**10** were characterized by HRMS (ESI) in positive mode (see table 3, SII and figures S1-S20). Analogue **2** was also characterised by NMR (S IV, table S1, figures S22-23.). The homogeneity of HPLC purified fractions were analyzed by mass spectroscopy. All the teixobactin analogues used were purified to >95% purity as indicated by HPLC.

Table 3: Compound number, name, chemical formula, mass calculated and mass observed for teixobactin analogues **1-10.**

Compound	Name	Chemical formula	Mass Calcd (Da)	Mass obsd (Da)
1	Arg$_3$-Leu$_{10}$-teixobactin	C$_{61}$H$_{104}$N$_{15}$O$_{14}$	1270.7887	1270.7913
2	D-Arg$_4$-Leu$_{10}$-teixobactin	C$_{59}$H$_{101}$N$_{14}$O$_{14}$	1229.7622	1229.7650
3	Arg$_9$-Leu$_{10}$-teixobactin	C$_{61}$H$_{104}$N$_{15}$O$_{15}$	1286.7836	1286.7843
4	Arg$_3$-D-Arg$_4$-Leu$_{10}$-teixobactin	C$_{62}$H$_{108}$N$_{17}$O$_{13}$	1298.8313	1298.8325
5	Arg$_3$-Arg$_9$-Leu$_{10}$-teixobactin	C$_{64}$H$_{111}$N$_{18}$O$_{14}$	1355.8527	1355.8606
6	D-Arg$_4$-Arg$_9$-Leu$_{10}$-teixobactin	C$_{62}$H$_{108}$N$_{17}$O$_{14}$	1314.8262	1314.8263
7	Arg$_3$-D-Arg$_4$-Arg$_9$-Leu$_{10}$-teixobactin	C$_{65}$H$_{115}$N$_{20}$O$_{13}$	1383.8952	1383.8943
8	Arg$_3$-Ile$_{10}$-teixobactin	C$_{61}$H$_{104}$N$_{15}$O$_{14}$	1270.7887	1270.7896
9	D-Arg$_4$-Ile$_{10}$-teixobactin	C$_{59}$H$_{101}$N$_{14}$O$_{14}$	1229.7622	1229.7607
10	Arg$_9$-Ile$_{10}$-teixobactin	C$_{61}$H$_{104}$N$_{15}$O$_{15}$	1286.7836	1286.7780

Supporting information

Supplementary Information (ESI) available: Peptides HPLC, LC-MS analysis, NMR analysis, *in vitro* antibacterial assay (MIC, MBC, time kill kninetics), *in vitro* cytotoxicity assay, *in vivo* cytotoxicity assay and *in vivo* antibacterial efficacy.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

Corresponding author isingh@lincoln.ac.uk

† These authors have contributed equally to this work. The project was designed by Ishwar Singh with the help of all authors. The manuscript was written by Abhishek Iyer, Anish

Abbreviations used

AA, Amino acid; Ac_2O, Acetic anhydride; Alloc, Allyloxycarbonyl; AMR, Antimicrobial Resistance; ATCC, American Type Cell Culture; Boc, tert-butyloxycarbonyl; CFU, Colony-forming Unit; DCM, Dichloromethane; DIC, Diisopropylcarbodiimide; DIPEA/DIEA, diisopropylethylamine; DMAP, 4-Dimethylaminopyridine; DMF, *N,N*-Dimethylformamide; Et_3N, Triethylamine; ESI, Electrospray Ionization; HATU, N-[(dimethylamino-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; hDFs, Human Dermal Fibroblasts; HRMS, High Resolution Mass Spectroscopy, HPLC , High-performance Liquid Chromatography; MeOH, Methanol; MIC, Minimum Inhibitory Concentration; MRSA, Methicillin-resistant *Staphylococcus aureus*; NMR, Nuclear Magnetic Resonance; *P. aeruginosa, Pseudomonas aeruginosa*; PBS, Phosphate Buffered Saline; $[Pd(PPh_3)_4]^0$, Tetrakis(triphenylphosphine)palladium(0); PG, Protecting Group; $PhSiH_3$, Phenylsilane; *S.*

aureus (SA), *Staphylococcus aureus*; TFA, Trifluoroacetic acid; TIS, Triisopropylsilane; Trt,

Trityl; VRE, Vancomycin-resistant Enterococci

References: (1) *AMR Review*, http://amr-review.org, accessed Dec 11 2017.

(2) Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.;

Mueller, A.; Schaberle, T. F.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.;

Steadman, V. A.; Cohen, D. R.; Felix, C. R.; Fetterman, K. A.; Millett, W. P.; Nitti, A. G.;

Zullo, A. M.; Chen, C.; Lewis, K. A new antibiotic kills pathogens without detectable

resistance. *Nature* **2015,** *517*, 455-459.

(3) Giltrap, A. M.; Dowman, L. J.; Nagalingam, G.; Ochoa, J. L.; Linington, R. G.; Britton,

W. J.; Payne, R. J. Total synthesis of teixobactin. *Org. Lett.* **2016,** *18*, 2788-2791.

(4) Jin, K.; Sam, I. H.; Po, K. H. L.; Lin, D. a.; Ghazvini Zadeh, E. H.; Chen, S.; Yuan, Y.;

Li, X. Total synthesis of teixobactin. *Nature Communications* **2016,** *7*, 12394.

(5) Jad, Y. E.; Acosta, G. A.; Naicker, T.; Ramtahal, M.; El-Faham, A.; Govender, T.;

Kruger, H. G.; Torre, B. G. d. l.; Albericio, F. Synthesis and biological evaluation of a

teixobactin Analogue. *Org. Lett.* **2015,** *17*, 6182-6185.

(6) Parmar, A.; Iyer, A.; Vincent, C. S.; Van Lysebetten, D.; Prior, S. H.; Madder, A.;

Taylor, E. J.; Singh, I. Efficient total syntheses and biological activities of two teixobactin

analogues. *Chem. Commun.* **2016,** *52*, 6060-6063.

(7) Yang, H.; Chen, K. H.; Nowick, J. S. Elucidation of the teixobactin pharmacophore.

ACS Chemical Biology **2016,** *11*, 1823-1826.

(8) Abdel Monaim, S. A. H.; Jad, Y. E.; Ramchuran, E. J.; El-Faham, A.; Govender, T.;

Kruger, H. G.; de la Torre, B. G.; Albericio, F. Lysine scanning of Arg10–teixobactin:

deciphering the role of hydrophobic and hydrophilic residues. *ACS Omega* **2016,** *1*, 1262-

1265.

 (9) Parmar, A.; Prior, S. H.; Iyer, A.; Vincent, C. S.; Van Lysebetten, D.; Breukink, E.;

Madder, A.; Taylor, E. J.; Singh, I. Defining the molecular structure of teixobactin

analogues and understanding their role in antibacterial activities. *Chem. Commun.* **2017,** *53*,

2016-2019.

 (10) Wu, C.; Pan, Z.; Yao, G.; Wang, W.; Fang, L.; Su, W. Synthesis and structure-activity

relationship studies of teixobactin analogues. *RSC Advances* **2017,** *7*, 1923-1926.

(11) Parmar, A.; Iyer, A.; Lloyd, D. G.; Vincent, C.; Prior, S. H.; Madder, A.; Taylor, E. J.;

Singh, I. "Syntheses of potent teixobactin analogues against methicillin-resistant

Staphylococcus aureus (MRSA) through the replacement of L-allo-enduracididine with its

isosteres". *Chem. Commun.* **2017,** *53*, 7788–7791.

(12) Schumacher, C. E.; Harris, P. W. R.; Ding, X.-B.; Krause, B.; Wright, T. H.; Cook, G.

M.; Furkert, D. P.; Brimble, M. A. Synthesis and biological evaluation of novel teixobactin

analogues. *Organic & Biomolecular Chemistry* **2017,** *15*, 8755-8760.

(13) Abdel Monaim, S. A. H.; Ramchuran, E. J.; El-Faham, A.; Albericio, F.; de la Torre, B.

G. Converting teixobactin into a cationic antimicrobial peptide (AMP). *J. Med. Chem.* **2017,**

60, 7476-7482.

(14) Parmar, A.; Iyer, A.; Prior, S. H.; Lloyd, D. G.; Leng Goh, E. T.; Vincent, C. S.;

Palmai-Pallag, T.; Bachrati, C. Z.; Breukink, E.; Madder, A.; Lakshminarayanan, R.;

Taylor, E. J.; Singh, I. Teixobactin analogues reveal enduracididine to be non-essential for

highly potent antibacterial activity and lipid II binding. *Chemical Science* **2017,** *8*, 8183-

8192.

(15) Fiers, W. D.; Craighead, M.; Singh, I. Teixobactin and its analogues: a new hope in

antibiotic discovery. *ACS Infectious Diseases* **2017,** *3*, 688–690.

(16) Hughes, D.; Karlén, A. Discovery and preclinical development of new antibiotics.

Upsala Journal of Medical Sciences **2014,** *119*, 162-169.

(17) Le, C.-F.; Yusof, M. Y. M.; Hassan, H.; Sekaran, S. D. In vitro properties of designed

antimicrobial peptides that exhibit potent antipneumococcal activity and produces

synergism in combination with penicillin. *Scientific Reports* **2015,** *5*, 9761.

(18) Wu, X.; Wang, Z.; Li, X.; Fan, Y.; He, G.; Wan, Y.; Yu, C.; Tang, J.; Li, M.; Zhang,

X.; Zhang, H.; Xiang, R.; Pan, Y.; Liu, Y.; Lu, L.; Yang, L. In vitro and in vivo activities of

antimicrobial peptides developed using an amino acid-based activity prediction method.

Antimicrob. Agents Chemother. **2014,** *58*, 5342-5349.

(19) Mah, F. S.; Davidson, R.; Holland, E. J.; Hovanesian, J.; John, T.; Kanellopoulos, J.;

Shamie, N.; Starr, C.; Vroman, D.; Kim, T. Current knowledge about and recommendations

for ocular methicillin-resistant Staphylococcus aureus. *Journal of Cataract & Refractive*

Surgery **2014,** *40*, 1894-1908.

(20) Henry, C. R.; Flynn, H. W., Jr.; Miller, D.; Forster, R. K.; Alfonso, E. C. Infectious

keratitis progressing to endophthalmitis. *Ophthalmology* **2012,** *119*, 2443-2449.

Table of contents graphic

Peptide 2 decreased bacterial burden and corneal edema in *S. aureus* infected mice cornea