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Alanine Scan Reveals Modifiable Residues in Teixobactin

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Abstract

An alanine scan of Lys_{10}-teixobactin reveals that a cationic residue at position 10 is not necessary for antibiotic activity and that position 3 tolerates substitution without loss of activity. An unexpected correlation between poor aqueous solubility and better antibiotic activity of the teixobactin analogues is observed.

The report of teixobactin in 2015 sparked new interest in the discovery and development of new antibiotics.[1] This interest reflects the novel mode of discovery of teixobactin, its mechanism of action, and its therapeutic promise. The potential for teixobactin to impact medicine has lead to a race to elucidate the teixobactin pharmacophore through structure-activity-relationship (SAR) studies consisting of chemical synthesis of analogues in conjunction with minimum inhibitory concentration (MIC) assays.[2–9]

Teixobactin is a nonribosomal undecapeptide. It is composed of four D-amino acids and seven L-amino acids, including the nonproteinogenic amino acid *allo*-enduracididine at position 10 (Fig. 1).[1] Teixobactin may be thought of as having two sections: an N-terminal tail comprising residues 1–7, and a 13-membered macrolactone ring comprising residues 8–11. Residues from both sections have been modified to modulate the activity of teixobactin.[2–9]

Both the *N*-terminal tail and the 13-membered macrolactone ring are important in the activity of teixobactin.[8,9] The *N*-terminal tail is involved in binding to the bacterial lipid bilayer membrane, while the macrolactone ring is thought to bind to the pyrophosphate group of lipid II or its precursors and inhibit bacterial wall biosynthesis. X-ray crystallography reveals that the macrolactone ring adopts a conformation in which the amide NH groups align in a fashion that can bind the pyrophosphate group.[9] SAR studies have revealed that hydrophobic isoleucine residue at position 11 is sensitive to substitution, while the alanine residue at position 9 can tolerate substitution.[9]

A number of research groups, including our own, have demonstrated that other positively charged amino acids can be substituted for *allo*-enduracididine at position 10, with only moderate reduction in activity.[3,4,8,10,11,12] Arginine has been the most popular substitution, while lysine has proven somewhat more active. Both our research group and that of Singh *et al.* have independently demonstrated the necessity of D-stereochemistry at position 8.[7,8]

Structure-activity-relationship studies of the N-terminal tail have involved inversion of stereochemistry of the D-amino acids,[3,5,7,8] modification of *N*-methyl-D-Phe at position 1,[2,5,11,12] and modification of other positions.[2,8,9,11,12] Singh *et al.* and Albericio *et al.* both demonstrated that incorporating L-stereochemistry at positions 1, 4, and 5 yield an inactive Arg_{10}-teixobactin analogue.[3,5] Singh *et al.* later broadened the stereochemical analysis to individual D-residues.[7] Our research group demonstrated that the enantiomer of Arg_{10}-teixobactin is equally active.[8] Albericio *et al.*, Su *et al.*, and Li *et al.* showed that modifications to the side chain of *N*-methyl-D-Phe and the methylated *N*-terminus yield inactive teixobactin analogues.[2,5,11]

Several Arg_{10}-teixobactin analogues with substitutions to positions 4, 5, 6, and 7 gave a broad range of activity.[2,9,11,12] In a more aggressive approach, our research group replaced residues 1 to 5 with a dodecanoyl group to yield an Arg_{10}-teixobactin analogue we termed lipobactin, with only a two- to fourfold loss in activity.[8]

Albericio *et al.* systematically studied SAR through a lysine scan of Arg_{10}-teixobactin, in which each residue except 1, 8, and 10 was replaced with lysine or D-lysine.[6] Replacement of the hydrophobic residues at positions 2, 5, 6, and 11 (Ile and D-*allo*-Ile) resulted in complete loss of activity. Replacement of residues 7 and 9 (Ser and Ala) with lysine resulted in reduced activity. Replacement of the hydrophilic residues at positions 3 and 4 (Ser and D-Gln) had a modest effect on activity. Introduction of lysine at position 3 doubled the minimum inhibitory concentration (MIC) against *Streptococcus aureus* and *Bacillus subtilis*, while introduction of lysine at position 4 doubled the MIC against *Streptococcus aureus* and halved the MIC against *Bacillus subtilis*.

Gellman *et al.* have suggested that a lysine scan is best accompanied by a traditional alanine scan.[13] An alanine scan is complimentary to a lysine scan, because it truncates existing side chains into small, structurally non-perturbing methyl groups, without introducing charge or other molecular interactions. Thus, an alanine scan is commonly used to evaluate the relative contribution of each substituted side chain group to activity.

In the current study, we set out to perform an alanine scan on Lys_{10}-teixobactin. We chose Lys_{10}-teixobactin, because we previously found that it has activity superior to Arg_{10}-teixobactin. To perform the alanine scan, we sequentially replaced each residue with alanine while maintaining its native stereochemistry: *N*-methyl-D-alanine for position 1, L-alanine for positions 2, 3, 6, 7, 10, and 11, and D-alanine for positions 4 and 5. Position 8 (D-Thr) was not modified, because it is essential to the 13-membered macrolactone ring. Position 9 was not modified, because it is already alanine.

We synthesized Lys_{10}-teixobactin and the alanine scan analogues by our previously published synthetic route.[8] In this route, an acyclic peptide is prepared by Fmoc-based solidphase peptide synthesis on resin, the precursor is cyclized in solution, and the resulting cyclic peptide is deprotected and purified by reverse-phase HPLC. We compared the antibiotic activities of the alanine scan analogues to Lys_{10}-teixobactin, by performing MIC assays as we have done previously, with five types of Gram-positive bacteria, and with the Gram-negative bacterium *Escherichia coli* as a negative control and teixobactin as a positive

control.[8] Each assay was performed in a 96-well plate with concentrations of 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/mL peptide, diluted from a stock solution of 10 mg/mL in sterilized DMSO.

Of the nine alanine scan analogues, one proved comparable in activity to Lys_{10}-teixobactin (0.5–2 μg/mL), three were somewhat less active, three were weakly active, and two were inactive (Table 1). The analogues generally proved more active against *Streptococcus salivarius* and less active against *Enterococcus durans*. Teixobactin and all of the Lys_{10}-teixobactin alanine scan analogues were inactive against Gram-negative *Escherichia coli*.

Replacement of Ser_3 with alanine (Ala_3,Lys_{10}-teixobactin) resulted in comparable activity to Lys_{10}-teixobactin. This result shows that the hydroxyl group of Ser_3 is not essential for activity and that this position can tolerate a non-polar residue. This analogue is the second reported teixobactin analogue with a residue other than serine at position 3. Both analogues (Ala_3,Lys_{10}-teixobactin and Lys_3,Arg_{10}-teixobactin) are active,[6] suggesting that this position can tolerate diverse amino acid substitutions.

Replacement of D-Gln_4, Lys_{10}, and Ile11 with D-alanine and alanine (D-Ala_4,Lys_{10}-teixobactin, Ala_{10}-teixobactin, and Lys_{10},Ala_{11}-teixobactin) resulted in moderate reduction in activity. The activity of D-Ala_4,Lys_{10}-teixobactin is significant, because it shows that the polar amide group of D-Gln_4 is not essential for activity. The activity of Ala_{10}-teixobactin is especially significant, because it shows that a positively charged side chain at position 10 is also not essential for activity. Combined with our previous finding that Ala_9 can be replaced with lysine without loss in activity,[9] these results show that all three positions on the 13-membered macrolactone ring can tolerate substitutions.

Replacement of Ile_2, D-*allo*-Ile_5, and Ser_7 with alanine and D-alanine (Ala_2,Lys_{10}-teixobactin, D-Ala_5,Lys_{10}-teixobactin, and Ala_7,Lys_{10}-teixobactin) resulted in substantial reduction in activity. While the activities are significantly reduced, these alanine analogues still exhibit some activity. These results reflect that positions 2, 5, and 7 are sensitive to substitution, while leaving the door open to amino acid substitutions that are similar in chemical properties or size. Replacement of *N*-Me-D-Phe_1 and Ile_6 with *N*-methyl-D-alanine and alanine (*N*-Me-D-Ala_1,Lys_{10}-teixobactin and Ala_6,Lys_{10}-teixobactin) resulted in loss of activity (>32 μg/mL). The results show that positions 1 and 6 are highly sensitive to substitution.

Lys_{10}-teixobactin and Lys_{10}-teixobactin alanine scan analogues showed no significant cytotoxicity toward HepG2 cells in an LDH release assay, and no significant hemolysis of human red blood cells at 100, 50, and 25 μg/mL.§

In our previous experience handling teixobactin analogues, we observed poor aqueous solubility.[9] In synthesizing and purifying Lys_{10}-teixobactin and analogues, we routinely

§All Lys_{10}-teixobactin alanine scan analogues showed ≤ 2% cytotoxicity upon incubation with HepG2 for 24 hours and ≤ 2% hemolysis upon incubation with human red blood cells for 1 hour. Teixobactin exhibited no cytotoxicity at 100, 50, and 25 μg/mL. At 100 μg/mL, teixobactin exhibited 6% hemolysis. When the hemolysis assay is performed in the presence of 0.0005% Tween80 (polysorbate 80), instead of 1% DMSO, no significant hemolysis by teixobactin occurs at 100 μg/mL (personal communication with Dallas Hughes, Lucy Ling, and Kim Lewis).

have to dissolve the crude peptide product in 40% aqueous acetonitrile to solubilize and inject the peptide onto the preparative reverse-phase HPLC instrument. We qualitatively evaluated the aqueous solubility of the teixobactin analogues by adding the DMSO stock solutions to PBS buffer at pH 7.4 (Table 1). Three of the teixobactin analogues — Lys_{10}-teixobactin, Ala_3,Lys_{10}-teixobactin, and Ala_{10}-teixobactin — exhibited poor solubility in PBS buffer. These three analogues are also the most active, suggesting that there is a positive correlation between the poor aqueous solubility of teixobactin analogues and the antibiotic activity of the peptide. This poor solubility may impart enhanced binding of the peptide to the bacterial lipid bilayer membrane.‡

This alanine scan study reveals that position 3 of teixobactin tolerates modification without loss of antibiotic activity, and that positions 4, 10, and 11 can also tolerate modification. Positions 2, 5, and 7 only weakly tolerate modification, and positions 1 and 6 do not tolerate modification. Fig. 2 summarizes these findings. In conjunction with our previous result from modifying alanine at position 9,[9] these results show that all three positions of the 13-membered macrolactone ring can tolerate substitution. The observation that residue 10 of teixobactin (*allo*-enduracididine) can be replaced with alanine while retaining 1–8 μg/mL activity, runs contrary to popular belief and opens the door to developing many new teixobactin analogues.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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‡The poor solubility of Lys_{10}-teixobactin might explain why the MIC values we observe here (0.5–2 μg/mL) differ from those we have reported previously (0.25–1 μg/mL).[8]

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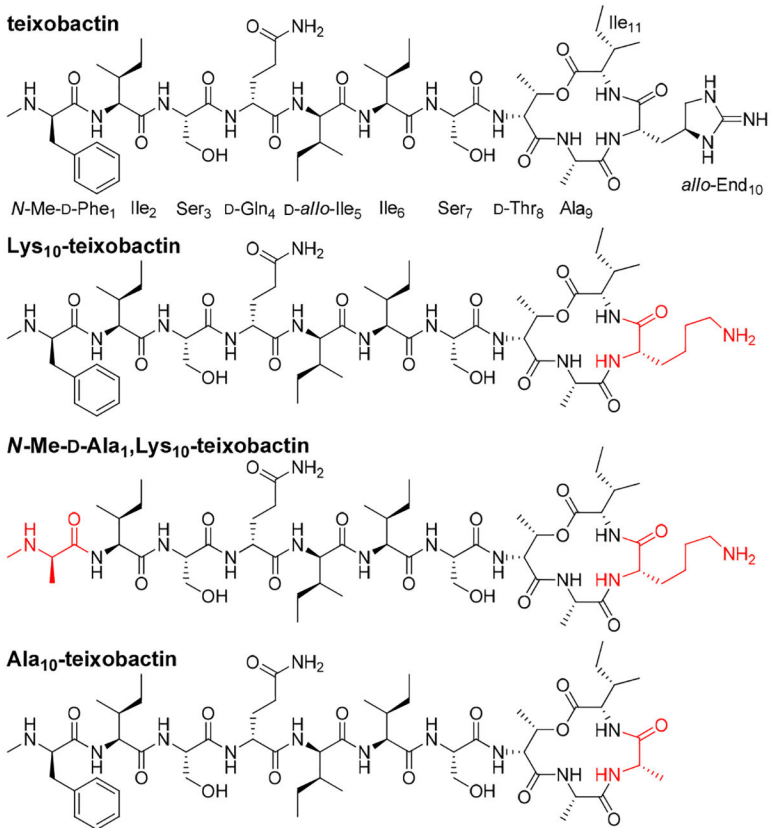

Fig. 1.
Teixobactin and analogues.

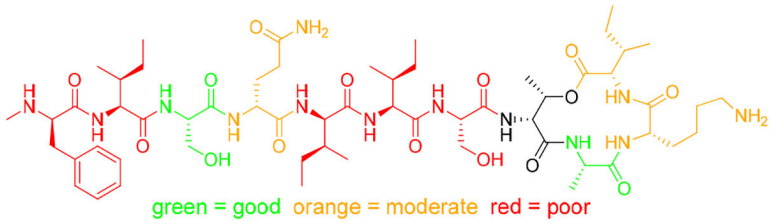

Fig. 2.

Tolerance of amino acids in Lys_{10}-teixobactin toward substitution.

Table 1

MIC values of teixobactin analogues in μg/mL.

	Staphylococcus aureus ATCC 29213	**Staphylococcus epidermidis ATCC 14990**	**Streptococcus salivarius ATCC 13419**	**Enterococcus durans ATCC 6056**	**Bacillus subtilis ATCC 6051**	**Escherichia coll ATCC 10798**	**antibiotic activity**	**solubility in PBS buffer[b]**
Lys_{10}-teixobactin[a]	0.5	0.5–1	0.5	2	1	>32	good	poor
N-Me-D-Ala_1,Lys_{10}-teixobactin[a]	>32	>32	>32	>32	>32	>32	poor	good
Ala_2,Lys_{10}-teixobactin[a]	>32	32	32	>32	32	>32	poor	good
Ala_3,Lys_{10}-teixobactin[a]	0.5	1	0.5	4	1	>32	good	poor
D-Ala_4,Lys_{10}-teixobactin[a]	1	4	2	16	4	>32	moderate	good
D-Ala_5,Lys_{10}-teixobactin[a]	>32	16	16	>32	16	>32	poor	good
Ala_6,Lys_{10}-teixobactin[a]	>32	>32	>32	>32	>32	>32	poor	good
Ala_7,Lys_{10}-teixobactin[a]	>32	32	16	>32	32	>32	poor	good
Ala_{10}-teixobactin[a]	1	4	1	8	2–4	>32	moderate	poor
Lys_{10},Ala_{11}-teixobactin[a]	4	8	2	>32	8	>32	moderate	good
teixobactin	0.125	0.06	0.03	0.5	0.06	>32	good	poor

[a]Trifluoroacetic acid (TFA) salt.

[b]Teixobactin analogues with poor solubility were observed to form a gelatinous mass upon addition of 1 μL of 10 mg/mL peptide stock solution in DMSO to 20 μL of PBS buffer at pH 7.4. A small amount of crystal violet was added to the PBS buffer to facilitate visualization of the gelatinous mass, which persisted even after stirring.