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CONCISE ARTICLE

Antimicrobial activity of Levofloxacin – M33 peptide conjugation or combination

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M33 is a branched peptide currently under preclinical characterization for development as a new drug against Gram-negative bacteria. Here we report its antibacterial activity in conjugation or combination with levofloxacin (LVFX), a fluoroquinolone antibiotic. Antibacterial assays showed no significant differences in activity when used in conjugation, while the combination of M33 and LVFX showed improved activity against Gram-negative bacteria. Combination treatment therefore opposes antimicrobial-resistance, restoring the effect of LVFX.

Introduction

Antimicrobial resistance (AMR) is an increasing problem as bacteria become more offensive.[1,2] It is therefore necessary to find new molecules with antimicrobial activity, new approaches and new applications of well-known compounds to fight infections. Several years ago, our research group identified, optimized and synthesized a tetra-branched peptide called M33[3,4,5] with good activity against Gram-negative bacteria: MICs against *P. aeruginosa* and *K. pneumoniae* are 1.5 μM.[6,7] In the present study, we investigated M33 activity after covalent conjugation and after combination with levofloxacin (LVFX), a broad-spectrum fluoroquinolone antibiotic. We chose LVFX for two reasons: first, Gram-negative bacterial pathogens have already developed resistance to fluoroquinolones (FQ);[8,9] second, LVFX only has one reactive moiety, a carboxylic group, that could be used orthogonally for solid-phase peptide synthesis (SPPS). Specifically, a lysine was linked to the C-terminus of the three-lysine-branched core, spaced with a beta-alanine, so that the side chain amine group could react with the LVFX carboxylic group. Unlike previous reports,[10,11] this system enables complete SPPS and the conjugate is obtained by HPLC purification. This synthesis approach is favourable because it avoids intermediate purifications that could reduce the final yield. Repeated washing of resins eliminates impurities and excess reagents, producing a crude lyophilized solid ready for HPLC purification. Antimicrobial assays against *P. aeruginosa* and *E. coli* were carried out with free M33, M33-LVFX conjugate, M33 combined with LVFX and free LVFX. The results showed that the activity of the conjugate was no better than that of M33, whereas the combination of the peptide with LVFX had a stronger effect than M33 and LVFX separately. These findings offer a good opportunity to fight AMR, restoring the activity of antibiotics currently challenged by AMR.

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Results and discussion

M33-LVFX conjugate synthesis

We designed a new approach to conjugating antibiotics with peptides using only the SPPS method. Specifically, we linked a well-known antibiotic, LVFX, to M33 with the purpose of improving or restoring its activity against Gram-negative bacteria.[12,13] LVFX structure is suitable for SPPS because it only has one carboxylic group as reactive moiety. LVFX was handled as an amino acid and added to the peptide in a further coupling step on the solid phase.
The synthesis of M33-LVFX included steps in which certain amino acids were added manually, i.e. the first amino acid, Fmoc-Lys(Dde)-OH, and the last one, Boc-Lys(Boc)-OH. The process then proceeded on the automatic synthesizer with Fmoc-βAla-OH as second step and two consecutive couplings of Fmoc-Lys(Fmoc)-OH to build the lysine core, followed by the nine sequential additions of Fmoc amino acids to complete the peptide KKIRVRLSA. At this point, the peptide, duly protected and still linked to the resin, was treated with hydrazine to append LVFX. **Figure 1** illustrates the final steps of conjugation. The crude material was worked up as a mixture of free peptides. M33-LVFX was characterized by analytical RP-HPLC and MALDI-TOF (**Figure 2 a,b**).

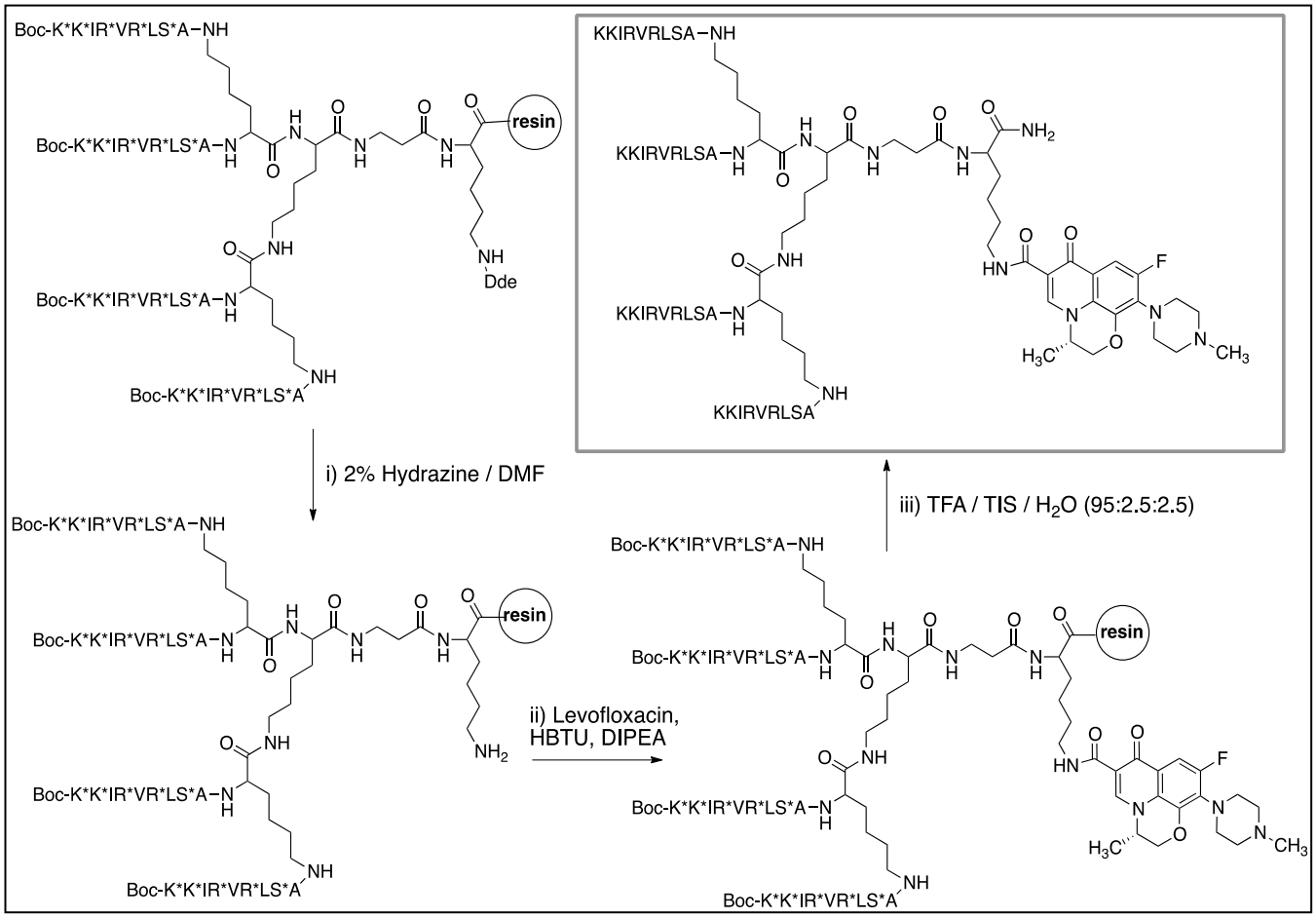

Figure 1 i) Deprotection of lysine-Dde by 2% hydrazine in DMF; 10 min at room temperature; ii) Conjugation of LVFX to M33 derivative; iii) Deprotection of side-chain groups and cleavage of conjugated peptide from resin. [(*) = side-chain protected amino acid]

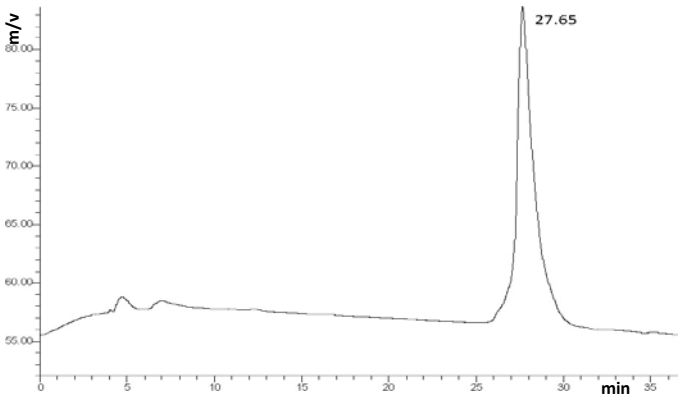

Figure 2 a. HPLC profile of M33-LVFX from 60:40 A/B to 45:55 A/B in 40 minutes (A: TFA 0.1% in water; B: MeOH).

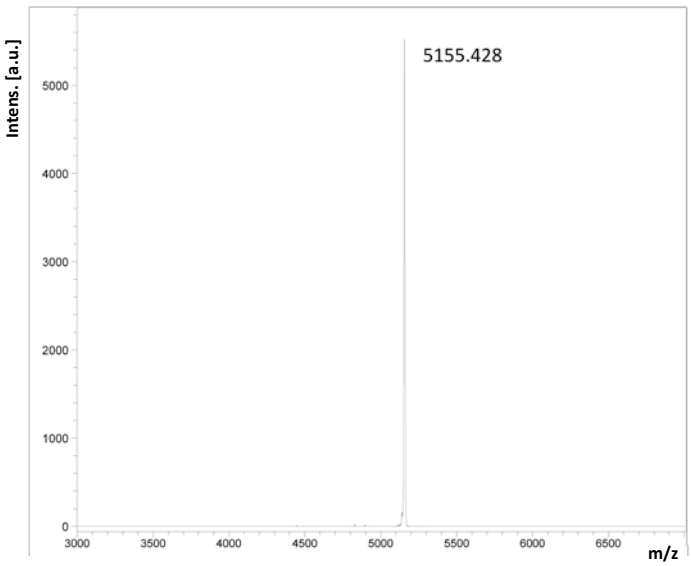

Figure 2 b. MS spectrum of M33-LVFX.

This new synthesis method is favourable for many reasons. First, the process is run completely on resin in an automatic synthesizer with great saving of time. Besides, solid phase synthesis allows the excesses of reagents needed to obtain a good yield, removing them together with the product without wasting time or peptide. The synthesis process is therefore practical and quick, providing easily purified crude peptides.

		MIC (μM)			
Species and strain	***Features***	**M33**	**M33-LVFX conjugate**	**Combination M33/LVFX [a]**	**LVFX**
P. aeruginosa PT5	*PAO1 (wild-type)*	2.3	1.15	0.58	1.15
P. aeruginosa PT629	*MexAB-OprM overexpression*	2.3	1.15	1.15	4.6
P. aeruginosa PT149	*MexEF-OprN overexpression*	2.3	2.3	1.15	9.2
E. coli ATCC 25922	*Reference strain, fluoroquinolone susceptible*	2.3	2.3	0.04	0.04
E. coli M6a	*fluoroquinolone resistant*	0.58	1.15	0.58	>9.2
E. coli V439a	*fluoroquinolone intermediate*	2.3	2.3	1.15	4.6
E. coli M103b	*fluoroquinolone susceptible (reduced susceptibility)*	0.58	0.58	0.58	0.58
E. coli V386a	*fluoroquinolone resistant*	0.58	2.3	1.15	>9.2

Table 1 The strains tested included either wild-type or reference strains (indicated) or isolates of human origin with different susceptibility profiles to fluoroquinolones. Improvements with respect of single M33 and LVFX is highlighted in grey. [a] M33 and LVFX were tested in equimolar amounts and the data refers to the minimum concentration of both that inhibited growth of the strain.

MIC assays on conjugation and combination of M33 with LVFX

The M33-LVFX conjugate was tested for its antimicrobial activity in comparison with peptide M33, LVFX and the physical mixture of M33 with LVFX, against a collection of *P. aeruginosa* and *E. coli* strains with different susceptibilities to M33 and LVFX. The MICs obtained are shown in **Table 1**.

The M33-LVFX conjugate showed MIC values that were on the whole similar to those of free M33. Altogether these results revealed that conjugation of M33 with LVFX did not affect the activity of M33 whereas it can impairthe activity of LVFX (*E. coli ATCC 25922)*. M33 is effective against LVFX-resistant strain.

Experimental Section

Materials and methods

All materials were obtained from commercial suppliers; (Fmoc)4-K2K-βAla-Wang Tentagel resin, Tentagel S Ram resin, Nα-9-fluorenylmethoxycarbonyl (Fmoc) amino acids, O-(benzotriazol-1-yl)-N,N,N,N-tetramethyluronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA) and piperidine were purchased from Iris Biotech GmbH and N,N-dimethylformamide (DMF), Methanol (MeOH) from Carlo Erba Reagents. N,N-diisopropylethylamine (DIPEA) was purchased from Merck SpA, Triisopropylsilane (TIS), Hydrazine and Levofloxacin from Sigma Aldrich. Milli-Q (Millipore) water was used for HPLC analysis. All coupling reactions were performed with 4 equivalents of amino acids pre-activated with HBTU (4 equiv), and DIPEA (8 equiv) in DMF. Coupling time was 40min. The washing solvent was DMF. Fmoc removal was performed with 40% piperidine in DMF (v/v) for 3 minutes and 20% piperidine in DMF for 20 minutes. Reaction volumes: a) for the 180mg resin scale: 2.2ml for the condensation, 1.2ml for the deprotection, 2.5ml x 5 times washing and 2.5ml x 2 times for the cleavage; b) for the 30mg resin scale: 0.6ml for the condensation step, 0.6ml for the deprotection step, 1ml x 5 times washing and 1ml x 2 for the cleavage. Peptides purification was performed on Perkin Elmer HPLC using a reverse phase C18 column Jupiter Phenomenex (10 μm 300Å 250x10 mm) using 0.1% TFA in water (A) and MeOH (B) as mobile phase, with a 220 nm absorbance detection. For analytical RP-HPLC a C18 column Jupiter Phenomenex (5 μm 300Å 250x0.46 mm) was used. Purified peptides were characterized by UltraflexIII MALDI TOF/TOF mass spectrometry (Bruker Daltonics, Bremen, Germany).

M33 synthesis

M33 peptide was synthesized on (Fmoc)4-K2K-βAla-Wang Tentagel resin (loading 0.43 mmol/g, 180mg) and carried out on a Syro automated synthesizer (MultiSynTech, Germany). Resin swelling and washing was followed by removal of the Fmoc protecting groups. The resin was washed again and the first amino acid (Fmoc-Ala-OH), preactivated with HBTU/DIPEA, was coupled for 40 minutes under shaking to ensure complete anchoring. The resin was washed, followed by removal of the Fmoc group and washed again. The rest of the peptide chain was then synthesized with subsequent coupling reactions, washings, deprotections, and washing until the peptide was fully elongated. Side-chain-protecting groups were tert-butoxycarbonyl for Lys, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg, and tert-butyl

ether for Ser. Cleavage from the resin and removal of the side-chain protecting groups was accomplished simultaneously by treating the peptidyl-resin with the mixture TFA/H_2O/TIS (95:2.5:2.5) for two hours. Then the resin was filtered off, and cold diethyl ether was added to the filtrate for peptide precipitation. The resulting peptide was purified by preparative RP-HPLC and then lyophilized. Yield: 22,34 mg (6.2%). MS (MALDI TOF/TOF): M+ calculated for $C_{209}H_{399}N_{75}O_{45}$ is 4683.88 Da; found (m/z) is 4683.75 Da. HPLC method (analytic): 0.1% TFA in water (A) - MeOH (B), from 70:30 A/B to 40:60 A/B in 30 minutes (r.t. 26.4min).

Synthesis of M33-LVFX conjugate

M33-LVFX conjugate was synthesized on Tentagel S Ram resin (loading 0.25 mmol/g, 30mg). Resin swelling and washing was followed by removal of the Fmoc protecting groups. Following the procedure described above for M33, Fmoc-Lys(Dde)-OH was introduced as first amino acid. The second amino acid added was Fmoc-βAla-OH, then Fmoc-Lys(Fmoc)-OH was added twice to build the lysine core. The rest of the peptide chain was synthesized with subsequent coupling reactions , washings, deprotections, and washings until the peptide was fully elongated. Finally Boc-Lys(Boc)-OH was used as N-terminals of the branched peptide. The Dde protecting group was then removed from N-ε of C-terminal lysine, by washing the peptidyl-resin with 2% hydrazine in DMF (v/v) (3x500µL). The free ε amino group was coupled with LVFX (75 µmol; 10:1 LVFX:ε amino group) , activating the LVFX carboxylic group with HBTU (75 µmol) and DIPEA (150 µmol), overtnight. After draining and washing M33-LVFX conjugate was cleaved from the resin and deprotected, following the same procedure described for M33. Yield: 2.5 mg (6.5%). MS (MALDI TOF/TOF): M+ calculated for $C_{233}H_{430}FN_{81}O_{48}$ is 5154.42 Da; found is 5155.428 Da. HPLC method (analytic) 0.1% TFA in water (A) - MeOH (B), from 60:40 A/B to 45:55 A/B in 40 minutes (r.t. 27.6 min).

MIC assays

MICs were determined using a standard microdilution assay as recommended by the Clinical and Laboratory Standards Institute.[14] Assays were performed in triplicate using cation-supplemented Mueller-Hinton (MH) broth (Becton Dickinson, Franklin Lakes, NJ, USA) and a bacterial inoculum of $5x10^4$ CFU/well, in a final volume of 100 µl. Results were recorded after 18–20 h of incubation at 37°C. Tested strains included the wild-type PT5 *P. aeruginosa* strain and its isogenic mutants PT629 and PT149 that overexpress efflux pumps MexAB–OprM and MexEF–OprN, respectively,[15] and *E. coli* strains with different susceptibility profiles to fluoroquinolones.[16]

Conclusions

The need for innovative antimicrobial drugs is driving the search for new antibiotics or new strategies for antimicrobial therapy. We evaluated the use of the antimicrobial peptide M33, currently in preclinical development as a free compound, to improve the activity of LVFX against clinically relevant Gram-negative bacteria including LVFX-resistant strains. M33 was used in conjugation and in combination with LVFX. For the conjugation procedure we set up an original synthesis method, which can also be used for the conjugation of different peptides and small molecules. The results of the present study with resistant strains suggest that in this case a combination approach is more effective than conjugation for reinforcing antimicrobial therapy. Since many bacteria have already developed resistance to FQ, this finding indicates a possibility of recovering some of the antibiotics discarded due to AMR.

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Conflict of interest

The patents covering the intellectual property of peptide M33 are owned by or licensed to SetLance. Chiara Falciani, Alessandro Pini and Luisa Bracci are partners of SetLance.

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