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SHORT COMMUNICATION

Site-specific pegylation of an antimicrobial peptide increases resistance to *Pseudomonas aeruginosa* elastase

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Abstract M33 is a branched peptide currently under preclinical characterization for the development of a new antibacterial drug against gram-negative bacteria. Here, we report its pegylation at the C-terminus of the three-lysine-branching core and the resulting increase in stability to *Pseudomonas aeruginosa* elastase. This protease is a virulence factor that acts by destroying peptides of the native immune system. Peptide resistance to this protease is an important feature for M33-Peg activity against *Pseudomonas*.

Keywords Antimicrobial peptides · Branched peptides · Pegylation · Peg · Elastase · Peptide stability

Introduction

A common strategy for resolving protein-based drug instability is drug modification by pegylation. The number of pegylated products on the market is increasing (Pasut and Veronese 2012), together with the number of new conjugates entering clinical trials (Bailon and Won 2009; Pasut and Veronese 2009). Polyethylene glycol (PEG) reduces rapid kidney clearance of proteins by increasing their hydrodynamic volume. It also prevents immunogenicity, reduces protein aggregation by repulsion between pegylated surfaces and increases protease stability (Sang-Heon et al. 2005).

The antimicrobial peptide M33 (Pini et al. 2010) is a synthetic molecule obtained by improving a phage library-derived peptide (Pini et al. 2005, 2007). It is currently under preclinical study for efficacy, pharmacokinetics and toxicity in animals. The molecule is strongly active against gram-negative bacteria, including several *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients (Pini et al. 2010; Falciani et al. 2012). M33 is synthesized in the branched form described by Tam (1988), in which identical peptide sequences are linked by a lysine core. This branched structure makes peptides highly resistant to the action of circulating peptidases and particularly suitable for in vivo use (Bracci et al. 2003; Falciani et al. 2007a, b; Pini et al. 2006, 2008; Lozzi et al. 2003).

Here, we report the C-terminal modification of the branched peptide M33 with a Peg4 molecule and its increased resistance to the *Pseudomonas* virulence factor, elastase, a peptidase used by the bacterium to degrade peptides and proteins of the host's native immune system. This modification may improve peptide performance against the bacterium *P. aeruginosa*, a major pathogen studied worldwide on account of the dramatic increase in its antibiotic resistance (Pendleton et al. 2013; Ciofu et al. 2013).

Materials and methods

Peptide synthesis: M33 and M33-Peg

Peptides (M33 and M33-Peg) were produced on a solid support in tetra-branched form. M33 was produced as acid

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peptide by solid-phase synthesis through standard Fmoc chemistry on Fmoc4-Lys2-Lys-β-Ala Wang resin with a Syro multiple peptide synthesizer (MultiSynTech, Witten, Germany). M33-Peg was synthesized as amide peptide on TentaGel S RAM resin with Fmoc-NH-Peg4-COOH as first coupling step, and Fmoc-Lys(Fmoc)-OH was then used to build the tetrameric core. In both syntheses, side chain protecting groups were 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for R, *t*-butoxycarbonyl for K and *t*-butyl for S. The final products were cleaved from the solid support, deprotected by treatment with a solution containing 95 % TFA, 2.5 % triisopropylsilane and 2.5 % water, and precipitated with diethyl ether. Crude peptides were purified by reversed-phase chromatography on a column for medium-scale preparation, in linear gradient form for 30 min, using 0.1 % TFA/water as eluent A and methanol as eluent B. Purified peptides were obtained as trifluoroacetate salts (TFacetate). The exchange from TFacetate (toxic by-product) to acetate form was carried out using a quaternary ammonium resin in acetate form (AG1-X8, 100–200 mesh, 1.2 meq/ml capacity). The resin-to-peptide ratio was 2,000:1, resin and peptide were stirred for 1 h, the resin filtered off, washed extensively and the peptide recovered and freeze-dried (Pini et al. 2012). Final peptide purity and identity were confirmed by reversed-phase chromatography on a Phenomenex Jupiter C18 analytical column (300 Å, 5 µm, 250 × 4.6 mm) with a linear gradient from 70:30 A/B to 40:60 A/B in 30 min and by mass spectrometry (MS) MALDI TOF/TOF.

M33 stability to *Pseudomonas aeruginosa* elastase

Tetra-branched M33 or M33-Peg peptides (0.170 mM) were incubated at 37 °C with *P. aeruginosa* elastase 0.27 µM (Calbiochem) in 20 mM Tris–HCl, 1 mM $CaCl_2$ at pH 7.8. The concentrations indicated corresponded at a peptide-to-enzyme ratio of 630:1. At different time intervals (T0, T3, T6 h), 40-µl aliquots were removed, diluted with 960 µl of 0.1 % trifluoroacetic acid (TFA)/water and analyzed by HPLC and mass spectrometry. The percent of uncleaved peptide at T3 and T6 respect to T0 was calculated as integration units obtained from the intact peptide

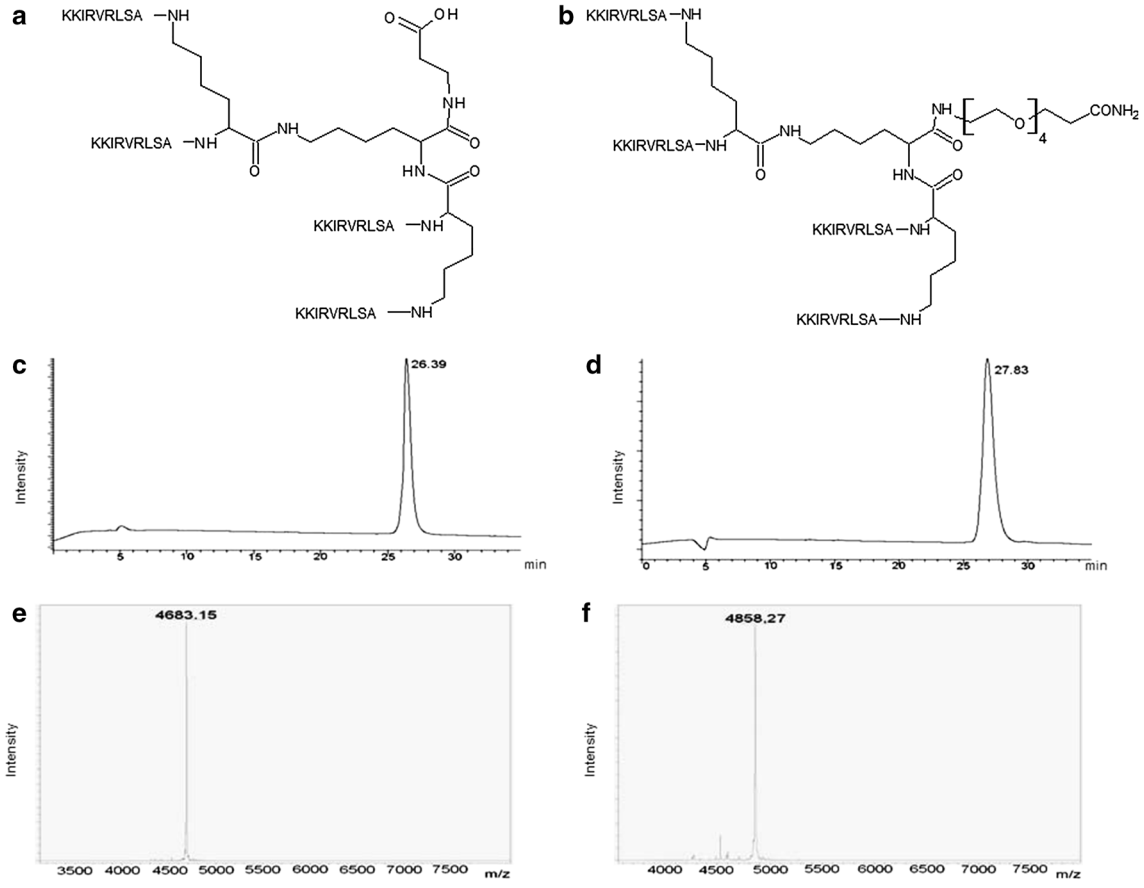

Fig. 1 **a** Structure of tetra-branched M33; **b** structure of tetra-branched M33-Peg. For both peptides, the amino acids of the four peptide sequences are indicated in one-letter code, and the three lysines of the branching core are drawn as structural formula. For M33, the C-terminus is an acid, for M33-Peg, an amide. HPLC profiles of M33 (**c**) and M33-Peg (**d**) after purification. Retention times are indicated. MS profiles of M33 (**e**) and M33-Peg (**f**). MWs are indicated

peak. Liquid chromatography was performed on a Phenomenex Jupiter C18 analytical column (300 Å, 5 µm, 250 × 4.6 mm) using 0.1 % TFA/water as solvent A and methanol as solvent B with a linear gradient from A/B 95:5 to A/B 5:95 in 30 min. MS analysis of samples was performed with a Bruker Daltonic ultraflex MALDI TOF/TOF mass spectrometer.

Results

M33 pegylation, HPLC evaluation and MICs

In the tetra-branched structure, the four peptide sequences are linked by a three-lysine core. Thus, the molecule has four N-terminal ends and a single C-terminal end (Fig. 1a). The C-terminal end can generally be used for further modification without affecting peptide activity (Falciani at al. 2007b, 2010, 2011, 2013). Figure 1b shows the peptide M33 linked to a Peg4 molecule through its C-terminal end. This site-specific conjugation is obtained during solid-phase synthesis as described in the methods section. This strategy produces very uniform and stable preparations, as shown by HPLC and MS profiles (Fig. 1c–f), readily applicable to an industrial process.

This pegylation strategy does not affect the stability to circulating proteases (M33 and M33-Peg resulted equally intact after 24 h of incubation in serum, not shown), and does not worsen the antibacterial activity. The following MICs were obtained: *Klebsiella pneumonia* ATCC13833, M33 1.5 µM, M33-Peg 0.7 µM; *P. aeruginosa* ATCC27853, M33 1.5 µM, M33-Peg 0.7 µM; *P. aeruginosa* PAO-1, M33 0.7 µM, M33-Peg 0.7 µM.

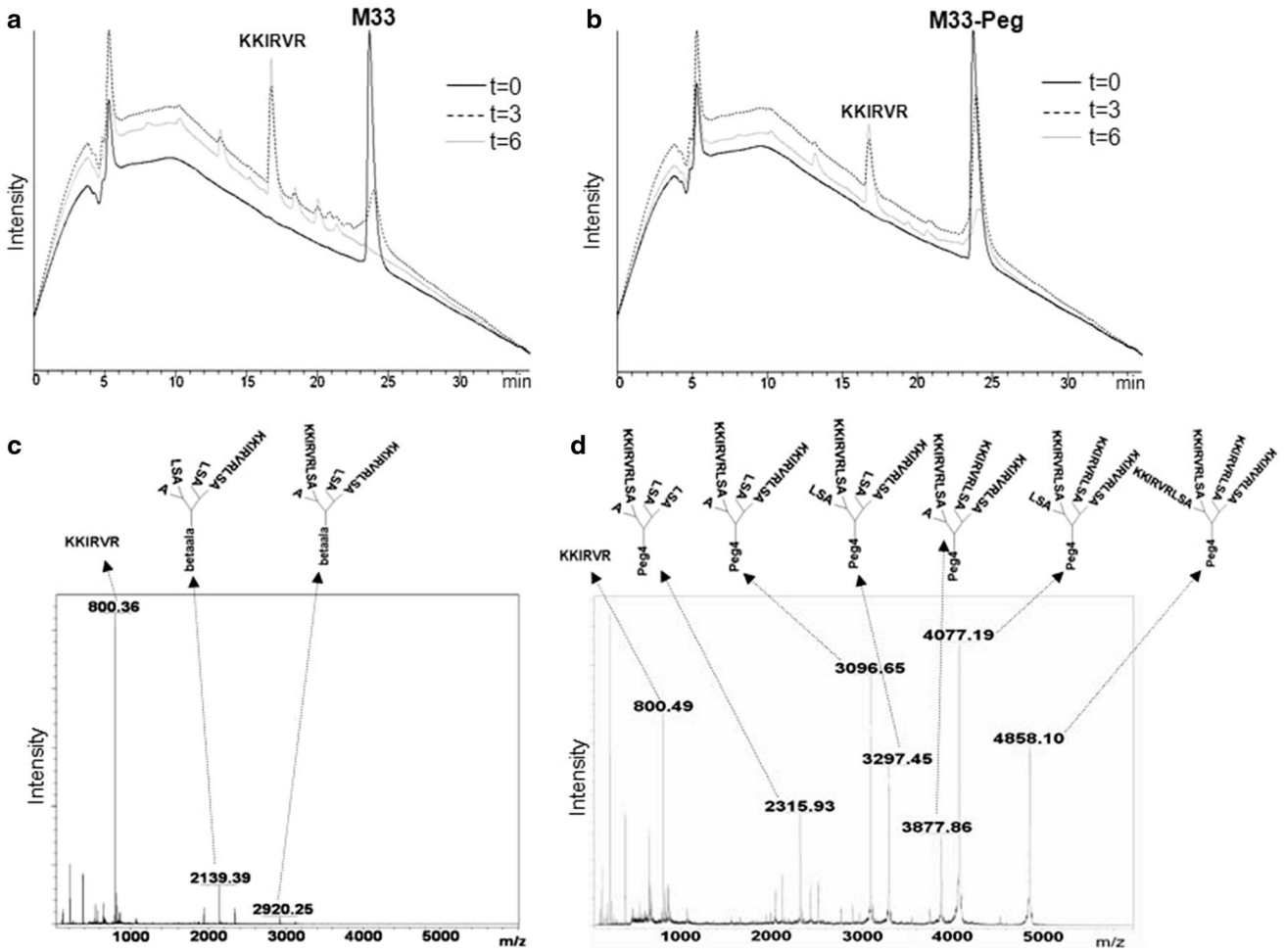

Fig. 2 HPLC profiles of M33 (**a**) and M33-Peg (**b**) at time 0 (*continuous lines*) and after 3 (*dotted lines*) and 6 (*grey lines*) hours of incubation with elastase. The names of intact peptides and the main proteolytic fragment are indicated. Retention times of intact molecules are slightly different from Fig. 1, because a different gradient was used in these analyses. MS profiles of M33 (**c**) and M33-Peg (**d**) after 6 h of incubation. Expected MWs for intact molecules are 4,683 (M33) and 4,858 (M33-Peg). All other peaks correspond to proteolytic fragments produced by elastase. Examples of proteolytic fragments are indicated in the *upper part* of panels **c** and **d**

Stability to *Pseudomonas aeruginosa* elastase

Pseudomonas aeruginosa elastase is a member of the family of M4 metallopeptidases (thermolysin family) (de Kreij et al. 2000) and hydrolyzes peptide bonds preferentially at the N-terminal of hydrophobic residues. Metallopeptidases play a key role in bacterial virulence by breaking down the natural host-defense peptides produced by infected individuals (Schmidtchen et al. 2002; Hornef et al. 2005). We previously demonstrated that these proteins may influence peptide activity in vitro and in vivo (Falciani et al. 2012). To determine whether M33 and M33-Peg were affected differently by elastase from *P. aeruginosa*, the two peptides were incubated with the purified protease and after appropriate time intervals, the crude solutions were analyzed by HPLC and mass spectrometry.

After 3 h of incubation, 38 % of M33 and 79 % of M33-Peg resulted uncleaved, at 6 h the M33 was completely proteolysed while 35 % of M33-Peg remained intact (Fig. 2a, b).

After 6 h of incubation with elastase, the MS profile showed an absence of the peak corresponding to M33 (4683 Da) (Fig. 2c), whereas that of M33-Peg confirmed the presence of a peak corresponding to the molecular weight of the intact peptide (4858 Da) (Fig. 2d). M33 and M33-Peg have the same cleavage sites at R6-L7 and S8-A9 peptide bonds.

Discussion

Development of new drugs is a long and complex process that includes identification of a lead molecule, optimization, preclinical evaluation in animals and clinical trials in humans. The antimicrobial peptide M33 was identified some years ago for its strong antibacterial activity against gram-negative bacteria, including multi-resistant strains of the pathogens *P. aeruginosa*, *K. pneumonia*e, *Acinetobacter baumannii* and other enterobacteriaceae. It is currently under preclinical characterization in animals and is expected to enter clinical trials in the next few years. The optimization phase that produced the latest version of M33, currently under investigation, started a few years ago with modification of the peptide sequence (Pini et al. 2010), followed by improvement of standard procedures for synthesis and purification (Pini et al. 2012), and with site-specific pegylation at the C-terminal of the branched structure, described in this article. To our knowledge, this is the first report of site-specific pegylation of a therapeutic peptide in tetra-branched form.

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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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