Amino Acids (2012) 43:467–473
DOI 10.1007/s00726-011-1103-z

ORIGINAL ARTICLE

Efficacy and toxicity of the antimicrobial peptide M33 produced with different counter-ions

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Received: 26 July 2011 / Accepted: 22 September 2011 / Published online: 8 October 2011

Abstract The tetra-branched peptide M33 (Pini et al. in FASEB J 24:1015–1022, 2010) is under evaluation in animal models for its activity as antimicrobial agent in lung infections and sepsis. The preclinical development of a new drug requires medium-scale manufacture for tests of efficacy, biodistribution, pharmacokinetics and toxicity. In order to produce the most suitable peptide form for these purposes, we evaluated the behaviour of the peptide M33 obtained with different counter-ions. We compared activity and toxicity in vitro and in vivo of the peptide M33 produced as trifluoroacetate salt (TFacetate) and as acetate salt. The two forms did not differ substantially in terms of efficacy in vitro or in vivo but showed different toxicities for human cells and in animals. M33-TFacetate proved to be 5–30% more toxic than M33-acetate for cells derived from normal bronchi and cells carrying ΔF508 mutation in the CFTR gene, the most frequent variant in cystic fibrosis. M33-TFacetate produced manifest signs of in vivo toxicity immediately after administration, whereas M33-acetate only generated mild signs, which disappeared within a few hours. The peptide M33-acetate proved more suitable for the development of a new drug, and was therefore chosen for further characterization.

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Keywords Antimicrobial peptides · Trifluoroacetic acid · Branched peptides · Gram-negative bacteria · Cystic fibrosis · Peptide toxicity

Introduction

A non-natural peptide sequence with strong antimicrobial activity, particularly against Gram-negative bacteria, was identified previously (Pini et al. 2005, 2007, 2010). The best peptide variant obtained through several optimization steps was called M33 (KKIRVRLSA). It was synthesized and used in multiple antigen peptide (MAP) form (Tam 1988), with a core of three-branched lysine residues onto which four copies of the same peptide sequence were synthesized. This branched form proved to be particularly resistant to peptidase activity, making MAP peptides more suitable for clinical applications than the respective linear peptides (Bracci et al. 2003; Pini et al. 2006, 2008, 2010; Falciani et al. 2007, 2009, 2010). The tetra-branched peptide M33 neutralized bacterial lipopolysaccaride and prevented septic shock in vivo, qualifying for development as a new antibacterial drug. M33 is currently under evaluation for its activity in *Pseudomonas aeruginosa* lung infections and development as a new drug for cystic fibrosis (CF)-related infections.

M33 peptide was initially synthesized using standard Fmoc solid phase peptide synthesis (SPPS) (Fields and Noble 1990), purified by reversed-phase HPLC and obtained as trifluoroacetate (TFacetate) salt. Under standard conditions, the TFacetate salt was obtained because cleavage of the peptide from the resin support and deprotection of the side-chain-protecting groups were carried out simultaneously by treating the peptidyl resin with trifluoroacetic acid (TFA). TFA was also used during purification

of the synthetic peptide by reversed-phase HPLC (Rivier et al. 1984), using a solvent system with TFA as acidic ion pairing reagent that interacts with basic positively charged amino acid side-chains of lysine and arginine residues, as well as the free N-terminal amino group.

Before starting preclinical studies on the efficacy, biodistribution, pharmacokinetics and toxicity of M33, we evaluated the effect of counter-ions on peptide antimicrobial activity and in vitro and in vivo toxicity. We produced M33 peptide as TFacetate and as acetate salts in order to test the antibacterial activity and toxicity of the same peptide with different counter-ions (Verbeeck et al. 2006).

Various approaches can be used to replace TFacetate counter-ion by acetate (Roux et al. 2008). A common procedure is to use an excess of an acid stronger than TFA, such as HCl, reprotonating TFacetate to form the free acid, which is easily removed by freeze-drying (Andrushchenko et al. 2007; Sani et al. 2007; Beck et al. 2001). Since a very strong acid solution may damage the peptide, we chose an ion-exchange procedure, which ensures efficient removal of TFA under mild acid conditions. The TFacetate/acetate exchange was followed by quantitative ^{19}F-NMR and ^{1}H-NMR (Little et al. 2007; Preiss et al. 1998) in order to determine the exact amount of both counter-ions. The two peptide forms were then tested against a panel of relevant bacteria for their activity, against eukaryotic cells for toxicity (Cornish et al. 1999) and in vivo for activity and toxicity.

Materials and methods

M33 peptide synthesis

Solid-phase synthesis was carried out by standard Fmoc chemistry on $Fmoc_4$-Lys_2-Lys-β-Ala Wang resin with a Syro multiple peptide synthesizer (MultiSynTech, Witten, Germany). 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 product was cleaved from the solid support, deprotected by treatment with TFA containing triisopropylsilane and water (95/2.5/2.5), and precipitated with ethyl ether. Crude peptide was then purified by reversed-phase chromatography on a semipreparative Phenomenex Jupiter C18 column (300 Å, 10 µm, 250 × 10 mm) in linear gradient form for 30 min, using 0.1% TFA/water as eluent A and methanol as eluent B. 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) and by mass spectrometry with a Bruker Daltonics ultraflex MALDI TOF/TOF.

Purification by ion-exchange resin

The exchange from TFacetate to acetate was carried out using a quaternary ammonium resin in acetate form (AG1-X8, 100-200 mesh, 1.2 meq/ml capacity, Bio-Rad). We used a resin-to-peptide ratio of 2,000:1 to ensure high anion exchange yield. The resin (8.6 g) was added directly to the peptide solution (47 mg in 172 ml water) and the suspension was stirred for 1 h. The peptide sample was filtered from the resin and recovered. The resin was washed extensively with water and the pooled solutions were evaporated and freeze-dried, yielding 39 mg of peptide.

Nuclear magnetic resonance

NMR instrumentation

^{19}F and ^{1}H NMR spectra were recorded on a Bruker DRX-600 spectrometer operating at 14.1 T. The data was processed and analysed using the XWIN-NMR software package (Bruker Biospin). All spectra were recorded in automatic mode using a 34-unit automatic sample changer (BACS-120).

Preparation of calibration standards and samples for ^{19}F and ^{1}H NMR

Stock solutions of TFA and acetic acid (AcOH) were first prepared by accurately weighing a few drops of each acid into a previously weighed volume of DMSO-d6 solvent to a final concentration of 403.54 and 439.22 mM for TFA and AcOH, respectively. Seven standard samples of known concentration for each acid were then prepared by first diluting the stock solution to 20 mM followed by further dilutions to produce standards having 20, 10, 5, 2.5, 1.25, 1 and 0.5 mM concentrations, each in 600 µl of DMSO-d6 solvent. Samples of peptide, treated and not treated with AG1-X8 resin, for which the TFacetate and acetate content was to be determined, was accurately prepared by weighing 9.04 mg (not exchanged) and 10.21 mg (exchanged) of material, into a previously weighed volume of DMSO-d6 solvent, followed by dilution to obtain 1 mg for each 600 µl sample.

NMR data acquisition

All ^{19}F and ^{1}H NMR spectra were recorded at 300 K in DMSO-d6 solvent (Sigma) using a single pulse sequence. ^{19}F spectra were acquired with the addition of 64 scans over a spectral width of 56 KHz, while 128 scans and a spectral width of 10 KHz were used for ^{1}H spectra. A relaxation delay of 7 s and data digitalization over 64 K data points were employed in both cases. No processing

prior to Fourier transform was necessary. All NMR spectra were recorded in triplicate for each of the calibration standards and averaged integrals were employed to create a calibration curve. For samples in which the TFacetate and acetate content was to be determined, spectra were also acquired in triplicate and integrals averaged. Calibration curves enabled determination of the TFacetate and acetate contents of each sample, and inference of actual peptide content in weighed material. Peptide/counter-ion molar ratio was also determined.

Cell culture and treatment with M33

16HBE14o- (human bronchial epithelial cells) and CFBE41o- (cystic fibrosis bronchial epithelial cells with ΔF508 mutation in the CFTR gene) were provided by Dieter Gruenert, University of California, San Francisco, under a material transfer agreement signed in 2010. 16HBE14o- and CFBE41o- were plated at a density of 2.5×10^4 per well in 96-well microplates, previously incubated with coating solution (88% of LHC basal medium, 10% bovine serum albumin, 30 µg/ml bovine collagen type I and 1% human fibronectin).

Different concentrations of M33-TFacetate or M33-acetate, from 0.05 to 50 µmol/L, were added 24 h after plating. Cells were grown for 48 or 72 h at 37°C. Growth inhibition was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

In vivo toxicity

Animal procedures were approved by the Ethical Committee of the Azienda Ospedaliera Universitaria Senese on 21 September 2009.

C57BL/6NCr mice (20–22 g) were inoculated with peptide M33-acetate or M33-TFacetate (25 mg/Kg) intraperitoneally (IP) and subcutaneously (SC). Each animal was treated three times (at time 0 and 12 and 24 h after the first injection). Signs of toxicity were checked by visual inspection. A toxicity score (indicated in Fig. 3) was attributed to the following signs: wiry coat and poor motility = mild signs; very wiry coat and poor motility even under stimulation = manifest signs. Animals were monitored for 7 days after the first inoculation.

In vivo efficacy

Animal procedures were approved by the Ethical Committee of the Azienda Ospedaliera Universitaria Senese on 18 November 2010.

Balb-c mice (20 g) were infected by IP injection (500 µl PBS solution containing bacteria) with the indicated lethal amounts of bacteria (see "Results"). Bacteria were cultured overnight, centrifuged, diluted in sterile PBS and measured by spectrophotometer. Further dilutions in PBS were sometimes necessary to obtain the right amount of bacteria.

Groups consisted of eight animals. Moribund animals were killed humanely to avoid unnecessary distress. Surviving mice were monitored for 7 days. Thirty minutes after bacterial administration, peptide was inoculated by IP injection of 1 ml PBS solution containing the indicated amount of peptide (see "Results"). *P* value was calculated using GraphPad Prism software.

Results

Synthesis and purification

Synthesis and purification processes of the tetra-branched peptide M33 resulted in more than 95% pure product, as shown by analytical reverse phase chromatography on a Jupiter C18 column (Fig. 1a) and by mass spectrometry (MS) revealing a single peak at the expected molecular mass of the tetrabranched peptide M33 (4,683 Da) (Fig. 1b). Both techniques confirmed that the peptide was integral after the resin-ion exchange procedure (not shown). TFacetate and acetate counter-ions cannot be identified by these characterization procedures. Their small molecular weight makes them unsuitable for detection by C18-reverse phase HPLC and MALDI MS spectrometry.

Quantification of counter-ion content

TFacetate and acetate content of peptide batches was assessed by ^{1}H and ^{19}F quantitative NMR spectroscopy. For peptide batches not exchanged from TFacetate to acetate, TFacetate was as high as 31.5% of weight. This translates into a TFacetate/peptide molar ratio of ~19, accounting for neutralization of the peptide net charge of 19+. Ion exchange purification was effective in lowering TFacetate content to a TFacetate/peptide molar ratio of ~1. Acetate content increased to a molar ratio of ~18, maintaining a counter-ion count of 19 (Table 1).

Minimum inhibitory concentrations (MIC) of M33-TFacetate and M33-acetate

M33 is mainly active against Gram-negative bacteria as described in Pini et al. (2010). When M33-TFacetate and M33-acetate were tested against Gram-negative bacteria of pathogenic species (Table 2), the antimicrobial profiles and potency were on the whole similar for the two peptide forms, showing that the counter-ions did not affect peptide activity in vitro.

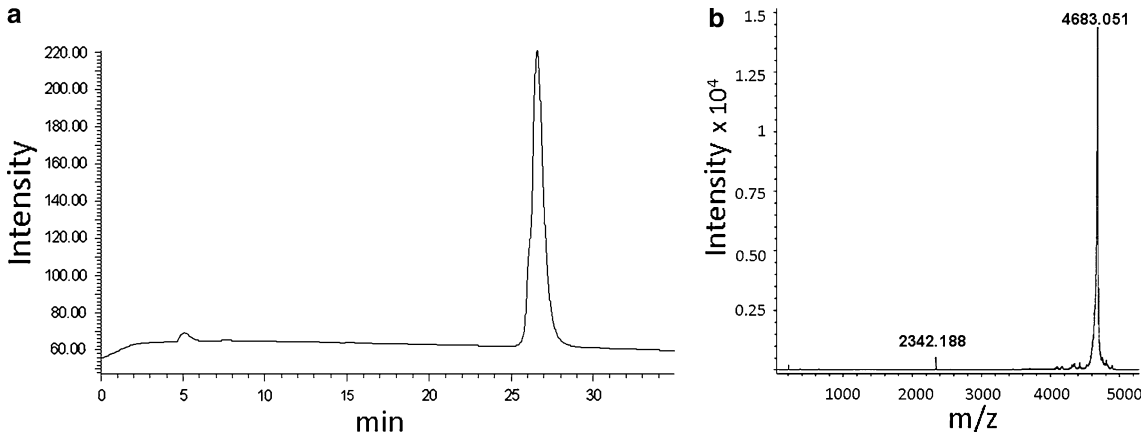

Fig. 1 HPLC (**a**) and MS (**b**) profiles of branched peptide M33. UV chromatogram at 220 nm of the purified peptide M33 using reverse phase HPLC on a Jupiter C18 analytical column. A single peak is present at the retention time corresponding to the molecular mass of M33 (**a**). M33 MALDI-TOF spectrum after purification by reverse phase chromatography. A main peak at the molecular mass of M33 is evident together with a small peak of the bi-charged molecule (**b**). Counter-ions cannot be identified by these two characterization procedures

Table 1 Weight contents and molar ratios (in parentheses) obtained for TFacetate (by ^{19}F NMR) and acetate (by ^{1}H NMR) in batches of peptides exchanged or not exchanged from TF-acetate to acetate

M33	TF-acetate % *w/w* (TF-acetate/peptide molar ratio)	Acetate % *w/w* (acetate/peptide molar ratio)
Peptide NOT exchanged from TF-acetate to acetate	31.5 (18.9)	0
Peptide exchanged from TF-acetate to acetate	2.4 (1.3)	18.3 (18.2)

Table 2 MIC of M33-TFacetate and M33-acetate

Bacterial species and strains	M33-TFacetate (μM)	M33-acetate (μM)
K. pneumoniae ATCC 13883	3.4	3.4
E. coli ATCC 25922	6.8	3.4
A. baumannii ATCC 17978	6.8	6.8
C. freundii CGUG 418	3.4	6.8
P. mirabilis CGUG 26767	>14	>14
P. aeruginosa PAO-1	3.4	3.4
P. aeruginosa ATCC 27853	3.4	1.7

Peptides were tested against Gram-negative bacterial strains of several pathogenic species

Toxicity to eukaryotic cells

The toxicity to eukaryotic cells is an important feature for new pharmacological entities. Cystic fibrosis (CF) is a possible therapeutic application of peptide M33, in which case M33 needs to be active against bacteria in the lower airways. We therefore analysed the peptide's toxicity against bronchial epithelial cells derived from CF patients (CFBE410) and bronchial epithelial cells from healthy individuals (16HBE14). CFBE410 cells showed similar percentage survival when incubated with M33-TFacetate and M33-acetate (Fig. 2a), whereas M33-TFacetate was 10–20% more toxic than M33 acetate to 16HBE14 cells (Fig. 2b). CFBE410 cells appeared more sensitive than 16HBE14 to the toxicity of both peptide forms. This is not surprising since CFBE410 are impaired in certain biological functions by the genetic mutation that causes the disease, and this might make these cells more sensitive to any kind of treatment.

Toxicity in vivo

Preliminary acute toxicity of purified M33-acetate and M33-TFacetate peptides was tested by intraperitoneal (IP) and subcutaneous (SC) administrations. No difference in acute toxicity (not shown) was recorded for both peptide forms and the LD50 was in line with Pini et al. (2007). However, we noted that substantial differences in toxicity signs were shown by mice immediately after peptide administration. Healthy mice treated three times IP with M33-acetate at 25 mg/Kg, which was the higher dosage used for efficacy experiments in Pini et al. (2010), showed very mild signs of toxicity only after the second treatment (Fig. 3a). These signs (wiry coat and poor motility) disappeared after 30–60 min. No apparent signs were shown

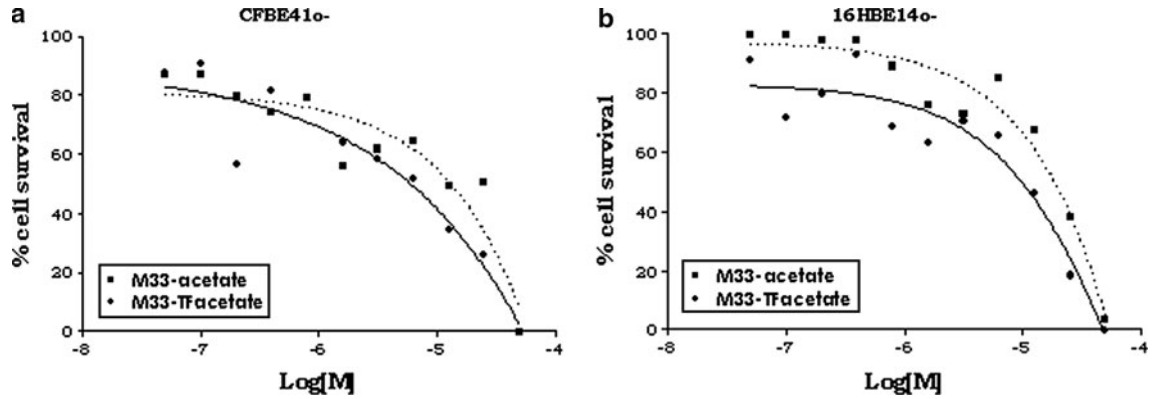

Fig. 2 Cytotoxicity of M33-TFacetate and M33-acetate in CFBE410 (**a**) and 16HBE14 (**b**) cells. Percentage cell survival (*Y* axis) is plotted against peptide concentrations (*X* axis). The data were analysed by non linear regression using GraphPaD prism 3.02

after the first and third administrations. In contrast, M33-TFacetate at the same IP dose caused manifest signs of toxicity (very wiry coat and very poor motility even under stimulation) after the first treatment and mild signs after the second and third administrations (Fig. 3b). Manifest signs disappeared within 2–4 h, mild signs within 30–60 min.

The SC treatment seemed less toxic with both peptide forms. No signs were shown by mice treated three times with M33-acetate 25 mg/Kg SC (Fig. 3c). Mild signs were shown by mice after each injection with M33-TFacetate 25 mg/Kg SC (Fig. 3d) but all disappeared within 30 min.

In vivo efficacy

In order to verify whether M33-acetate still protected mice from lethal infection as described in Pini et al. (2010), where M33-TFacetate was used, we carried out new efficacy experiments in vivo. The minimum number of bacteria causing 100% lethal infection (LD100) after IP injection was 1.5×10^9 *Escherichia coli* TG1. Bacterial LD100 killed mice in 18–24 h. Mice were infected with the LD100 of bacteria and treated IP with M33 30 min later. M33-acetate protected 100% of animals from signs of sepsis and death (7-day survival) when administered in a single dose at a concentration of 10 mg/Kg (Fig. 4).

Discussion

The development of a new drug is a long process including a research phase, preclinical experimentation, clinical study and set-up for large-scale production. When the new molecule enters preclinical experimentation, its physico-chemical

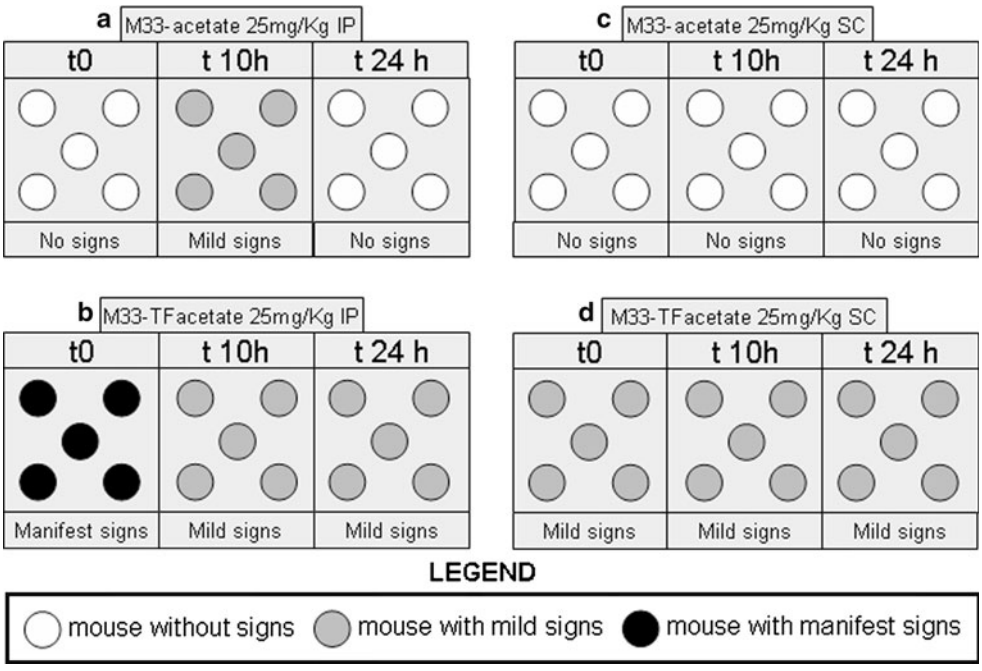

Fig. 3 M33 toxicity in vivo. Five mice per group (*each circle* represents a mouse) were inoculated three times with M33-acetate (**a**, **c**) or M33-TFacetate (**b**, **d**) at the times indicated in the figure. In **a** and **b** mice were inoculated IP with M33-acetate and M33-TFacetate, respectively. In **c** and **d**, mice were inoculated SC with M33-acetate and M33-TFacetate, respectively. *Different scales of grey* indicate severity of signs shown by animals after injection. Mice inoculated with saline did not shown any signs of toxicity (not shown)

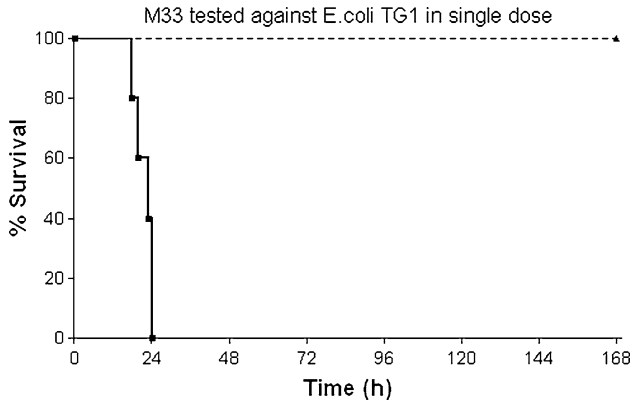

Fig. 4 In vivo antibacterial activity of M33-acetate peptide. Balb-c mice (20 g) were injected IP with a lethal amount of *E. coli* TG1cells. *Continuous line* (control), inoculation with bacteria and no M33; *broken line*, inoculation with bacteria and a single injection of M33 peptide (10 mg/kg) 30 min later

profile should already be characterized to avoid repetitions at later phases of development.

The branched peptide M33 had already proved to be a strong antibacterial agent, mainly against Gram-negative bacteria, with special activity against *P. aeruginosa* strains isolated from CF patients (Pini et al. 2010). M33 is currently under evaluation for its efficacy in the care and eradication of lung infections caused by *P. aeruginosa*, and late preclinical characterization is soon expected to begin.

Most synthetic peptides are nowadays produced using Fmoc-solid-phase procedures (Merrifield 1963). Due to cleavage and purification conditions, synthetic peptides are mainly obtained as TFacetate salts, so TFacetate may be present in relatively high quantities in cationic peptide preparations. The most popular procedure used to get rid of TFacetate among peptide synthesizers is TFacetate-acetate ion exchange by the law of mass action, i.e. displacing TFacetate by repeated addition of acetic acid and freeze drying. Since TFacetate can be a toxic contaminant for some eukaryotic cells (Cornish et al. 1999), it is important to evaluate its actual content and if necessary, consider an ion-exchange procedure before setting up large-scale production. We therefore decided to study its chemical profile in terms of counter-ion analysis after synthesis and purification. M33 was previously tested without a specific evaluation of the TFacetate content and was used in vivo directly after the standard HPLC purification procedure, repeated addition of acetic acid and freeze drying. Unexpectedly, this peptide form carried 18 TFacetate ions, as we now demonstrated by NMR analysis, and was effective in vivo, though possible toxicity due to TFacetate had not hitherto been evaluated. The present study compared the general behaviour of the same peptide M33 when produced as TFacetate and acetate. M33-acetate was obtained with an additional step based on ion-exchange chromatography as described in "Materials and methods". This procedure almost completely replaced TFacetate with acetate (1:18 ratio) as confirmed by NMR.

The efficacy of the peptide appeared essentially identical in both forms, as shown by MICs against different species and strains of bacteria (Table 2), and by comparison of in vivo efficacy using M33-acetate in the present study with that in Pini et al. (2010), where M33-TFacetate was used. In terms of in vitro toxicity, we demonstrated that the two peptide forms did not differ substantially when tested on human cells. We used immortalized airway epithelial cells, described as critical in the development of genetic and pharmacological therapies and important in the high-throughput screening strategies now being employed to identify new drugs for the treatment of CF (Gruenert et al. 2004). Since a possible therapeutic application of peptide M33 is for CF, we analysed potential peptide toxicity against immortalized epithelial cells derived from CF patients (CFBE410) and epithelial cells from healthy individuals (16HBE14).

Contrary to the results of in vitro toxicity experiments, we noted a major difference in vivo, where M33-TFacetate caused more evident signs of general toxicity. Elimination of a factor that could increase peptide toxicity is a crucial aspect for the development of this new candidate drug (Abraham et al. 2008; Arnold et al. 2007; Michalopoulos and Falagas 2008).

This work can be useful not only for the development of peptide M33 but also for the many new peptides currently being developed as monomeric or branched molecules (Bragonzi 2010; Pini et al. 2008; Reichert et al. 2010), for which Fmoc-solid phase synthesis is the most widely used production system. The possible toxicity of TFacetate counter-ions, which are not eliminated with standard procedures, should be considered by all peptide producers and developers.

Acknowledgments This research was supported by the Italian Foundation for Cystic Fibrosis (project FFC#14/2009 adopted by "Un Natale da Fiaba" with Kiwanis Club, LIFC-Associazione Toscana-ONLUS and Famiglia Papini Prato), the Tuscan Regional Administration (Project SPAC: "POR CReO FESR 2007-2013) and PROGETTO PRIN (2008KCLR7M_004). The authors thank Gianni Pozzi and Tiziana Braccini for their help about in vivo toxicity study.

Conflict of interest The authors declare that they have no conflict of interest.

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