PEGylation of the peptide Bac7(1–35) reduces renal clearance while retaining antibacterial activity and bacterial cell penetration capacity

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ABSTRACT

The proline-rich antibacterial peptide Bac7(1–35) protects mice against *Salmonella typhimurium* infection, despite its rapid clearance. To overcome this problem the peptide was linked to a polyethylene glycol (PEG) molecule either via a cleavable ester bond or via a non-hydrolysable amide bond. Both the PEGylated conjugates retained most of the *in vitro* activity against *S. typhimurium.* In addition, the ester bond was cleaved in human serum or plasma, releasing a carboxymethyl derivative of Bac7(1–35) which accounts for a higher activity of this peptide with relative to the other, non-hydrolysable form. Both PEGylated peptides maintained the capacity of the unconjugated form to kill bacteria without permeabilizing the bacterial membranes, by penetrating into cells. They exploited the same transporter as unmodified Bac7(1–35), suggesting it has the capacity to internalize quite sizeable cargo if this is linked to Bac7 fragment. PEGylation allows the peptide to have a wide distribution in mice, and a slow renal clearance, indicating that this strategy would improve the bioavailability of Bac7, and in principle of other antimicrobial peptides. This can be an equally important issue to reducing cytotoxicity for therapeutic use of these antibacterials.

1. Introduction

Antibiotic resistance has become a major public health problem within the lifetime of most people living today. The number of bacterial species and strains no longer susceptible to antibiotics is growing up day by day. To counteract this situation new antimicrobials with novel mechanism of action are greatly needed. Antimicrobial peptides (AMPs) from innate immunity of animals are among the most interesting candidates for this role [1,2].

Proline-Rich Antimicrobial peptides (PR-AMPs) are a widespread group of antimicrobial peptides (AMPs) present in mammalian neutrophils, as peptides belonging to the cathelicidin family, and in the hemolymph of several invertebrate species [3,4]. Despite their different origin and evolution, they have common hallmarks, such as *i*) a high content of arginine and proline residues, *ii*) activity directed mainly against Gram-negative bacteria, *iii*) a remarkably low cytotoxicity towards eukaryotic cells, *iv*) the lack of extensive membrane damaging effects, *v*) and reduced activity of the all-*D* enantiomers, consistent with a mode of action based on recognition of stereospecific molecular targets and/or transporters [4,5].

Studies on the bovine PR-AMPs Bac7 have revealed important aspects of the mode of action and the antimicrobial activity of this peptide [6,7]. The 1–35 fragment has *in vitro* antimicrobial activity against Gram-negative *Escherichia coli, Salmonella typhimurium, Klebsiella pneumonia* and *Enterobacter cloacae* at concentrations ranging from 1 to 10 μM, comparable to that of the natural peptide. Furthermore, it remains active against antibiotic-multi-resistant clinical isolates due to a mechanism of action that is different to those of currently used antibiotics [6].

Abbreviations: Boc_2O, Di-t-butyl dicarbonate; Et_2O, ethyl ether; CFU, colony forming unit; Cmc, S-carboxymethyl-cysteine; DIEA, N,N-Diisopropylethylamine; HCTU, 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; OH-PEG-OMe, methoxy-polyethylene glycol; HRP, horseradish peroxidase; IE, ion exchange; kDa, kilodalton; MeOH, Methanol; $N_2H_4 \cdot H_2O$, hydrazine hydrate; NCL, native chemical ligation; NMP, N-methylpyrolidone; PA, petroleum ether (fraction 40–60 °C); Ph_3PBr_2, triphenyl phosphite dibromide; Phth, phthaloyl; PI, propidium iodide; PrOH(2), 2-propanol; Py, pyridine; PyBOP, (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reversed phase high-performance liquid chromatography; SPB, sodium phosphate buffer; SPPS, solid phase peptide synthesis; TA, thioanisole; TCEP.HCl, Tris(2-carboxyethyl)phosphine hydrochloride.

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Bac7(1–35) has the capacity to translocate into both bacteria [5,7] and eukaryotic cells without cell damage [8,9]. Uptake into *E. coli* and other Gram–negative bacteria is mediated, at least in part, by the SbmA transporter [5,10], a dimeric inner membrane protein involved in the transport of different types of peptides, in cooperation with the cognate outer membrane protein YaiW [11,12], and has been shown to target proteins involved with protein production (the chaperone DnaK and ribosomal subunits) [3,4,13].

The therapeutic potential of Bac7(1–35) has been assessed in mice infected with *S. typhimurium*, resembling a model of typhoid fever infection [14]. No toxic effects were observed when the peptide was administered to the mice i.p. up to 75 mg/kg and it significantly increased the survival rates and reduced the bacterial load in liver and spleen of infected animals [14]. However, its circulating lifetime was low as it was easily removed by murine kidneys due to its small size and/or was degraded. The peptide reached kidneys and bladder by 1 and 3 h, respectively, after injection and was totally excreted within 24 h [14].

Among the several antimicrobial peptides that are currently undergoing clinical trials, most are being tested for topical use [15]. Very few are being considered for systemic therapy because of the many hurdles that must be overcome [16]. First, peptide drugs have short circulating half-lives, due to proteolytic digestion and rapid kidney clearance, and are often antigenic [17,18]. They also tend to show low therapeutic indices *in vivo* [16,19] in part due to a reduced activity in the presence of serum and plasma components [20,21].

The polyethyleneglycol (PEG) moiety is frequently attached to peptide and protein drugs (PEGylation) in order to improve the *in vivo* efficacies of these drugs, by reducing their cytotoxicity and immunogenicity or by prolonging the *in vivo* half-life [17,22,23]. It has also been successfully used to modify AMPs [24–27]. For example, a 5 kDa PEG linked to the N-terminus of tachyplesin resulted in a compound with reduced cytotoxicity and sensitivity to serum inhibition [26]. The cytotoxicity of the peptide magainin 2 was also significantly reduced after PEGylation [25]. N-terminally PEGylated AMPs consisting of fragments belonging to human LL-37 and insect cecropin A also showed reduced toxicity towards lung epithelial primary cell cultures [28]. However, to the best of our knowledge, there are no studies relating to its use simply to improve the pharmacokinetics of an internally acting AMP without altering its activity.

Here, we describe the modification of Bac7(1–35) by linking it to a long 20-kDa PEG chain to reduce the clearance rate of the peptide. The different derivatives, one hydrolysable and the other stable, were tested for their *in vitro* activity against *S. typhimurium* cells, and the effects of human serum and plasma on the kinetics of peptide release and its stability were investigated. Our linkage strategy also allowed preparation of a fluorescent variant of the PEGylated peptide, for use in optical imaging analysis to monitor its biodistribution and permanence in the body of mice.

2. Materials and methods

2.1. Materials

20 kDa mPEG-OH was purchased from Nektar therapeutics (Huntsville, AL, USA, Lot. 307360) or from Sunbio Chemicals Co., Ltd. (South Korea, Lot. C1OH-020-09135) and was dried before use by azeotrope distillation from toluene. As indicated in the technical specifications the polydispersivity is 452 ± 36 residues.

Activating reagents, HCTU and PyBOP, were from Calbiochem–Novabiochem AG (Switzerland). Anhydrous DMF, NMP and PIP were from Biosolve Ltd (The Netherlands); DIPEA, TFA, TA, TIPS from Fluka Chemie AG (Switzerland). All other reagents and solvents were reagent grade and were purchased from Fluka, Sigma–Aldrich (USA), Biosolve Ltd (Netherlands), Alexis (USA) and Advanced ChemTech (USA). Fmoc-protected amino acids were obtained from Novabiochem (Switzerland), Inbios (Italy), Iris Biotech GmbH (Germany) or Bachem AG (Switzerland).

2.2. Purification methods

Analytical RP-HPLC was carried out on a Gilson HPLC System. Samples were eluted with a linear gradient from A = 0.1% TFA in water to B = 0.1% TFA in MeCN.

Preparative RP-HPLC was performed on a Waters RCM with PrepPak Cartridge Delta-Pak 300 15RP18 (100 × 25 mm I.D.) at a flow rate of 7 ml/min or on a Waters Prep LC universal base module with a PrepPak Cartridge Delta-Pak 300 15RP18 (100 × 40 mm I.D) column at a flow rate of 18 ml/min. Samples were injected manually and eluted from the column with a gradient slope from 0.6% to 0.8% B/min. Pure fractions, according to analytical RP-HPLC or ESI-MS analysis, were pooled and freeze-dried. TFA was removed after lyophilizing three times with 10 mM HCl solution.

IEX-HPLC was carried out on AKTA Basic 10 (Amersham Pharmacia, Sweden) using one or two column in series, HiTrap™ SP HP (5 mL, Pharmacia) equilibrated in 20 mM Na phosphate buffer pH 6.5. Elution was carried out with a NaCl gradient.

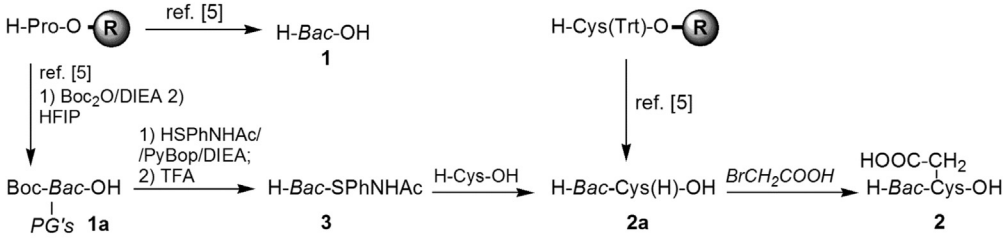
The eluent from analytical RP-HPLC or preparative RP-HPLC was collected as fractions and aliquots analysed on Applied Biosystems Sciex API 150EX or ion trap mass spectrometer (Amazon SL, Bruker) for the correct peptide conjugate.

2.3. Peptide synthesis

All the peptides used in this study are shown in Scheme 1, Scheme 2 and Table 1. The synthesis of Bac7(1–35) [Bac, in Table 1] and its further conjugation to BODIPY® FL N-(2-aminoethyl) maleimide fluorophore [Bac-BY in Table 1] are also described in the Supplementary data. Small portions of the crude peptide resins were cleaved, deprotected with a modification of the procedure as described in the Supplementary data and purified by RP-HPLC to furnish Bac7(1–35) (**1**) (Scheme 1 and Table 1). The C-terminally extended Bac-Cys(H)–OH (**2a**) could be obtained in two ways, directly synthesizing it in the solid state from Cys-substituted chlorotrityl resin, or in solution from **1a** via intermediate **3** (Scheme 1). In the latter case, N-terminally and side-chain protected crude peptide Boc-Bac(1–35)-OH (**1a**) was cleaved from the resin with HFIP, and Bac-arylthioester (**3**) was prepared according to the general method of Beyermann [29,30]. The free thiol group of **2a** was than reacted with excess bromoacetic acid in the presence of base to form the S-carboxymethylated Bac-derivative (**2**) (Scheme 1 and Table 1).

2.4. PEGylation of Bac7(1–35)

Two sets of C-terminally PEGylated Bac7(1–35) were synthesized: C-terminal esters – left column on Scheme 2, and C-terminal amides – right column on Scheme 2. mPEG-$OCOCH_2Br$ (**4**) was prepared by bromoacetylation of commercial HO-PEG-OMe (mPEG-OH) with $BrCH_2COBr$/DIEA, according to published procedures [31,32]. NH_2-PEG-OMe (mPEG-NH_2) (**5**) was prepared in 3 steps from mPEG-OH. Bromoacetylation of **5** with the activated ester of bromoacetic acid $BrCH_2COOSu$ (Scheme 2) produced amide **6**. Coupling of **5** with N, S-protected cysteine led to H-Cys(H)–NH-PEG-OMe (**7**). Thioether ligation of H-Bac7(1–35)-Cys(*H*)–OH (**2a** – Scheme 1) with excess of bromoacetyl-PEG-ester (**4**) or bromoacetyl-PEG-amide (**6**) produce the expected C-terminal PEGylated Bac7(1–35) ester [**8**, Bac_E-PEG] or amide [**9**, Bac_A-PEG, see Table 1], with good yield.



Scheme 1. Preparation of peptide precursors.

H-Cys(H—OC(O)—OPEG—OMe (**10**) was prepared with excellent purity and yield by activation of an excess of Boc-Cys(Trt)-OH with Boc_2O/pyridine [33], followed by coupling to mPEG-OH, deprotection and purification of the ester. Similarly, mPEG-NH_2 was coupled to Boc-Cys(Trt)-OH, activated with HCTU/DIEA to obtain H-Cys(H)—NH-PEG-OMe (**7**). An excess of H-Cys-OPEG-OMe (**10**) or H-Cys—NH—PEG-OMe (**7**) were then reacted with Bac7(1—35)-SPhNHAc (**3**) under conditions of native chemical ligation [34] and respectively formed the C-terminal PEGylated ester Bac7(1—35)-Cys-OPEG-OMe (**11**) and PEGylated amide Bac7(1—35)-Cys-NHPEG-OMe (**12**). Michael addition of Alexa Fluor 680 maleimide or BODIPY® FL N-(2-aminoethyl) maleimide to the free thiol group in **11** or **12** formed the corresponding succinimide adducts (**13**, **14**) and (**15**), according to modifications of a published procedure [14] or further explained in the Supplementary data. The free thiol in Bac7(1—35)-Cys(H)-OPEG-OMe (**11**) was reacted with an excess of bromoacetic acid in the presence of base (as already described for **2** → **2a**) and formed S-carboxymethylated Bac7(1—35)-Cmc-OPEG-OMe (**17**).

A detailed description of all the reactions reported in Scheme 2 is available in the Supplementary data. The concentration of peptides was determined using three methods: *i*) by accurate weighing after lyophilisation; *ii*) using the method of Waddell [35]; *iii*) by analytical RP-HPLC using an internal standard. PEGylated peptides were also checked by acetic acid-urea PAGE for any eventual release of free peptide (see Supplementary data).

2.5. Serum and plasma preparation

Murine serum and plasma were drawn from a pool of mice. Human serum and plasma were taken from a pool of healthy donors. Human and murine plasma was obtained with 2% (v/v) Na-citrate as anticoagulant. Fluid samples were centrifuged at 13,000 g for 5 min and stored at −20 °C until use.

2.6. Bacterial strains and growth conditions

The strains used in this study were *Salmonella enterica* serovar Typhimurium ATCC 14028 (*S. typhimurium*), *E. coli* BW25113 and *E. coli* BW25113Δ*sbmA*, a deletion mutant for the *sbmA* gene. The inoculum was incubated overnight at 37 °C with shaking. For the assays the overnight bacterial cultures were diluted 1:30 in fresh Mueller—Hinton Broth (MHB) and incubated at 37 °C with shaking for approximately 2 h.

2.7. Bac7 stability and its release from PEGylated forms in serum and plasma

Bac and Bac_E-PEG (120 μg) were incubated in 200 μl of 25% (v/v) murine or human serum/plasma in PBS at 37 °C. At different times, aliquots were withdrawn, diluted 1:5 in sample buffer (12% SDS, 6% dithiothreitol, 40% glycerol, 0.05% bromophenol blue, 150 mM Tris—HCl, pH 7), incubated for 15 min at 60 °C and analysed on a 16% Tricine/SDS gel. Proteins were then blotted onto nitrocellulose membrane and incubated overnight with shaking in 40 mM Tris—HCl, pH 7.5, 5% non-fat milk, 0.1% Tween 20, 200 mM NaCl (Blocking Solution, BS) at 4 °C. Samples were incubated at room temperature for 2 h with rabbit anti-Bac7(1—35) IgG diluted 1:1000 in BS, followed by an HRP-conjugated anti-rabbit IgG (1 h incubation). The detection of the chemiluminescence was performed using the commercially available kit ECL Plus Western Blotting Detection Reagent (Pierce).

2.8. In vivo biodistribution by time-domain optical imaging

In vivo biodistribution studies have been performed as previously described [14]. Two mice were injected intraperitoneally with 200 μl of Bac_E-PEG-Alexa680 in PBS corresponding to 6.9 nmol AlexaFluor® 680, one monitored in the abdominal region and the other in the renal region for 24 h. A blank image was acquired before treatment of each animal and this was subtracted from the images of the treated animal. The experiment was repeated twice.

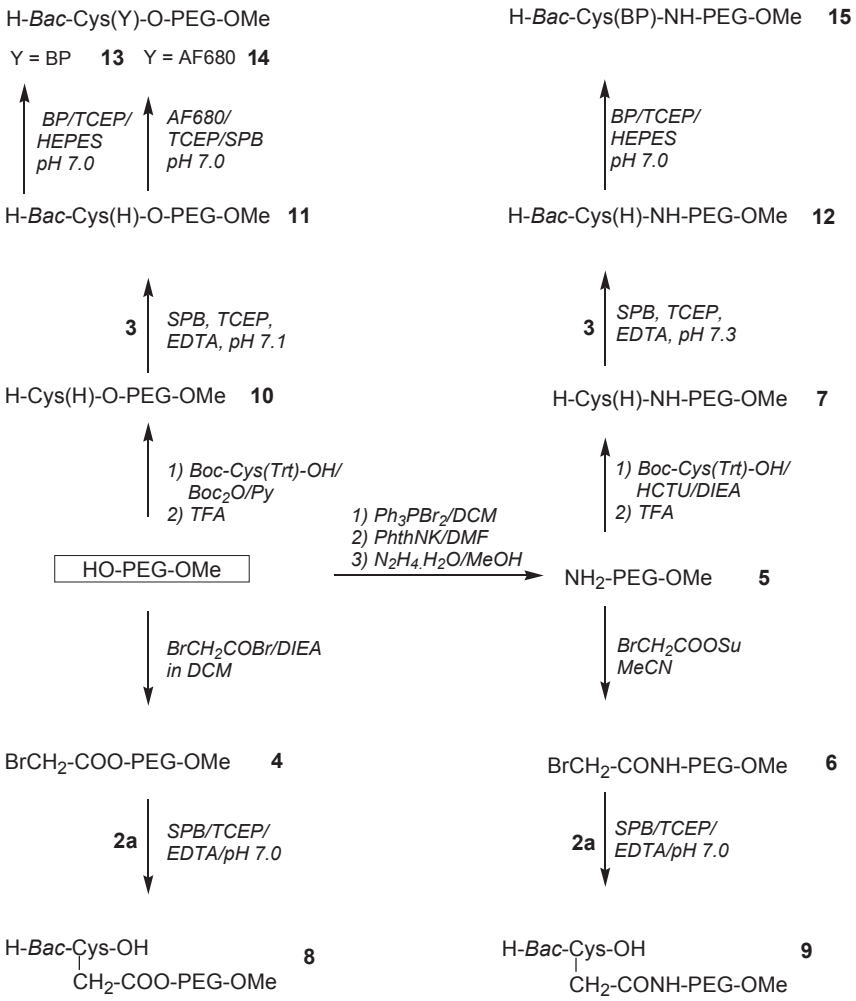
Two-dimensional scanning regions of interest (ROI) were selected and the laser power, integration time and scan step were optimized according to the signal emitted. The data were recorded as temporal point-spread functions, and the images were reconstructed as fluorescence intensity and lifetime. All the experimental procedures were performed according to the guidelines of the European (86/609/EEC) and the Italian (D.L.116/92 and subsequent addenda) laws and approved by the Italian Ministry of University and Research as well as by the Animal Experimentation Committee of the University Animal House.

2.9. Minimum inhibitory concentration

Broth microdilution susceptibility assay was performed as previously described [6]. Briefly, two-fold serial dilutions of peptides were prepared in 96-well microplates in MHB with the addition of human or murine serum or plasma to reach the final concentration of 25% after bacteria addition, and 100% MHB as control. To study the effect of the preincubation on Bac_E-PEG activity, bacteria were added to each well containing peptide in 25% serum or plasma after 24 h at 37 °C. The MIC (Minimum Inhibitory Concentration) was taken as the lowest concentration of antimicrobial peptide resulting in the complete inhibition of visible growth after 24 h of incubation at 37 °C.

2.10. Growth kinetics assay

Bacterial growth inhibition tests were performed using mid-log phase bacterial cultures diluted in MHB to 1×10^6 CFU/ml,



AF680: Alexa Fluor 680 Maleimide (Cys-succinimide adduct); **BP**: BODIPY® FL N-(2aminoethyl)maleimide (Cys-succinimide adduct); **HO-PEG-OMe** = HO-CH_2CH_2-$(OCH_2CH_2)n_{av}$-OCH_2CH_2OMe, where n_{av} = 452± 36

Scheme 2. Amide or ester bond C-terminal PEGylation of Bac7(1–35).

in presence or absence of different concentrations of peptides. Bacterial growth was followed at 37 °C with intermittent shaking for 4 h by measuring every 10 min the absorbance at 620 nm with a microplate reader (Tecan Trading AG, Switzerland). To evaluate if the culture medium causes the release of free peptide, Bac_E-PEG was incubated in MHB at 37 °C for different times up to 4 h. At the end of incubation, 10 μl of each sample were loaded on a 16% Tricine/SDS gel. As control, Bac in water or MHB was loaded.

The gel was stained by Coomassie blue and iodine staining for protein bands and PEG species, respectively. For iodine staining, the gel was stained with a 5% Barium chloride solution in milliQ water (5% w/v $BaCl_2$ in 100 ml milliQ water, ~240 mM HCl) for 15 min, washed in distilled water for 30 min, floated in 0.1 M iodine solution (2% w/v KI, 1.27% w/v I_2 in milliQ water) for 15 min and washed in distilled water again [36].

Table 1
List of peptides used in this study.

Compound	Name used	Number[a]
Bac7(1–35)	Bac	1
Bac7(1–35)-Cys(BODIPY-maleimide)	Bac-BY	–
Bac7(1–35)-Cys(H)–NH-PEG-OMe	Bac_A-PEG	9
Bac7(1–35)-Cys(BODIPY-maleimide)–NH–PEG-OMe	Bac_A-PEG-BY	15
Bac7(1–35)-Cys(–CH_2CO–OPEG-OMe)-OMe	Bac_E-PEG	8
Bac7(1–35)-Cys(-CH_2COOH)	Bac_{CMC}	2
Bac7(1–35)-Cys(BODIPY-maleimide-)-O–CH_2CO–OPEG-OMe	Bac_E-PEG-BY	13
Bac7(1–35)-Cys(ALEXAFluorC2-maleimide-)-O–CH_2CO–OPEG-OMe	Bac_E-PEG-Alexa	14

[a] Numbers indicate the corresponding compounds described in Scheme 1 and 2.

2.11. Flow cytometric analysis

Integrity of the cell membrane in *Salmonella* cells was assessed by measuring the PI uptake by flow cytometry, as previously described [7]. For the uptake evaluation, we used a cytofluorimetric method described in Benincasa et al. [37]. Data analysis was performed with the FCS Express3 software (De Novo Software, Los Angeles, CA).

2.12. Confocal scanning laser microscopy (CSLM)

CSLM analysis were performed by using a Nikon C1–SI confocal microscope. An oil immersion objective lens was used. The optimum photomultiplier setting was determined in preliminary experiments performed *ad hoc*, and then the same setting was used for all samples. *Salmonella typhimurium* ATCC 14028 cells treated for 2 h with 0.25 μM Bac-BY, 1 μM Bac_E-PEG-BY and 4 μM Bac_A-PEG-BY, or for 10 min with 0.25 μM Polymyxin B, were prepared following the same protocol used for the flow cytometric uptake assay without any fixation. Ten μl of each bacterial suspension were placed between two cover-glasses to obtain an unmovable monolayer of cells. The image stacks collected by CSLM were analysed with the EZ-C1 Free Viewer (Nikon Corporation) and the Image J 1.40 g (Wayne Resband, National Institutes of Health, USA) software.

3. Results

3.1. Preparation of PEGylated forms of Bac7(1–35)

Bac7(1–35) (hereafter named Bac) was conjugated to 20 kDa poly(ethylene glycol) (PEG) using two different strategies (Scheme 2). In one case the peptide was linked to the polymer through an amide bond (Bac_A-PEG) (compound **9** in the Scheme), and in the other via an ester bond (Bac_E-PEG) (compound **8**) that in principle allows the peptide to be released by blood esterases producing a carboxymethyl derivative of Bac7(1–35) (Bac_{CMC}) (compound **2**). Corresponding fluorescent derivatives of Bac_A-PEG and Bac_E-PEG have also been prepared to allow detection of the PEGylated peptides in mice or when internalized into bacterial cells (Scheme 2). All the compounds and their names are listed in Table 1.

3.2. Antimicrobial activity of PEGylated Bac

Both amide- and ester-linked forms of the PEGylated peptide showed a good antibacterial activity against *S. typhimurium* (Table 2). In particular, when assayed in Mueller–Hinton broth (MHB), Bac_E-PEG showed MIC values of 4–8 μM and Bac_A-PEG of 8–16 μM, respectively 4- and 8-fold less active than that of the unconjugated peptide. The anti-salmonellae activity was evaluated also by growth inhibition assays. At 2 μM, both Bac_E-PEG and Bac_A-PEG reduced bacterial growth by 75% after 4 h (Fig. 1A and B) while unmodified Bac showed a similar growth inhibition at 0.5 μM (Fig. 1C), confirming a difference in activity in line with the MIC. The rapid antibacterial effect of the modified peptides, which initiated without delay, suggested that the PEGylated forms *per se* were at least in part responsible for the antibacterial activity in spite of the steric hindrance due to the presence of the PEG group. To further support this hypothesis, Bac_E-PEG was incubated in MHB for 4 h, and the products analysed by SDS-PAGE. No free peptide was detected (data not shown), confirming that short-term

Table 2
Microdilution susceptibility assay of Bac7(1–35) and its PEGylated forms against *Salmonella typhimurium*.

Peptide	MIC (μM)				
	Mueller–Hinton	Human		Murine	
		Serum	Plasma	Serum	Plasma
Bac	1–2	0.5–1	0.06–0.12	32	≥32
Bac_A-PEG	8–16	8–16	2	>32	>32
Bac_E-PEG	4–8	2–4	1–2	64	16
Bac_E-PEG (24 h)[a]	2	1	0.25	nd	nd
Bac_{CMC}	0.5–1	0.5	0.03–0.06	nd	nd

MIC values are representative of three independent experiments with comparable results.
[a] Peptide was incubated for 24 h in the indicated media before the addition of bacteria (see Materials and methods Section).

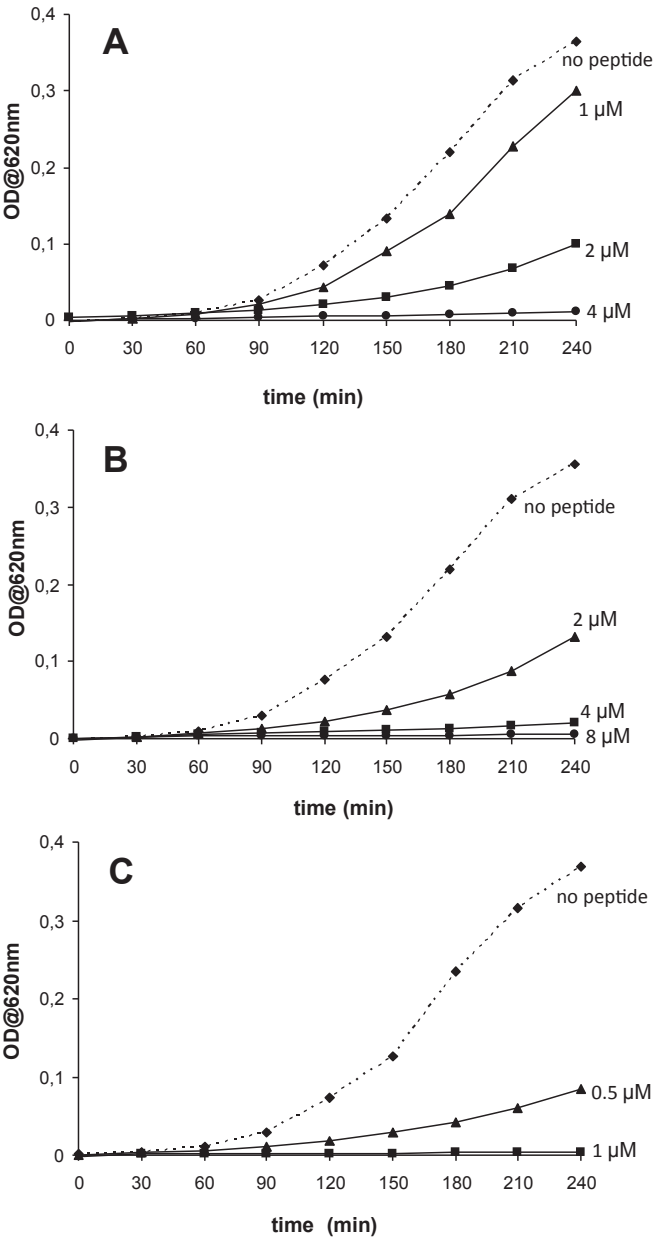


Fig. 1. Growth kinetics of *Salmonella typhimurium* in the presence of BacE-PEG, BacA-PEG and Bac. Bacterial growth in MHB was monitored at 620 nm. The dotted line indicates the growth rate of the untreated bacterial cells, the continuous lines indicate the growth rate of bacterial culture in the presence of the indicated concentrations of BacE-PEG (A), BacA-PEG (B) and Bac (C). Growth kinetics are representative of three independent experiments with identical results.

antimicrobial activity was due to the conjugated forms.

3.3. Release of Bac in human body fluids and stability of the native peptide

The kinetics of release of Bac from Bac_E-PEG in the presence of human serum and plasma is shown in Fig. 2. A peptide with the expected size was detected using an anti-Bac7 antibody after 8 h of incubation with either plasma or serum and release was nearly complete within 72 h (Fig. 2A and B). On the other hand Bac_A-PEG did not release free Bac within the same time period (data not shown). To verify if Bac is stable and not degraded in the body fluids after being released from PEG, a Western blot analysis of unmodified Bac incubated with human serum or plasma was carried out (Fig. 2C). Results indicated that the Bac was not degraded in both body fluids up to at least 72 h incubation.

3.4. Effects of serum and plasma on the antibacterial activity of PEGylated Bac

Bacterial susceptibility testing was performed in the presence of human serum or plasma to evaluate their effects on the antibacterial activity of all the peptides (Table 2). Different results were obtained depending on the type of body fluid. The addition of 25% human serum to the medium did not modify the anti-salmonellae activity of Bac_A-PEG (MIC = 8–16 μM) or of unconjugated Bac (MIC = 0.5–1 μM). Conversely, it resulted in an appreciable increase in the activity of Bac_E-PEG (MIC = 2–4 μM) likely due to the partial release of the peptide (Table 2). In addition, when the assay was carried out using an aliquot of Bac_E-PEG preincubated for 24 h with 25% human serum, the activity showed a further 2 fold increase, reaching a MIC of 1 μM. This value is similar to that measured for Bac_{CMC}, the carboxymethyl peptide that would be obtained by ester bond hydrolysis of Bac_E-PEG (0.5–1 μM) (Table 2). Overall these results show that in human serum an active peptide is released from the polymer explaining the somewhat higher activity of Bac_E-PEG than Bac_A-PEG.

Interestingly, in the presence of plasma (25%) the activity of all the peptides against *S. typhimurium* was increased 4–8 fold (Table 2). It is significant that the increase in activity occurred to the same extent for both PEGylated and non PEGylated peptides and we are currently unable to propose a reason for this effect, so that further investigations are required to clarify it.

As *in vivo* experiments were carried out in a mouse model, the effects of murine serum and plasma were also tested. In contrast to what was observed in the presence of human body fluids, the antibacterial activity of unconjugated Bac and of both the pegylated forms was scarce in the presence of murine serum or plasma (Table 2).

3.5. Internalization into S. typhimurium cells

In order to evaluate whether the PEGylated derivatives retained the same mechanism of action as the native peptide, we monitored the cellular uptake using the fluorescent derivatives Bac_A-PEG-BY and Bac_E-PEG-BY. First, however, we performed a permeabilization assay to exclude that the PEGylated forms could damage the bacterial membranes. The percentage of propidium iodide (PI)-positive bacterial cells treated with the PEGylated peptides at their MIC, or slightly above MIC values, did not increase when compared to the free peptide (<2% of PI positive cells, data not shown), suggesting membrane permeabilization does not occur.

When Bac_A-PEG-BY and Bac_E-PEG-BY were incubated with *S. typhimurium* at 1 μM concentrations, the detected fluorescent signal indicated that they had been internalized into the cells, albeit at a lower level than unPEGylated Bac-BY (Fig. 3A). Furthermore, Bac_E-PEG-BY exhibited a higher level of internalization than Bac_A-PEG-BY. Uptake assays were also carried out using Bac-BY, Bac_E-PEG-BY and Bac_A-PEG-BY at a concentration corresponding to ¼ the MIC values. Under this condition a similar amount of each peptide was found inside the bacterial cells and with an analogous time-dependence (Fig. 3B).

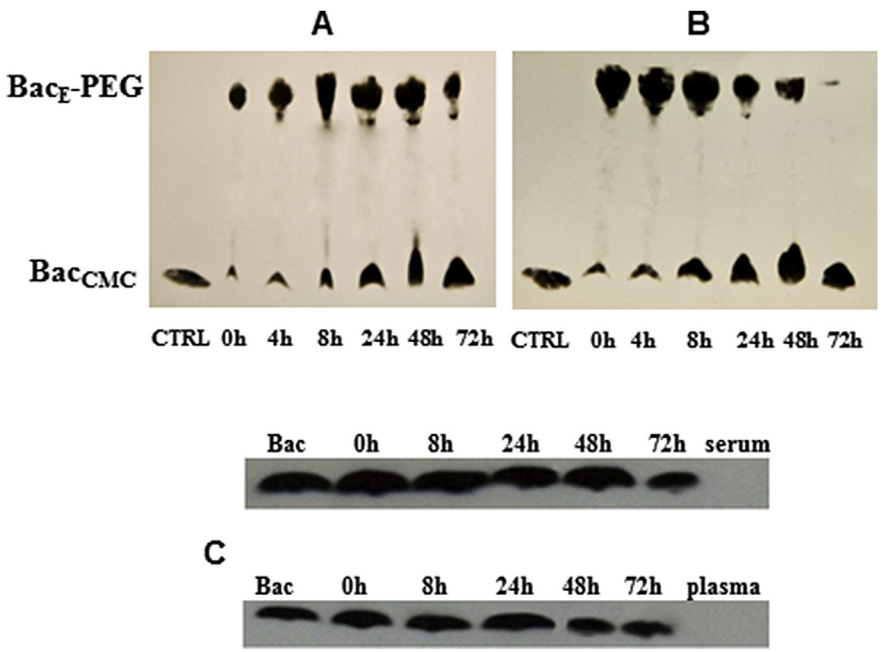


Fig. 2. Evaluation of Bac release from PEG conjugates in the presence of human biological fluids and Bac7 stability in human blood fractions. Western blot analyses of BacE-PEG in human plasma (A) and human serum (B). BacE-PEG was incubated in 25% human plasma or serum up to 72 h at 37 °C. Samples were then loaded on the gel to allow peptide detection with a specific anti-peptide antibody. Western blot analysis of Bac incubated for different times at 37 °C in 25% human serum or plasma (C). Lane 1: 0.5 μg Bac; lanes 2–6: Bac after incubation with human or murine serum and plasma for the indicated times; lane 7: serum or plasma alone.

The localization of the peptides in *S. typhimurium* cells was examined by confocal scanning laser microscopy (CSLM) (Fig. 3C). A homogeneous distribution of Bac and of both the PEGylated derivatives in the cell cytoplasm was observed. In contrast, the membrane-acting antibiotic polymyxin B was visible mainly on the cellular surfaces, as expected.

To evaluate whether the internalization of the PEGylated compounds involves the same inner membrane protein transporter SbmA responsible for Bac translocation, we repeated the uptake assays using the *E. coli* BW25113Δ*sbmA* mutant strain with deleted *sbmA* gene. The susceptibility of this mutant strain was significantly decreased to both the PEGylated forms with respect to the wild-type strain (Fig. 4A and B), and corresponded to a parallel reduction in the level of uptake of both Bac_A-PEG-BY and Bac_E-PEG-BY by the mutated strain (Fig. 4C). This confirms the involvement of the SbmA protein also in the internalisation of the 20 kDa-PEGylated form of the peptide.

3.6. In vivo biodistribution

In vivo biodistribution analyses and evaluation of the permanence of the peptide in mice were performed using another fluorescent derivative of Bac_E-PEG. For this purpose, the maleimide group of Alexa Fluor680© was linked to the free thiol group of the cysteine residue located between the peptide and the PEG tail to give Bac_E-PEG-Alexa. Biodistribution analyses were performed on healthy mice by monitoring the availability of the labelled, PEGylated peptide up to 24 h after its intraperitoneal administration and performing *ex vivo* analysis at 24 h. Results showed that the 24 kDa PEGylated form of Bac had a much wider distribution than the unPEGylated peptide and was detected in different organs (kidney, liver and peritoneal cavity) (Fig. 5A and B). Furthermore, it was still present at 24 h post-injection, suggesting a slower renal clearance compared to the unmodified peptide [14] and clearly due to the increased molecular mass. *Ex vivo* analysis showed a significant concentration of the PEGylated Bac in liver even at 24 h after injection, as well as in kidney (Fig. 5C), indicating a prolonged *in vivo* half-time for the peptide after its conjugation to PEG.

4. Discussion

AMPs are a promising class of antimicrobial biomolecules that originated millions of years ago as part of innate immunity [38]. As pharmaceuticals, however, AMPs have several disadvantages including toxic side effects observed at higher doses (especially for lytic AMPs targeting the membranes), and short circulation time in blood, due to their degradation by blood-borne proteases and/or a rapid clearance through the urinary tract. We have previously shown that Bac is not toxic for eukaryotic cells [8] and well tolerated in a mouse toxicity model [14] but rapidly cleared by the

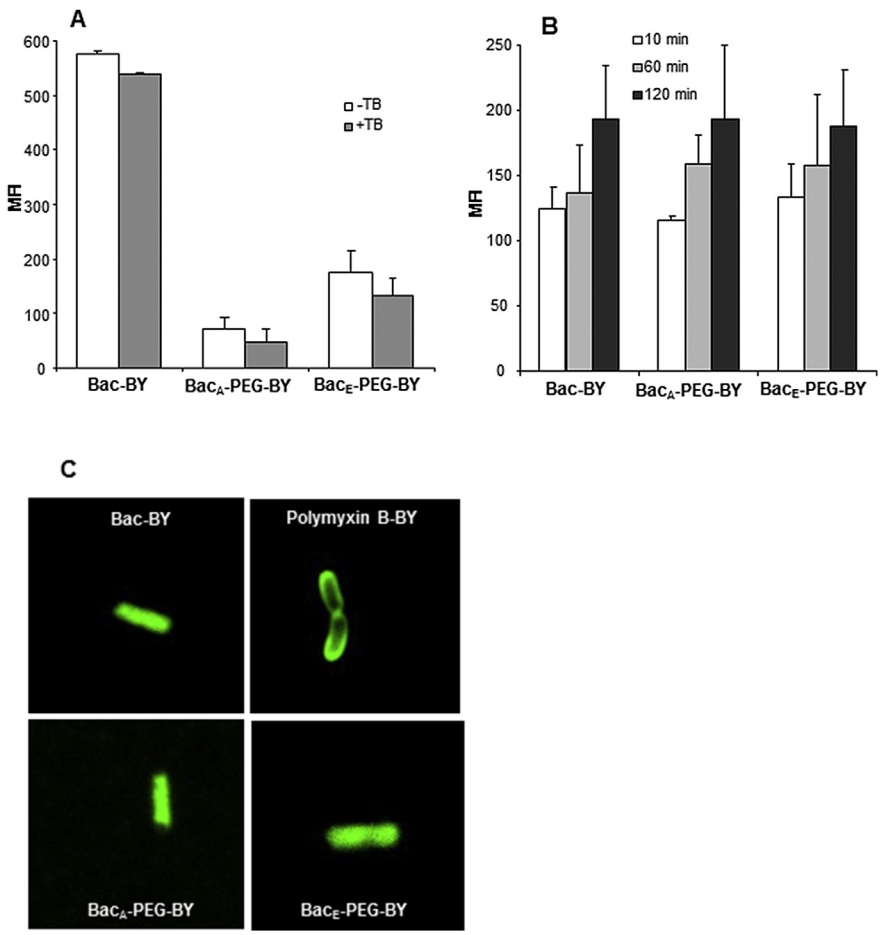


Fig. 3. Uptake studies of PEGylated forms of Bac. (A) Fluorescence of *S. typhimurium* cells exposed for 10 min to 1 μM Bac-BY, BacA-PEG-BY and BacE-PEG-BY, and analysed by flow cytometry with (grey histograms) or without (empty histograms) incubation with Trypan Blue (TB) quencher for 10 min at 37 °C. (B) Fluorescence of *S. typhimurium* cells exposed for the indicated times to 0.25 μM Bac-BY, 4 μM BacA-PEG-BY and 1 μM BacE-PEG-BY (¼ the respective MIC values), and analysed by flow cytometry after incubation with Trypan Blue (TB) quencher for 10 min at 37 °C. For both plots, the Mean Fluorescence Intensity (MFI) value, with SD as error bars, is reported in the y axis. (C) Confocal microscopy images of *S. typhimurium* treated with Bac-BY, Polymyxin B, BacE-PEG-BY and BacA-PEG-BY. All images are representative sections from the middle of the bacterial cell. Many fields were examined and, for each experiment, over 95% of the cells displayed the pattern of the respective representative cell shown here.

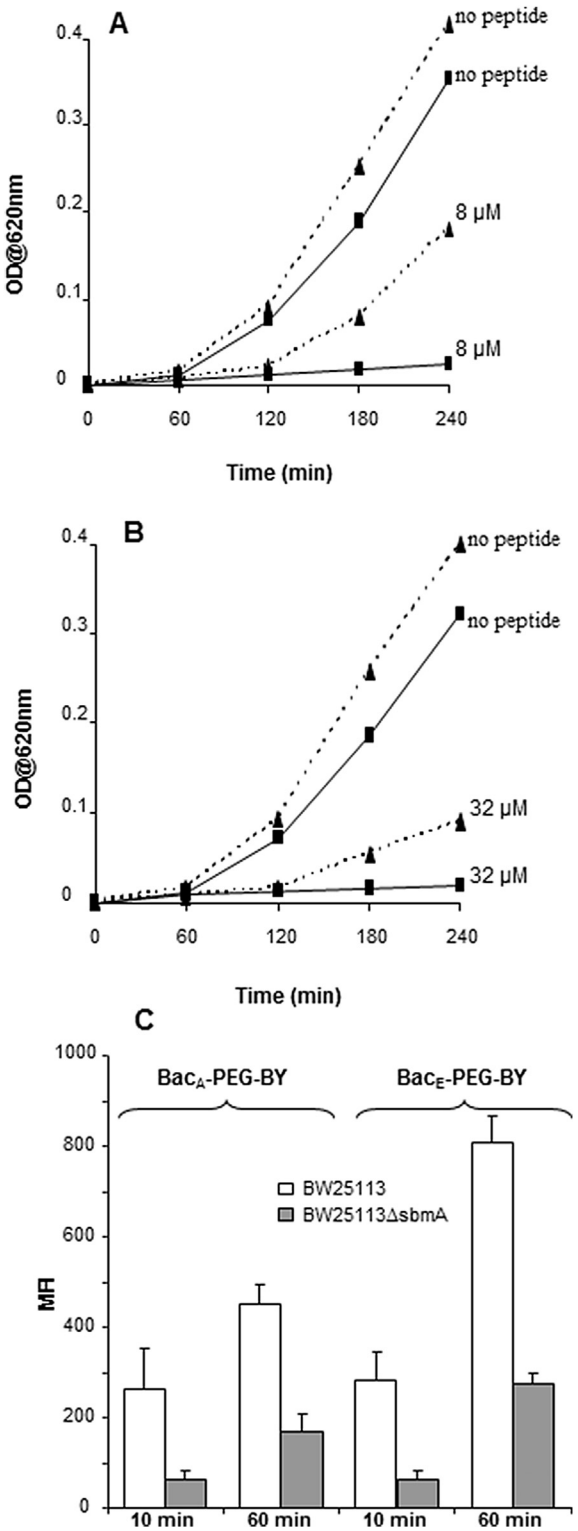


Fig. 4. The involvement of the membrane protein SbmA in the activity/uptake of the PEGylated peptides in *E. coli*. Growth kinetics of *E. coli* BW25113 and *E. coli* BW25113ΔsbmA in the presence of BacE-PEG-BY (A) and BacA-PEG-BY (B) at their MIC value. In the figure the continuous line indicates the wild-type *E. coli* BW25113 strain, and the dotted line indicates the mutant *E. coli* BW25113ΔsbmA. Growth kinetics are representative of three independent experiments with identical results. (C) Uptake of 4 μM BacA-PEG-BY and 2 μM BacE-PEG-BY into *E. coli* BW25113 and BW25113ΔsbmA strains. The Mean Fluorescence Intensity (MFI) value, with SD as error bars, is reported in the y axis.

kidney when injected intraperitoneally into mice.

In this work we have demonstrated that PEGylated derivatives of Bac [i.e. Bac7(1–35)] retain an appreciable antimicrobial activity and have substantially the same mode of action as the unmodified peptide, while exhibiting a lower clearance rate. This chemical modification could therefore considerably enhance its *in vivo* efficacy. It is a further example of the usefulness of covalent attachment to poly(ethyleneglycol) (PEG) chains of potential therapeutic agents as a leading approach for overcoming limits, such as physical and chemical instability, immunogenicity and rapid body clearance [39].

In selecting the best strategy for linking PEG to Bac we considered the particular mode of action of this peptide. Bac has to penetrate into bacterial cells to kill them [7] and the modification of amino acids at its N-terminus markedly reduces its activity [40]. For this reason we preferred to link the PEG tail to the C-terminus. Furthermore, we decided to use an ester bond in order to obtain a conjugate which was susceptible to blood esterases. As expected, while stable in MH medium, the unPEGylated peptide was progressively released by Bac$_E$-PEG on a time-scale of several hours in the presence of human serum or plasma. The released Bac peptide, being very rich in proline, then itself remains stable in serum for at least 72 h, and showed MIC values comparable to those of the native peptide (Table 2).

The mechanism of action of unmodified Bac involves transport by the SbmA membrane protein, so it was quite encouraging that the PEGylated conjugates should continue to have an appreciable anti-salmonella activity, and somewhat surprising for the Bac$_A$-PEG conjugate which is linked via a stable amide bond. This suggested that even in the ~24 kDa PEGylated form they could exert some antimicrobial activity. In the case of Bac$_E$-PEG it is likely that the this activity was due to both the PEGylated form and free released peptide. The difference in anti-salmonella activity between Bac$_E$-PEG and Bac$_A$-PEG is effectively only observed in presence of serum and plasma, but not medium, and thus likely due to the partial release of the peptide. The contribution to the antibacterial activity of the intact form is suggested by that of Bac$_A$-PEG, showing an 8-fold reduction of activity against *S. typhimurium* relative to Bac (Table 2).

Both PEGylated peptides exhibited loss of activity when assayed in murine plasma and serum with respect to human fluids. This result is in agreement with previous observations showing significantly reduced *in vitro* and *in vivo* antibacterial activity in presence of serum and plasma of mice, also due to peptide degradation [14]. The different influence of human and murine biological fluids on the antibacterial activity of Bac could find an explanation in the different composition of blood enzymes and/or proteins in the two species. A number of proteases and carboxy esterases have in fact been identified in murine blood that seem to be absent in human blood [41].

Despite an at least five-fold increased in size, both Bac$_E$-PEG and Bac$_A$-PEG conserve a mechanism of action mediated by their uptake into target cells. The detection of cytoplasmic fluorescence in salmonellae cells, particularly after incubation with the esterase-stable Bac$_A$-PEG-BY, indicates that the PEGylated forms can still translocate across the bacterial membranes. Internalization of the PEGylated peptides was understandably lower than that of unconjugated Bac, in agreement with a reduced, but not abrogated, activity. The fluorophore in the labelled derivatives was linked to the peptide and not to the PEG molecule (Scheme 2), so for Bac$_E$-PEG-BY it could not be excluded that the fluorescence inside the bacterial cells was due only to peptide released from PEG on the surface of the target cells and subsequently internalized, but this is quite unlikely for a stable derivative such as Bac$_A$-PEG-BY.

We have conclusively shown that the inner membrane protein

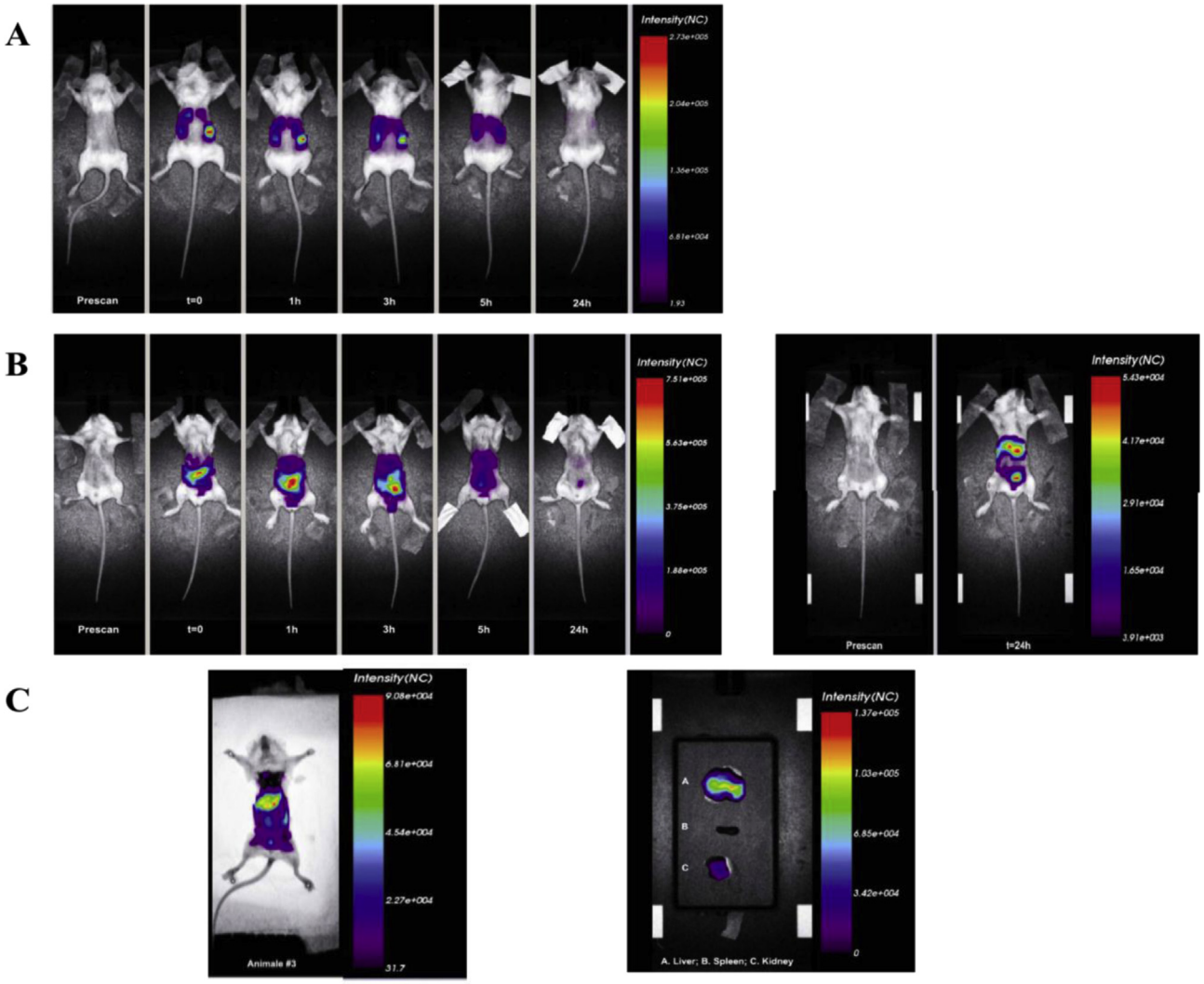


Fig. 5. Biodistribution of BacE-PEG-Alexa in healthy mice after i.p. injection. The animals were placed in a prone position (A) and supine position (B); fluorescence emission in the regions of interest encompassing respectively the kidneys, the thorax and abdomen, were acquired at the indicated times. Panel B on the right shows the fluorescence emission of the mouse in supine position at 24 h post injection and normalized with respect to the prescan acquired before peptide injection. (C) Ex vivo images: post-mortem images of mice at 24 h after i.p. injection of PEGylated BacE-PEG-Alexa. Imaging of the mice was performed immediately after sacrifice, after removal of the abdominal wall, and normalized. A specific signal was detected in kidney, liver and the peritoneal cavity (panel on the left); *ex vivo* images of organs at 24 h after i.p. injection of PEGylated BacE-PEG-Alexa. Imaging of the organs was performed immediately after sacrifice and normalized. The images are representative of one of three mice that showed comparable results.

SbmA, a secondary transporter with low-specificity for substrates previously shown to translocate Bac, is also involved in the uptake of Bac_E-PEG and Bac_A-PEG [5,10,12]. This protein is required for the uptake of structurally quite different molecules including proline-rich peptides, bleomycin, bacterial mycrocins and peptide nucleic acids [42]. Our results suggest that SbmA's transport capacity extends to the uptake of even quite large molecules ($\approx$24 kDa), albeit with a somewhat reduced efficiency, making it a potentially useful for transporting cargo into bacteria that express it.

The reduced excretion of the peptide is one of the most important effects of Bac7 PEGylation. A fluorescent derivative of Bac_E-PEG was still detectable at 24 h post-injection while the unmodified peptide disappeared within 3 h after injection [14]. Despite the molecular weight cutoff for glomerular filtration is thought to be 30–50 kDa [43], the increased mass of Bac7(1–35) to $\approx$ 24 kDa in the PEGylated form strongly delays the excretion of the peptide. The slower renal clearance increases peptide biodistribution compared with the unPEGylated form, which included the liver and peritoneal cavity other than kidney in agreement with previous observation. This result supports the possibility of increasing the therapeutic potential of Bac7.

In conclusion we have demonstrated that the addition of a 20 kDa tail of PEG to Bac is a workable strategy to enhance the *in vivo* properties of the peptide, improving its pharmacokinetics, while only marginally affecting its antibacterial activity and not substantially modifying its mode of action. PEG has already been approved for use in the formulation of many drugs and cosmetics [44] and the number of PEGylated products on the market is continuously increasing [39]. For this reason this kind of modification is promising for the future therapeutic application of anti-infective agents based on Bac7, and may be applied also to a vast repertoire of other AMPs with relative ease.

Author contributions

Monica Benincasa performed most of the experiments on the antibacterial activities of the peptides and wrote the initial draft of the manuscript; Sotir Zahariev designed, synthesized and purified

all the compounds some of which with the help of Chiara Pelillo; Chiara Pelillo and Annalisa Milan performed part of the *in vitro* assays and data analysis; Renato Gennaro contributed to conceptual advices and to the writing of the manuscript; Marco Scocchi supervised the experiments and edited the manuscript with the contribution of all the other authors. All authors have given approval to the final version of the manuscript.

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