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Original article

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$_2$Br (**4**) was prepared by bromoacetylation of commercial HO-PEG-OMe (mPEG-OH) with BrCH$_2$COBr/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$_2$COOSu (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.