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Novel human bioactive peptides identified in Apolipoprotein B: Evaluation of their therapeutic potential

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ABSTRACT

Host defence peptides (HDPs) are short, cationic amphipathic peptides that play a key role in the response to infection and inflammation in all complex life forms. It is increasingly emerging that HDPs generally have a modest direct activity against a broad range of microorganisms, and that their anti-infective properties are mainly due to their ability to modulate the immune response. Here, we report the recombinant production and characterization of two novel HDPs identified in human Apolipoprotein B (residues 887–922) by using a bioinformatics method recently developed by our group. We focused our attention on two variants of the identified HDP, here named r(P)ApoB$_L$ and r(P)ApoB$_S$, 38- and 26-residue long, respectively. Both HDPs were found to be endowed with a broad-spectrum antimicrobial activity while they show neither toxic nor haemolytic effects towards eukaryotic cells. Interestingly, both HDPs were found to display a significant anti-biofilm activity, and to act in synergy with either commonly used antibiotics or EDTA. The latter was selected for its ability to affect bacterial outer membrane permeability, and to sensitize bacteria to several antibiotics. Circular dichroism analyses showed that SDS, TFE, and LPS significantly alter r(P)ApoB$_L$ conformation, whereas slighter or no significant effects were detected in the case of r(P)ApoB$_S$ peptide. Interestingly, both ApoB derived peptides were found to elicit anti-inflammatory effects, being able to mitigate the production of pro-inflammatory interleukin-6 and nitric oxide in LPS induced murine macrophages. It should also be emphasized that r(P)ApoB$_L$ peptide was found to play a role in human keratinocytes wound closure *in vitro*. Altogether, these findings open interesting perspectives on the therapeutic use of the herein identified HDPs.

1. Introduction

The excessive and sometimes improper use of antibiotics has been responsible for the development of resistant bacterial isolates, the so-called 'superbugs', such as methicillin-resistant *Staphylococcus aureus* (MRSA) [1], vancomycin-resistant enterococci (VRE), and multidrug-resistant *Pseudomonas*, *Klebsiella*, and *Acinetobacter* [2]. This made the search for novel antimicrobial therapies and approaches imperative. In this scenario, the broad immunomodulatory properties of naturally occurring host defence peptides (HDPs) have attracted considerable attention. HDPs, also known as antimicrobial peptides (AMPs), are evolutionarily conserved molecules of the innate immune system. They are a

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; HDPs, host defence peptides; AMPs, antimicrobial peptides; LPS, lipopolysaccharide; ApoE, Apolipoprotein E; ApoB, Apolipoprotein B; LDL, low-density lipoprotein; IL-10, interleukin-10; MIC, minimal inhibitory concentration; TSA, Tryptic Soy Agar; AS, absolute score; MHB, Muller Hinton Broth; NB, Nutrient Broth; IPTG, isopropyl-β-D-thiogalactopyranoside; TFE, trifluoroethanol; SDS, sodium dodecyl sulfate; FIC, fractional inhibitory concentration; EDTA, ethylenediaminetetraacetic acid; IL-6, interleukin-6; NO, nitric oxide; CATH-2, cathelicidin-2; ONC, onconase; PBS, phosphate-buffered saline; CD, circular dichroism; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MALDI-MS, matrix assisted laser desorption ionisation mass spectrometry; RBCs, red blood cells; WH, wound healing.

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key element of the ancient, nonspecific innate defence system in most multicellular organisms, representing the first line of defence against invading microbes [3,4]. Found in all complex living organisms, HDPs have first attracted considerable attention for their modest antimicrobial activity directed towards a broad spectrum of pathogens including bacteria, viruses, fungi, and protozoa [3,4]. Natural HDPs have a size ranging from 12 to 50 amino acids, are mostly cationic owing to the presence of high levels of lysine and arginine residues, and contain over 50% hydrophobic amino acids [3,5]. These properties are at the basis of HDPs' ability to interact with membranes, and, in some cases, to penetrate cell membranes. In model systems, HDPs associate preferentially with negatively charged membranes of bacteria-like composition, but many peptides are also able to translocate into host cells. To date, the molecular bases of their selectivity towards bacterial membranes are still poorly understood [6]. It has been suggested that HDPs tend to translocate into bacterial cells owing to the presence of a large electrical potential gradient [7]. However, although HDPs' direct antimicrobial mechanism of action against bacteria mainly involves interaction with the bacterial membrane, multiple targets have been identified, such as cell wall peptidoglycans, cytosolic RNA, proteins, or cytosolic enzymes/chaperones [6,8]. Hence, the selection of resistance mechanisms in bacteria is improbable, since the removal of a single target, *e.g.* by mutation, would still allow other targets to mediate HDPs direct killing activity [8]. However, it is becoming increasingly evident that these peptides are endowed with a wide range of biological activities, such as multispecies anti-biofilm properties, modulation of innate immune response, and anticancer, analgesic, antioxidant and anti-inflammatory activities [3,9–13]. Therefore, although these bioactive peptides were often named AMPs, more recently they have been termed as HDPs to describe more appropriately the breadth of their activities [14]. Due to HDPs' multifunctional properties, as well as to the increased bacterial resistance to conventional antibiotics, these peptides have great chance to be used as anti-infective and immunomodulatory therapeutics. Although very few HDPs are currently in use in the market, many of them are progressing through clinical trials for the treatment of diseases including microbial infections, organ failure, immune disorders, wound healing, diabetes and cancer [15,16]. Currently, most of the therapies based on HDPs that have entered clinical trials were designed for topical applications [17], presumably due to issues concerning their stability and toxicity [18].

Natural cationic HDPs are encoded by genes from many organisms. In mammals, HDPs are expressed in a variety of cell types including monocytes/macrophages, neutrophils, epithelial cells, keratinocytes, and mast cells [19–21]. They are usually synthesized as pro-peptides from which mature and biologically active HDPs are released by bacterial and/or host proteases [20]. Apolipoproteins are a source of bioactive peptides. Previous reports have shown that peptides derived from the cationic receptor binding region of Apolipoprotein E (ApoE141-149) are endowed with broad anti-infective activity [22]. Apolipoprotein B (ApoB) also contains two LDL (low-density lipoprotein) receptor binding domains, namely region A (ApoB3147-3157) and region B (ApoB3359-3367). Region B, more uniformly conserved across species and primarily involved in receptor binding, has been found to be endowed with a significant antiviral activity [22]. Moreover, peptides derived from ApoB have been already used in vaccine preparations to treat atherosclerosis [23]. When ApoE deficient mice have been immunized with ApoB661–680 and ApoB3136–3155 peptides, a significant increase of the levels of peptide-specific immunoglobulins was detected accompanied by a concomitant increase of secreted interleukin-10 (IL-10) levels, with no effect on IFN-γ expression levels, thus indicating that ApoB derived peptides are able to modulate the immune response [23].

Recently, our group developed an *in silico* method [K. Pane et al. submitted, 24] to identify HDPs in protein precursors and to predict quantitatively their antibacterial activity. This method assigns to any given peptide an antimicrobial score, called "absolute score" (AS), on the basis of net charge, hydrophobicity and length of the peptide and of two bacterial strain-dependent weight factors defining the contribution of charge and hydrophobicity to the antimicrobial activity. We demonstrated that AS is directly proportional to the antimicrobial activity of HDPs expressed as Log(1000/MIC), where MIC is the minimal inhibitory concentration of the peptide. Score values lower than 6.5 are considered not significant as they correspond to predicted MIC values higher than 200 μM, while for score values higher than about 10 the linear relationship is no longer valid, and an increase in the score does not necessarily correspond to a concomitant increase in the antimicrobial activity. In order to analyse a protein potentially bearing hidden antimicrobial regions, the AS values of all the peptides of the desired length contained in a precursor protein can be plotted as a function of peptide sequence and length, thus obtaining an accurate map of the antimicrobial activity determinants. This method allows the identification of novel HDPs within the sequence of known proteins ("cryptic" HDPs), as demonstrated by the identification of a novel cationic HDP endowed with antibacterial and immunomodulatory activities in human ApoE [24], and in the transcription factor Stf76 from the archaeon *Sulfolobus islandicus*. In the last case, peptide VVL-28 represents the first antimicrobial peptide derived from an archaeal protein [25].

On the basis of the interesting results obtained in the case of human ApoE, we applied our *in silico* analysis method to a human Apolipoprotein B (ApoB) isoform [26,27]. In Fig. 1, the isometric plot of region 882–929 of this ApoB variant is shown. An absolute maximum, corresponding to region 887–922 (AS = 12.0), and a relative maximum, corresponding to residues 887–909 (AS = 10.6), are shown (Fig. 1). Even if several ApoB functional regions have already been analysed, and in some cases biologically active peptides were obtained [22,23], to the best of our knowledge, this is the first report identifying ApoB region 887–922 as a source of HDPs.

Here, we report the recombinant production and characterization of two variants of the putative HDP identified by our bioinformatics method in human ApoB, *i.e.* peptides ApoB887-923 and ApoB887-911. Both peptides include at the C-terminal side one (as in the case of ApoB887-923), or two (as in the case of ApoB887-911) small uncharged residues (serine, glycine or threonine), which are not present in the regions highlighted in the AS plot (Fig. 1). These residues have been arbitrarily included to avoid that the negatively charged C-terminus of the peptide is adjacent to the antimicrobial region.

To evaluate the therapeutic potential of these peptides, we analysed their structure, antimicrobial and anti-biofilm activities, the ability to act in synergy with conventional antibiotics, their anti-inflammatory and wound healing properties, and their possible toxic effects on eukaryotic cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Eight bacterial strains were used in the present study, *i.e. E. coli* ATCC 25922, methicillin-resistant *Staphylococcus aureus* (MRSA WKZ-2), *Salmonella enteriditis* 706 RIVM, *Bacillus globigii* TNO BM013, *Bacillus licheniformis* ATTC 21424, *Staphylococcus aureus* ATTC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Pseudomonas aeruginosa* PAO1. All bacterial strains were grown in Muller Hinton Broth (MHB, Becton Dickinson Difco, Franklin Lakes, NJ)