Radiopharmaceuticals: new antimicrobial agents

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Small antimicrobial peptides are good candidates for new antimicrobial agents. A scintigraphic approach to studying the pharmacokinetics of antimicrobial peptides in animals has been developed. The peptides were safely and reproducibly labelled with technetium-99m and, after intravenous injection of the radiolabelled peptides into infected animals, scintigraphy allowed real-time quantification of the peptide in the various body compartments. Antimicrobial peptides rapidly accumulated at sites of infection but not at sites of sterile inflammation, indicating that radiolabelled antimicrobial peptides could be used in detection of infection. These radiopharmaceuticals enabled the efficacy of antibacterial therapy in animals to be monitored. The scintigraphic approach provides a useful method for investigating the pharmacokinetics of small peptides in animals.

Antimicrobial peptides, produced by phagocytes, epithelial cells, endothelial cells and many other cell types, are an important component of innate immunity against infection by a variety of pathogens [1–4]. They can be expressed constitutively or induced during inflammation or microbial challenge. The main features of antimicrobial peptides are described in Box 1, and an online catalogue of all reported molecules can be consulted at http://www.bbcm.univ.trieste.it/~tossi/antimic.html. Antimicrobial peptides display antibacterial, antiviral and antifungal activities *in vitro* [5–10] and are effective in experimental infections with multidrug resistant *Staphylococcus aureus* [11] and *Mycobacterium tuberculosis* [12]. Interestingly, the antibacterial effect of antimicrobial peptides in experimentally infected animals might also be attributed to synergistic effects with endogenous antimicrobial peptides and proteins (such as lysozyme and secretory leukoprotease inhibitor (SLPI) [13]), reactive oxygen intermediates [14], or other local factors (such as pH, Ca^{2+} and Zn^{2+} concentrations) or to interactions with host cells, leading to enhanced antibacterial activities of the cells. In view of the emergence of pathogens with increased resistance to conventional anti-infectives, the use of antimicrobial peptides alone or in combination with current antifungal drugs could lead to the development of new therapies to combat otherwise resistant infections [15].

Inserting the peptides into the bacterial cytoplasmic membrane under the influence of the transmembrane electrical potential gradient results in transient permeability of membranes and leakage of cellular constituents, such as potassium ions, thus destroying the proton gradient across the membrane [2] and resulting in bacterial cell death. Although membrane permeabilisation is an essential step in cell death, several lines of evidence indicate that, in the process of killing *Candida*, some antimicrobial peptides (i.e. histatin- and lactoferrin-derived peptides) have an effect on membranes through an action on the energized mitochondrion [10,16–18]. Although antimicrobial peptides have different chemical structures, the basis of their antimicrobial activities is the interaction of the cationic (positively charged) domains of the peptides with the (negatively charged) surface of microorganisms (Fig. 1) [3]. Given that microbial membranes expose negatively charged phospholipids, such as lipopolysaccharide (LPS) or teichoic acids, on their surface, whereas mammalian cells segregate lipids with negatively charged headgroups into the inner leaflet, it is conceivable

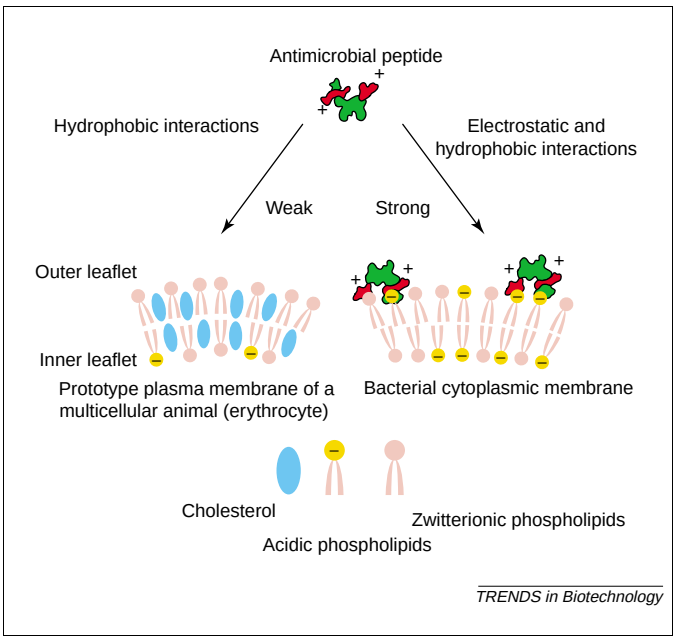

Fig. 1. The membrane target of antimicrobial peptides and the basis of their specific binding. Adapted, with permission, from [3].

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Box 1. Key features of antimicrobial peptides

- Antimicrobial peptides usually contain <50 amino acids with a net positive charge created by an excess of basic residues, such as lysine and arginine, and ~50% hydrophobic amino acids.
- Antimicrobial peptides are essential components of the innate host defence because of their ability to kill a wide range of pathogens.
- They have a wide distribution throughout the animal and plant kingdoms.
- They are effectors of local and systemic immune responses. The latter is essentially found in insects [a].
- Although they share basic features such as small size, hydrophobicity and cationic character, antimicrobial peptides have a great structural diversity. For the sake of simplicity they can be categorized into three main families:
  - linear peptides that adopt an amphipathic α-helical structure such as cecropin, magainins, bee mellitin and human ubiquicidin and histatins;
  - peptides with disulfide bridges (1 to 4) that may adopt a loop or a β-sheet structure. Interestingly, the core of some 3-disulfide bonded peptides, such as plant and insect defensins, combines α-helical regions and β-sheet structures connected by two of the three disulfide bridges, forming a cysteine-stabilized α/β motif;
  - peptides that are particularly rich in one amino acid (beside lysine and arginine) such as the tryptophan-rich indolicidin of bovine neutrophils and the proline-arginine-rich peptide PR39 of pig neutrophils.
- The majority of antimicrobial peptides are derived from larger precursors that harbor a signal sequence, whereas other peptides are generated by proteolysis from larger proteins (such as lactoferricin).
- In addition, some antimicrobial peptides such as mammalian defensins and LL-37 have other activities contributing to host defences by mediating an acute inflammatory reaction [b,c] and linking the innate with the acquired immune response [d,e].

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that antimicrobial peptides bind preferentially to pathogens over mammalian cells.

Large-scale production of antimicrobial peptides

Large quantities of purified microbial peptides are required to investigate their possible applications further. Difficulties arising in purifying natural antimicrobial peptides from various sources have prompted the recombinant production of antimicrobial peptides by genetically engineered bacteria [19,20] or by peptide synthesis [21,22]. Such methods result in sufficient amounts of antimicrobial peptides produced under good laboratory practice conditions, which is essential for future approval to use the peptides in clinical trials. Peptide synthesis also allows the production of chemical variants, such as D-enantiomers, peptides that have amino acid substitutions at various positions, and peptide libraries. Furthermore, synthetic peptides are usually small, rapidly removed from the circulation and other body compartments, and flexible, because they do not hold a particular structure in a hydrophilic environment, and they display a favourable adverse effect profile. Alternatively, yeast recombinant technology can be used for large-scale production of cysteine-rich peptides that have several disulfide bridges, which make them naturally very resistant to protease degradation because of their compact structure. It is our belief that both synthetic and recombinant antimicrobial peptides are the candidates of choice for further development as antimicrobial agents.

Measuring the pharmacokinetics of antimicrobial peptides

The classic method for studying the pharmacokinetics of small peptides in animals is to measure their levels in different organs at various intervals after injection using biochemical (or immunological) assays. A major disadvantage of this approach is that it does not allow whole-body, real-time monitoring of the biodistribution of the peptide in an individual animal. To circumvent this drawback, peptides need to be labelled to assess the biodistribution of the labelled peptides in animals. In the case of small peptides, it is not feasible to analyse their pharmacokinetics by preparing a fusion protein of the peptide under study with, for example, green fluorescent protein or luciferase, both of which can be monitored in animals in real-time [23,24]. Alternatively, peptides can be tagged with radioisotopes, such as technetium-99m (^{99m}Tc), indium-111 and iodine-125, and scintigraphic techniques can be used to quantify the amount of radiolabelled peptides in different organs at various intervals. Because of its favourable radiation characteristics and ready availability, ^{99m}Tc is the preferred label for studying the pharmacokinetics of peptides.

Radiolabelling of peptides

The aim of radiolabelling techniques is to firmly attach or incorporate the radionuclide into the peptide without altering its biological functions, thus allowing a reliable evaluation of its pharmacokinetics after intravenous administration. The various methods of labelling peptides with ^{99m}Tc, including indirect labelling using the preformed chelate approach or bifunctional chelating agents, and the direct labelling method have been discussed extensively [25,26]. The direct labelling method is a simple procedure in which the peptide is labelled in absence of an exogenous chelator. Using such a technique [27] and the radionuclide ^{99m}Tc, an array of peptides was labelled including those with disulfide bridges without affecting

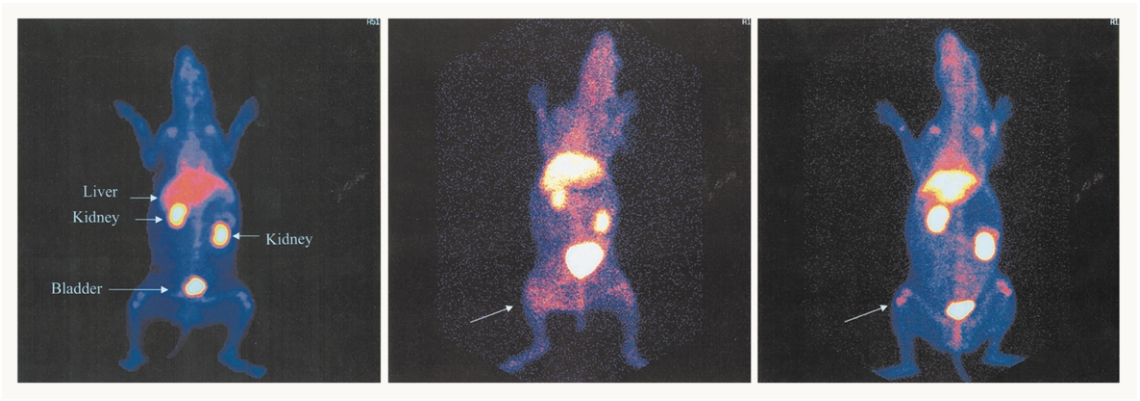

Fig. 2. (a) Biodistribution of ^{99m}Tc-labelled UBI 29–41 in a healthy rabbit 2 h after intravenous injection of the radiolabelled peptide. In short, ^{99m}Tc-UBI 29–41 injected into a normal rabbit was rapidly cleared from the circulation (half-life ~30–60 min) via the kidneys and bladder, with little activity in the liver and no deposits in the intestines. (b) Typical scintigram of ^{99m}Tc-labelled UBI 29–41 2 h after injection of the tracer into rabbits that had been infected intramuscularly with 10^7 colony forming units multidrug resistant *Staphylococcus aureus* or (c) injected with a 20-fold higher number of heat-killed multidrug resistant *S. aureus*. To determine whether the labelled peptide specifically accumulated at the site of infection or inflammation we calculated the ratio between the radioactivity in the affected and the contralateral thigh muscle. The ratios seen in infected animals, but not inflamed, were significantly higher than in normal rabbits. The infected or inflamed thigh muscle is indicated by an arrow. Adapted, with permission, from [29].

their biological functions [5]. In addition, radiochemical analysis showed that the labelling of antimicrobial peptides was rapid (within 10 min), effective (impurities $<5\%$ of the total radioactivity), stable (minimal release of radioactivity from the ^{99m}Tc-peptide in diluted human serum) and safe (no adverse effects in mice and rabbits). Unfortunately, the reaction mechanism underlying this ^{99m}Tc-labelling of peptides has not been elucidated. It may, however, involve the reduction of technetium, the production of a technetium intermediate, and the substitution reaction transferring the reduced technetium from this intermediate to the peptide. The end-product from this reaction could be a reduced metal(N4) complex, as reported for many tetrapeptides [28]. From preliminary data obtained using reverse-phase high-performance liquid chromatography (HPLC), it seems that this complex probably contains two peptide molecules around a reduced Tc core.

Application of labelled peptides for infection detection

Scintigraphic studies after intravenous injection of non-microbicidal concentrations of radiolabelled antimicrobial peptides into animals revealed that the peptides are rapidly cleared from the circulation (half-life 30–60 min) via the kidneys and bladder with little activity in the liver and no deposits in the intestines. Moreover, this favourable kinetic profile is accompanied by a limited radiation burden. An example of the scintigraphic data obtained with a ^{99m}Tc-labelled synthetic peptide corresponding to residues 29–41 of the antimicrobial peptide ubiquicidin (UBI 29–41) in a rabbit is shown in Figure 2a.

These studies also triggered the idea that this labelling technique could be useful for other applications, such as infection detection. Although most infections are diagnosed on the basis of clinical history, physical examination, medical imaging and identification of pathogens in body fluids and biopsies, in a select group of patients (transplant recipients and patients with prosthesis), fever could be owing to both infectious and inflammatory processes, such as allograft rejection. In such patients, radiopharmaceuticals that discriminate infections from sterile inflammations can therefore make an important contribution to the non-invasive diagnosis of infections by means of scintigraphy. Selected radiolabelled antimicrobial peptides accumulated rapidly (within 30 min) in the target (infected) tissues (1–2% of the injected dose) with little accumulation at sites of sterile inflammation, allowing bacterial and fungal infections to be distinguished from sterile inflammation [29,30]. An example of the uptake characteristics of ^{99m}Tc-labelled antimicrobial peptides in infected or inflamed thigh muscles in rabbits is given in Figures 2b and 2c, respectively. In agreement with the specificity of the *in vitro* binding of antimicrobial peptides to microorganisms, *in vivo* competition studies using unlabelled peptides as competitors indicated that, in addition to charge, the composition and sequence of peptides are key to their ability to accumulate at sites of infection [31]. For example, accumulation of ^{99m}Tc-labelled UBI 29–41 at the site of infection in mice could be inhibited by injecting an excess of the unlabelled UBI 29–41, but not by a scrambled version of this peptide, before injection of the tracer [31]. Interestingly, various ^{99m}Tc-labelled UBI peptides can be useful in infection detection in both immunocompetent and immunocompromised animals. Furthermore, a good correlation between the accumulation of ^{99m}Tc-labelled UBI29–41 peptides in bacterial infected thigh muscles of mice and the number of viable microorganisms present at the site of infection was found, indicating that our approach using radiolabelled peptides could be applied to monitor the efficacy of antimicrobial therapy of bacterial infections.

Outlook

Scintigraphic analysis of ^{99m}Tc-labelled peptides after intravenous injection is the method of choice for studying the pharmacokinetics of small antimicrobial peptides in animals because it allows reliable real-time, whole-body imaging and quantitative biodistribution studies without the need to kill animals at each interval. Using this

approach, quantitative data can be obtained within seconds after injection of the radiolabelled peptide up to ~12 h and the labelling procedure does not alter the biological functions of antimicrobial peptides [5]. In addition, our scintigraphic approach allows the analysis of the pharmacokinetics of not only peptides but also a wide range of probes, including anti-infectives [29,32,33], large proteins [34] and even cells [35]. For example, comparing the pharmacokinetics of ^{99m}Tc-labelled natural and transgenic human lactoferrin in mice indicated similar half-lives and both proteins were excreted by the liver, intestines, kidneys and bladder [34]. These results were in agreement with data for the transgenic human lactoferrin obtained in human volunteers.

As a spin-off from our research, we found that radiolabelled antimicrobial peptides can be useful in infection detection and monitoring of the efficacy of antibacterial therapy in animals. Currently, we are focusing on the discrimination between infection and sterile inflammation in (immunocompromised) animals that have an artificial implant, for example a prosthetic knee, hip or heart valve, using radiolabelled peptides. Clearly, more information, such as knowledge of the molecular structure and the toxicology of the ^{99m}Tc-labelled end-product, is required before considering the use of ^{99m}Tc-labelled peptides for detection of infectious processes in humans.

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