The Use of Technetium-99m Radiolabeled Human Antimicrobial Peptides for Infection Specific Imaging

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Abstract: Bacterial resistance to conventional antibiotics poses a challenge medicine to search for alternatives. Cationic antimicrobial peptides (AMPs) are promising for the development of a new class of antibiotics. This review focuses on the use of technetium-99m labeled synthetic AMPs, derived from human natural cationic AMPs, for target-delivery to and *in vivo* detection of infection sites caused by (drug-resistant) micro-organisms. The scintigraphic approach has proven to be a reliable method for evaluating AMPs in pharmacological studies and for optimizing target-delivery of radiolabeled AMPs to pathological sites in animals and humans. In addition, the effect of alterations in amphipathicity, amino acid substitution, and dimerization on the biological performance of AMPs is reported. Radiolabeled AMPs offer good perspectives for diagnosis of infections, for monitoring therapy, and, most importantly, for the ability to discriminate between infections and sterile inflammatory processes.

Key Words: Antimicrobial peptides, technetium-99m labeling, target-delivery, pharmacology, molecular imaging, infection detection, treatment monitoring.

INTRODUCTION

The emergence of infections with multidrug resistant micro-organisms has become a critical issue in medicine as they are responsible for the majority of health care-associated infections as well as for increasing lengths of hospital stay, severity of illness, deaths, and costs for treatment (www.idsociety.org). In many clinical situations, especially after surgery, the infection diagnosis remains difficult. Painful and/or febrile patients are exposed to a series of diagnostic studies to optimize treatment. These studies include laboratory tests that often reveal non-specific parameters like the erythrocyte sedimentation rate, white blood cell counts, and cytokine reactions. For obvious reasons these tests are not specific enough to differentiate between bacterial infections, sterile inflammation, and tumors. This differentiation is of crucial importance for further clinical analysis and/or treatment. Imaging studies such as x-ray, computed tomography (CT), and magnetic resonance imaging (MRI) show abnormalities caused by morphologic changes, and are therefore not suitable to provide reliable differentiation [1]. Moreover, most abnormalities can only be detected at advanced stages of disease. It is therefore obvious that morphologic imaging does not really contribute to an early diagnosis, whereas phase therapy can be more successful. Molecular imaging for diagnosis and monitoring of therapeutic response as well as development of new compounds for eliminating multi-drug resistant micro-organisms can play a leading role in new treatments as nuclear medicine imaging is based on changes in function, rather than on morphology. Cationic antimicrobial peptides (AMPs), belonging to the group of endogenous antimicrobial agents, have been proposed as infection seeking tracers and antimicrobial therapeutics because they have a natural ability to accumulate and interact at putative sites [2, 3]. Once they are radiolabeled their biodistribution and pharmacokinetics can be easily visualized and quantified at the site of pathology using scintigraphic techniques. This review highlights the radiochemical and pharmacological characteristics of radiolabeled AMPs in microbial pathology and their application as tracers to detect infections and in monitoring antimicrobial therapy.

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ENDOGENOUS AMPs

After invasion of micro-organisms, an inflammatory response of the host takes place aiming at the elimination of the pathogenic insult. Additionally, the inflammatory process tries to remove injured tissues and to stimulate regeneration of normal tissue architecture. Next, the immune system generates memory to recognize re-infections more effectively and dealing with them more adequately (adaptive immunity). Infection by pathogenic micro-organisms is usually followed by acute inflammatory responses. At acute inflammation various local events occur (Fig. **1**):

- Vascular and endothelial changes featuring vasodilatation and increased vascular permeability.
- Formation of exudate, containing extra cellular fluids with high protein content and cellular debris.
- Emigration of leukocytes from the smaller blood vessels and accumulation at the site of tissue injury (diapedesis), triggering of chemotaxis and recruitment of polymorphonuclear leukocytes (PMNs) and macrophages for ingesting foreign particles (phagocytosis). Cellular migration and triggering is mediated by chemokines and cytokines as well as by eruption of killed pathogens and host cells.

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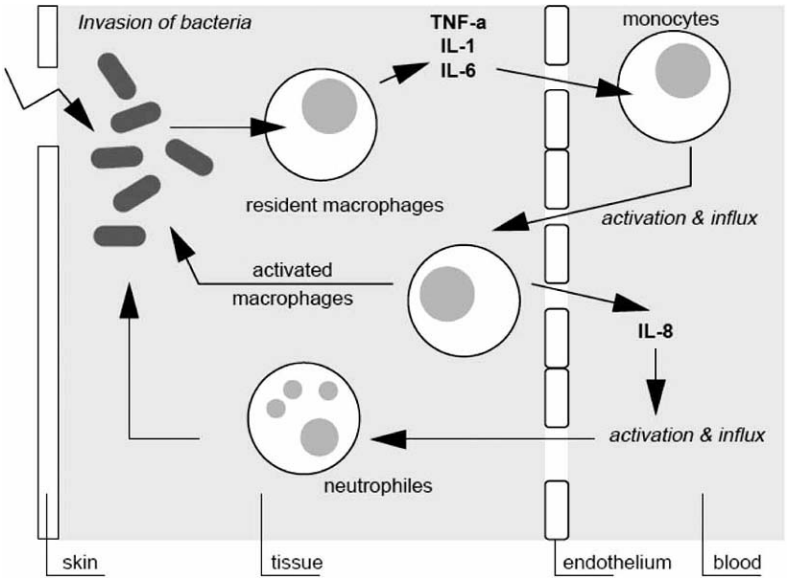


Fig. (1). Major events in bacterially infected tissues, designed to destroy invaded bacteria (see text). Bacterial invasions cause tissue damage which triggers a release of pro-inflammatory cytokines Tumor Necrosis Factor-a (TNF-α), Interleukin-1 (IL-1) and IL-6 by resident macrophages. These factors induce increased blood flow, increased capillary permeability, the expression of adhesion molecules on endothelial cells and on leukocytes in the circulation, which facilitates the influx of various types of leukocytes and the influx of various phagocytes (diapedesis). Infiltrating monocytes, which differentiate into macrophages, produce IL-8 that is chemoattractive to circulating neutrophils. At any stage phagocytes begin to engulf the bacteria.

- Generation of AMPs produced by phagocytes, epithelial cells, endothelial cells, and many other cell types. They can be expressed at a basic level or induced during inflammation or microbial challenge with an adaptive response depending on the invading pathogen.

Living organisms of all types have been found to produce a large repertoire of AMPs that play an important role in the defence against microbial invasion. After the discovery of antibiotics the bactericidal effect of cationic AMPs was ignored but the interest was restored after the emergence of micro-organisms resistant to the most widely used antibiotic agents. AMPs have attracted renewed attention as therapeutic drugs after the discovery that they have a wide distribution throughout the animal and plant kingdom [4]. Innate AMPs have existed for millions of years indicating that the preservation of this type of immunity is of importance in host defense even in higher species with well developed forms of adaptive immunity. Expression of genes for producing AMPs occurs in principle after contact with micro-organisms or microbial products like lipopolysaccharides, or after triggering by pro-inflammatory cytokines. Hundreds of antimicrobial peptides have been isolated and characterized so far and irrespective of their origin, spectrum of activity, and structure, most of these peptides share several common properties. They are generally composed of less than 60 amino acid residues, mostly L-amino acids, their net charge is positive, they are amphipathic, and in most cases they are membrane active. Cationic AMPs can be broadly categorized on the basis of their secondary structure into 4 classes: ***A***) amphipathic α-helices, such as cecropin, magainin, mellitin, ubiquicidin (UBI), ***B***) peptides with 2 or more disulphide bridges, such as α-and β-defensins, cathelicidins, protegrins, ***C***) peptides with one intermolecular disulphide bond having a loop/hairpin like structure, and ***D***) linear peptides lacking cystein with a high content of certain residues such as tryptophan-rich indolicidin or proline-arginine-rich PR39 [5].

In higher organisms antimicrobial peptides are mainly produced on epithelial surfaces and in phagocytic cells that play a crucial role in innate as well as in adaptive defense systems [6-8]. AMPs exhibit rapid killing and a broad spectrum of activity against Gram-positive and Gram-negative bacteria, fungi, parasites, and enveloped viruses [3, 7]. Their mode of action against invading pathogens is based upon the architectural and biochemical composition of the microbial cellular membrane [9]. These properties cause varying degrees of antimicrobial toxicity by perforation, membrane destabilization, metabolic inhibitors, and triggering of bacteriolysis [10, 11], and as such, they are a crucial component of the innate immunity against pathogenic infections. In laboratory animals, AMPs display microbicidal activities against experimental infections with bacteria, viruses, and fungi [12-18]. Besides their direct microbial killing properties some AMPs neutralize bacterial toxins and upregulate the host defense by triggering the release of chemoattractants or by other immunostimulatory effects [3, 5, 19]. Additionally, AMPs play an important role in anti-tumor activity [9], bone repair [20], and neoangiogenesis [21].

Cationic AMPs have a net positive charge due to an excess of basic residues lysine and arginine. The basis of the antimicrobial activity is the interaction of positively charged domains of the peptide with negatively charged molecules located on the surface of microbial membranes. Membranes of micro-organisms expose negatively charged lipoteichoic acid and phospholipids, while in normal mammalian cells negatively charged lipids face the cytoplasm (Fig. **2**). Also,

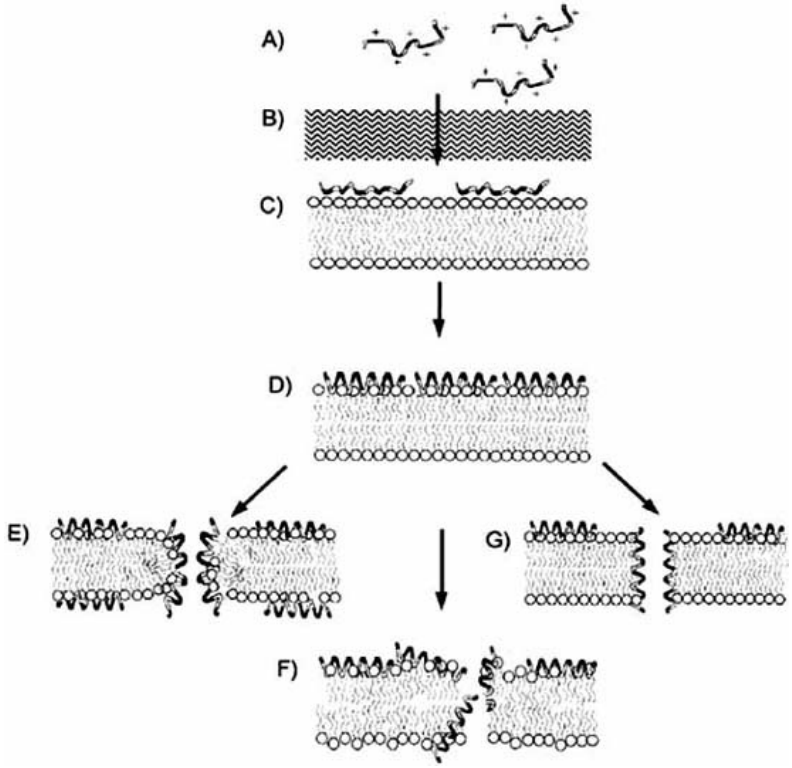


Fig. (2). The membrane target of antimicrobial peptides of multi-cellular organisms and the basis of specificity: a schematic representation of the proposed models for the mode of action of a-helical AMPs. (**A**) Electrostatic attraction to anionic microbial surface, (**B**) passage across the outer membrane and/or peptidoglican layer, (**C**) electrostatic attraction to anionic phospholipids in the cytoplasmic membrane, (**D**) structuring, membrane insertion and accumulation, (**E**) toroidal pore formation, (**F**) carpet mechanism for membrane permeabilization, (**G**) barrel stave pores formation. Reprinted with permission from Tossi *et al*. *Biopolymers (Peptide Science)* **2000**, 55, 4–30.

host cell membranes contain relatively large amounts of cholesterol thus protecting them from antimicrobial peptides-generated lysis [22, 23]. These properties explain the lack of electrostatic binding of cationic AMPs to mammalian cells and disruption of membranes at physiological conditions [7]. The interaction of these peptides with the microbial cytoplasmic membrane results in destabilization and pore forming in the membrane allowing leakage of cellular constituents such as potassium ions, thus destroying the proton gradient across the membrane which results into death. Also, intercellular activities leading to disturbed metabolic processes, binding to DNA, and inhibiting DNA synthesis, inhibition of protease and enzyme activity, and reducing mitochondrial activity has been reported [9, 13, 24-26]. The killing mechanism of antimicrobial peptides is so different from that of the conventional antibiotics that antimicrobial peptides are considered to be an attractive substitute or an additional drug. In view of the emergence of pathogens with increased resistance to conventional anti-infective drugs, the use of antimicrobial peptides alone or in combination with current antifungal drugs could lead to the development of new therapies to treat otherwise resistant infections [24]. It is generally assumed that the development of resistance against antimicrobial peptides is very limited in nature. This is conceivable as during evolution multiple antimicrobial activities of the host remained active and effective in withstanding invasions by micro-organisms. Nevertheless, pathogens can develop resistance by the modification of the lipid membrane composition, thereby reducing attractive electrostatic interactions [27] and increasing their virulence [28], or by releasing peptide degrading proteases or polyanionic components as decoys [29].

PRODUCTION OF ANTIMICROBIAL PEPTIDES

Large quantities of AMPs are required to investigate their possible applications such as therapeutical and infection imaging agents. Difficulties arising in reproducible purification of natural AMPs from various sources have prompted the recombinant production of antimicrobial peptides by (genetically engineered) bacteria [30], transgenic animals [31], or by peptide synthesis [32]. Such methods may yield sufficient amounts of antimicrobial peptides produced under good manufacturing practice conditions (GMP), which is essential for future approval to use the peptides in clinical trials. Peptide synthesis also allows the production of chemical variants, such as dimers for enhanced interaction with microbial membranes, D-enantiomers which are less easily degraded by enzymatic decomposition, peptides that have amino acid substitutions at various positions [33] or various linkers at desired places so that their pharmacology is altered or markers are incorporated [18, 34-36]. To study the role and character of different domains on natural molecules may result in the identification of biologically active regions e.g. those ones in antimicrobial cationic peptides which are responsible for binding and/or killing the target. Eventually, these techniques can be used to select and synthesize peptide frag-

ments that preferentially bind to specific species of pathogens and show favorable pharmacologic behavior.

SCINTIGRAPHIC IMAGING OF INFECTION

Radiopharmaceuticals have their medicinal use in diagnosis and therapy, especially for cancer and infections. Early identification and localization of the site of infection is crucial in the appropriate treatment of patients and isotopic imaging using radiopharmaceuticals has made significant contributions in this research field over the past decades. In many clinical situations it is of utmost importance to distinguish between aseptic inflammation and infection. Indeed, this is often the case in organ transplant rejection or aseptic loosening of implants where the preferred treatment is very different from antimicrobial therapy, but where the clinical signs are often similar. In order to be clinically useful, an imaging test should therefore be able to differentiate between both cases. Moreover it should be both case-sensitive and case-specific. It is especially important that the test is case-specific as an unspecific test can easily lead to unnecessary and costly operations and hospitalizations, in addition to the morbidity and unavoidable mortality sometimes associated with surgical interventions. In this context, it is clear that the clinical need for infection specific radiopharmaceuticals cannot be overemphasized. The majority of clinically used scintigraphic tests do not allow the distinction between inflammation and infection. Gallium-67 binds to bacteria, but also to proteins accumulating at both sites of sterile inflammation and bacterial infection. The metabolic marker ^{18}F-fluorodeoxyglucose (^{18}F-FDG) which is widely used to detect malignancies by positron emission tomography (PET) accumulates both in pathogens and in inflammatory cells and therefore cannot differentiate between infection and inflammation. Radiolabeled leukocytes and other agents that interact with receptors or domains on circulating and infiltrating leucocytes, such as radiolabeled immunoglobulin and monoclonal antibodies (or fragments thereof), exhibit similar properties [37]. Since AMPs often display preferential binding to micro-organisms, new radiopharmaceuticals for discriminating infections from inflammations may be recruited from the array of (human) antimicrobial peptides/proteins [38]. In general, peptides exhibit fast clearance from the circulation, rapid excretion and, most importantly, rapid extravascular tissue penetration, leading to fast accumulation in the target. With the aid of a gamma camera, the pathway of the radiolabeled peptide can be followed over time by scintigraphy *in vivo*. This tool is not only suitable for pharmacologic studies; it also has important applications in clinical patient care in diagnosis and therapy. The classic method of studying the pharmacokinetics of small peptides in animals is to measure their levels in different tissues and 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, early real-time monitoring of the distribution of the peptide *in vivo*. To circumvent this drawback, peptides need to be labeled with a marker to assess the biodistribution *in vivo*. In the case of small peptides, it is not feasible to analyze 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, because this conjugation affects the peptide characteristics dramatically [39]. Alternatively, peptides can be tagged with γ-emitting radioisotopes, such as technetium-99m (^{99m}Tc), indium-111 and iodine-125. These scintigraphic techniques can be used to quantify the amount of radiolabeled peptides in different organs at various intervals [40-42]. Recently, the solid phase synthesis of ^{18}F-labeled linear peptides enabled positron emission tomography (PET) with labeled peptides [36, 43]. Because of its favorable radiation characteristics, easy radiopharmacy, low costs, and availability ^{99m}Tc is the choice of isotope to determine the pharmacokinetics of radiolabeled AMPs.

LABELING OF AMPs WITH TECHNETIUM-99m

As mentioned earlier, the most suitable radionuclide for diagnostic patient studies is technetium-99m (^{99m}Tc) which is ready available, at low cost, from a molybdenum generator as ^{99m}Tc-pertechnetate. Moreover its relatively short half-life of 6 hours results in a low radiation burden for the patient and non-measurable environmental contamination. The photon energy emitted by this radionuclide is 140 keV, which is ideal for the scintigraphic detection with a gamma camera permitting high quality imaging. Another advantage is that the chemistry of ^{99m}Tc is comparable to that of β-emitter rhenium 188 (^{188}Re, half-life 17 hrs, 172 keV) which is applied for targeted radiotherapeutical purposes. ^{99m}Tc should be attached to the peptide in such a way that the complex is sufficiently robust to withstand radiolytic degradation and reactive labeling conditions that may affect biological properties of the peptides [44-46]. Technetium complexes, in which this element is in oxidation states –1 to +7 are known, although technetium +5 is the most suited oxidation state for incorporation into other molecules. Donor atoms may be sulphur, phosphorus, and nitrogen. A better understanding of the biological properties of ^{99m}Tc-radiopharmaceuticals can be expected if metal chelates with well-defined chemical structure and with a known stability are used in biological experiments [47, 48]. For this reason many small radiolabeled peptides are under study in various biological processes [40, 42]. On the other hand, it is difficult to orchestrate the chelators to a well defined position and attachment to the bioactive core of the peptide may lead to reduced biological activity [49]. An example of comparing biological properties of synthetic antimicrobial peptide UBI 29-41 after radiolabeling *via* various methods is given in Table **1**. UBI 29-41 labeled with technetium-99m *via* the BOC-hydrazino nicotinamide (BOC-Hynic) or the mercaptoacetyltriglycine (MAG_3) bifunctional ligands (both randomly attached to the amine residues on the peptide molecule and conjugated on the N-terminus of the peptide during solid-phase synthesis) significantly reduce the binding to bacteria and affect the uptake in bacterially infected tissues in mice compared to the direct method or the Hynic method [35, 50, 51]. In nuclear medicine few antimicrobial peptides have been studied that incorporate chelating groups for the technetium radionuclide during peptide synthesis. This approach makes it possible to attach this chelating group to the peptide at a remote position from the biologically active site. In this way, the metal complex is not supposed to disturb the peptide's biochemical actions. Most of these tetradentate ligands contain either one of two thiol groups and the remainder being N-donor atoms

Table 1. Biological Performance of Antimicrobial Peptide UBI 29-41, Labeled with Technetium-99m According to Various Methods, Against MRSA In Vitro and in Mice with MRSA-Infected Thigh Muscles

^{99m}Tc-Labeling Method	Labeling Yield *	Stability **	*In Vitro* Binding to *S. aureus*		Half-Life in Mice	T/NT Ratio	Main Organs of Accumulation
	(%)	(%)		Competitor (100 x)	(min)	1 hr p.i.	>(20%ID) #
Direct	93	97	42	9	98	3.3	U & K
BOC-Hynic	97	87	26	4	88	1.7	U
MAG_3	95	93	26	5	55	2.0	U & K
Solid-phase synthesis Hynic	95	92	46	6	113	2.8	U & K
Solid-phase synthesis N_2S_2	97	91	36	4	120	3.5	L & U & K
Iodine-123 chloramine-T method	94	72	43	6	240	2.5	K & T & S

Determined by ITLC, ** For 2 hrs in human serum at 37°C, determined by TCA precipitation. T/NT = target (infected thigh muscle)-to-non-target (contralateral muscle) ratio determined from ex vivo tissue counts. Competitor experiments were carried out with 100-times excess of unlabeled UBI 29-41 about 1 hr prior addition of the radiolabeled peptide. # K = Kidneys, L = Liver, U = Urinary bladder, T = Thyroid gland, S = Stomach at 1 hr p.i. in percentage of the injected dose (%ID).

as amine or amide groups. They are usually indicated as N_3S or N_2S_2 chelators (Fig. **3**) and the amine thiol based chelators have a well defined chemical structure of the technetium complex [52]. Another approach is the use of the Hynic system developed by Babich *et al*. [53]. Hynic conjugation uses a single tether to bind technetium to AMPs [35]. Besides the above mentioned labeling methods that require the attachment of a ligand on the peptide, direct ^{99m}Tc-labeling is also possible using the reducing conditions under which the +7 oxidation state in pertechnetate is reduced to the +5 oxidation state. This is problematic for peptides containing intramolecular disulfide bonds which are usually reduced during the labeling procedure resulting in altered chemical structure, biodistribution, and reduced biological activity as indicated in Table **1** for Iodine-123 (^{123}I, half-life 3.2 hrs, 159 keV) using the chloramine-T labeling method [50]. High

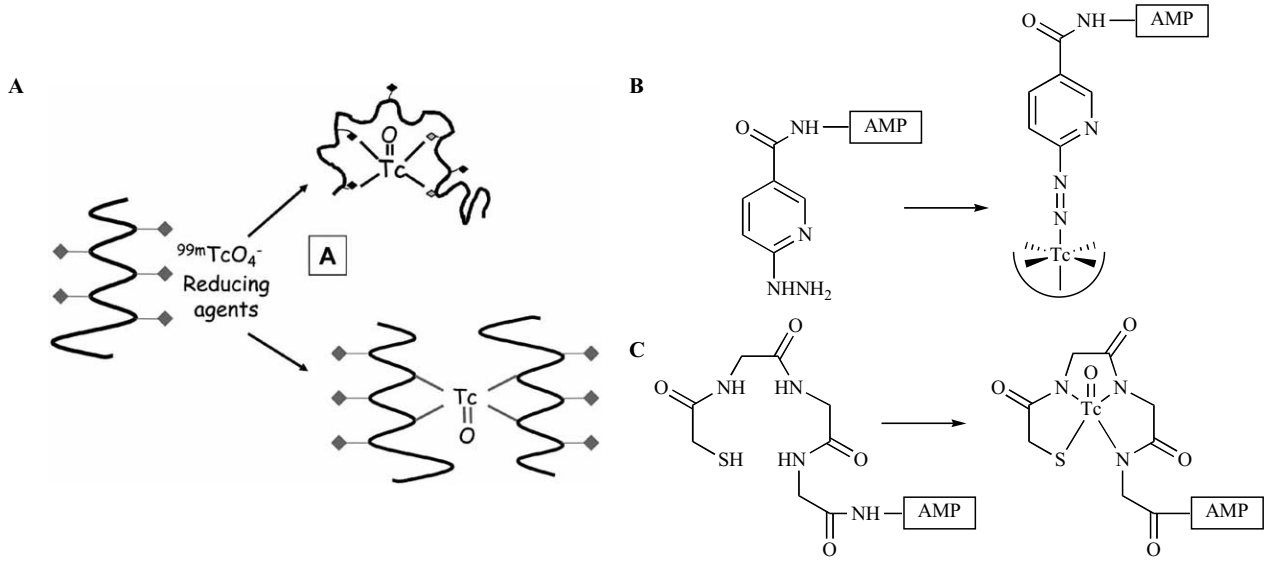


Fig. (3). Coordination chemistry of (**A**) direct labeled AMP within the peptide backbone (top panel) or as a dimer (bottom panel). The direct labeling requires coordination of technetium-99m with functional groups (♦) present in the peptide. Indirect labeling with the use of ligand/chelator HYNIC (**B**) or MAG_3 (**C**) attached to the peptide N-terminus during solid phase synthesis results in well-defined coordination chemistry.

concentrations of stannous ions, which are the most commonly used agents to reduce technetium, may reduce disulfide bonds as well. Methods using these ions at high concentration are not often suitable for ^{99m}Tc binding to proteins and peptides, although a recently developed method employs low amounts of stannous ions together with small amounts of KBH_4 which gives a mild radiochemical reaction leading to stable end products with high labeling yields (>90%) and intact biological characteristics [12, 45, 54]. This method has important value especially for linear peptides that do not contain disulfide bridges as donor atoms. This direct labeling method, without the need of a chelating agent, is a simple, fast, and cheap procedure that has been used to label an array of peptides successfully leaving their biological features intact, even in peptides containing one ore more disulfide bridges [12]. For example, the labeling method underwent extensive radiochemical and biological testing for synthetic antimicrobial peptide ubiquicidin fragment 29-41 (UBI 29-41, TGRAKRRMQYNRR, Mw 1,693 Da) in a coordinated research project (CRP) by the IAEA consisting of a multicenter study (http://www-pub.iaea.org/MTCD/publications/PDF/te_1414_web.pdf). It was concluded that the method was highly reproducible leading to a potentially clinical utility. The CRP is considered a major contribution because it provides the first validated specific ^{99m}Tc labeled infection imaging agent. The mechanism underlying this direct labeling method involves the reduction of ^{99m}Tc-pertechnetate by low concentrations of stannous ions, in the presence of another reducing agent KBH_4, the production of a stabilized TcO intermediate, followed by a substitution reaction [55]. According to a recent finding by the group of Ferro Flores *et al*., [56, 57] the labeling of a fragment of the synthetic cationic antimicrobial peptide UBI 29-41 results in a dimeric peptide complex with Lys^5 and Arg^7 being the most probable binding sites for ^{99m}Tc, indicating that residual amines are involved. Under alkaline conditions (final pH 9) and with a relatively short reaction time of 10-20 minutes a high labeling yield of more than 95% has been achieved [54, 58], as determined by techniques including high performance liquid chromatography (Fig. **4**). Also, it appeared that the stability of this complex in human serum albumin was excellent (less than 5% release of free ^{99m}Tc at 24 hrs with conserved biological activity) [54]. As an example, a comparison of the biological performance of radiolabeled UBI 29-41 between the various methods is given in Table **1**. As stated earlier, introducing bifunctional ligands at random on the peptide affects the biological performance of the UBI 29-41 in binding to bacteria and targeting infected lesions in mice. Although most labeling methods yield high labeling efficiency and good stability, most pre-clinical research with radiolabeled AMPs was carried out with AMPs directly labeled with technetium-99m because of simplicity of the method. So far, most clinical studies with ^{99m}Tc-UBI 29-41 have been carried out with kits using chemistry according to the direct method [59-61], although some kits for preparing ^{99m}Tc-Hynic-UBI 29-41 have been developed [51].

PHARMACOLOGICAL AND BIOLOGICAL TESTING OF ^{99m}TC-LABELED AMPs

In the last couple of years numerous AMPs, such as defensins, lactoferrins, ubiquicidins, and histatins have been labeled with ^{99m}Tc according to the direct method, and their biological features have been biologically and pharmacologically tested *in vitro* and in laboratory animals. An overview of the biological performance of ^{99m}Tc-labeled AMPs is given in Table **2**.

Defensins

Human neutrophil peptides (HNP), or defensins, are major constituents of the azurophilic granules of human neutrophils and have been shown to display broad-spectrum antimicrobial activities. Other activities of these defensins, which are released from stimulated neutrophils, include cytotoxic, stimulatory, and chemotactic activities towards a variety of target cells. First, the potential use of radiolabeled neutrophil

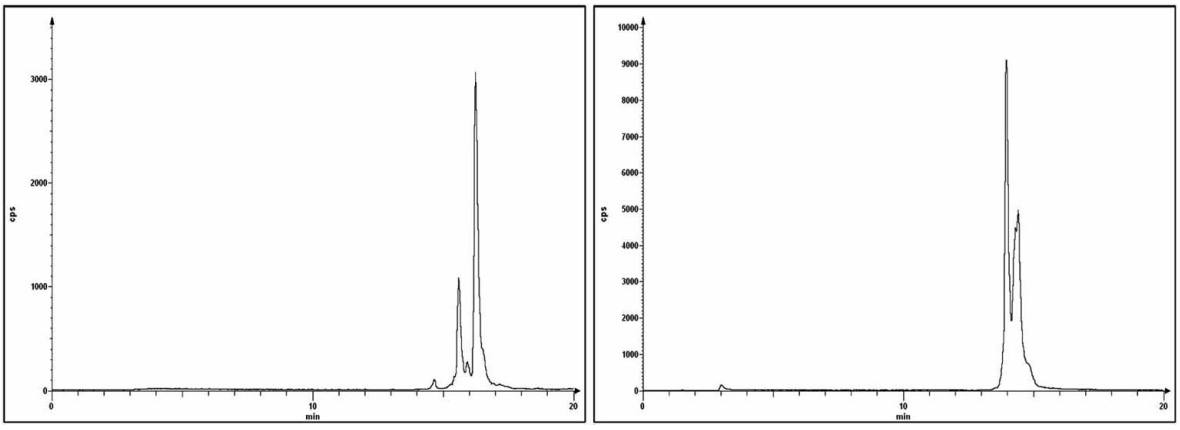


Fig. (4). Typical ^{99m}T-radioactivity profiles of direct (left panel) or Hynic (right panel) labeled UBI 29-41 analyzed by reverse-phase HPLC. The radiolabeled peptide is eluted in two peaks between 15-17 min for direct labeled UBI 29-41 and between 14-16 min for Hynic labeled UBI 29-41. For both labeled ^{99m}Tc-UBI 29-41 peptides the two peaks indicate monomeric (early peak) and dimeric (late peak) species. Methodology described by Welling *et al.* [51].

Table 2. Biological Performance Against MRSA In Vitro and in Mice with MRSA-Infected Thigh Muscles of ^{99m}Tc-Labeled Synthetic Peptides Derived from Human Cationic Antimicrobial Peptides

Peptide		Sequence	Mass	*In Vitro* Binding	*In Vitro* Killing	*In Vivo* Killing	Pharmacological Half-Life	T/NT Ratio	Main Organs of Accumulation
		Bold = aromatic side-chain **Underline = cationic side-chain**	**(D)**	**MRSA**	**IC 50 * (μM)**	**IC 50 * (μmol/ mouse)**	**(min)**	**1 hr p.i.**	**>(20%ID) #**
Defensins	HNP-1	AC**Y**CRIPACIAGER-RYGTCI**Y**QGRL**W**A**F**CC	3,442	47.8	4.9	0.3	98	2.4	K & U
Lactoferrins	hLF	692 amino acids	77,000	5.9	7.0	4.3	36	3.0	U & L
	hLF 1-11	GRRRRSVQ**W**CA	1,375	12.9	2.9	1.2	17	2.5	L & U
	hLF 2-11	RRRRSVQ**W**CA	1,318	13.0					
	hLF 3-11	RRRSVQ**W**CA	1,161	4.8					
	hLF 4-11	RRSVQ**W**CA	1,005	2.1	68.0	17.4	4	1.7	L & U
	hLF 5-11	RSVQ**W**CA	849	0.6					
	hLF 6-11	SVQ**W**CA	693	0.5					
	hLF 21-31	**F**Q**W**QRNMRKVR	1,549	5.6	21.0	8.9	3	4.1	U & L
Ubiquicidins	UBI 1-59	KV**H**GSLARAGKVRGQT PKVAKQEKKKKKTGRA KRRMQ**Y**NRRFVNVVPT **F**GKKKGPNANS	6,648	3.5	0.60	0.4	49	1.7	K & U
	UBI 1-18	KV**H**GSLARAGKVRGQT PK	1,890	31.0	24.5	8.2	126	1.9	U & K
	UBI 18-35	KVAKQEKKKKKTGRAK RR	2,169	66.9	23.5	2.9	49	2.4	K & U
	UBI 36-41	MQ**Y**NRR	867	17.2	>200	6.6	17	3.4	U & K
	UBI 42-59	**F**VNVVPTFGKKKGPNA NS	1,904	15.8	>200	8.0	21	3.1	U & K
	UBI 18-29	KVAKQEKKKKKT	1,444	34.0	82.5	68.0	38	2.9	U & K
	UBI 29-41	TGRAKRRMQ**Y**NRR	1,693	41.2	20.0	3.1	142	3.3	U & K
	UBI 31-38	RAKRRMQ**Y**	1,108	65.1	16.0	0.1	27	3.7	U & K
	UBI 22-35	QEKKKKKTGRAKRR	1,731	82.8	38.0	5.8	36	1.4	K & U
Histatins	Histatin-5	DS**H**AKR**HH**G**Y**KRK**FH**E K**HH**S**H**RG**Y**	3,036	5.9	2.8	3.5	10	2.4	U & K
	dH5	KRK**FH**EK**HH**S**H**RG**Y**	1,847	8.1	112.5	1.0	18	2.0	U & K
	dH5 di-mer	α,ε-(KRK**FH**EK**HH**S**H**RG**Y**)2 K-amide	3,803	15.8	8.2	2.0	9	1.8	U & K

(Table 1. Contd….)

Peptide		Sequence	Mass	*In Vitro* Binding	*In Vitro* Killing	*In Vivo* Killing	Pharmacological Half-Life	T/NT Ratio	Main Organs of Accumulation
		Bold = aromatic side-chain **Underline = cationic side-chain**	**(D)**	**MRSA**	**IC 50 * (μM)**	**IC 50 * (μmol/ mouse)**	**(min)**	**1 hr p.i.**	**>(20%ID) #**
	Dhvar 4	KRL**F**KKLLFSLRK**Y**	1,840	17.4	3.1	1.5	29	2.4	U & K
	Dhvar 4 dimer	α,ε-(KRLFKKLLFSLRK**Y**)2K-amide	3,789	33.8	0.4	1.0	34	2.2	K & L
	Dhvar 5	LLLFLLKKRKKRK**Y**	1,847	37.8	17.3	3.0	39	1.8	K & L & U
	Dhvar 5 dimer	α,ε-(LLL**F**LLKKRKKRK**Y**)2K-amide	3,803	25.7	0.4	2.0	23	2.0	K & L & U

* Inhibitory concentration that reduced the number of viable bacteria with 50% (IC 50) was determined after 2 h of incubation and was calculated from the results of at least 3 independent experiments. T/NT = target (infected thigh muscle)-to-non-target (contralateral muscle) ratio determined from ex vivo tissue counts. # K (Kidneys), L (Liver), U (Urinary bladder) at 1-2 hrs p.i. in percentage of the injected dose (%ID).

defensin-1, HNP-1, (ACYCRIPACIAGERRYGTCIYQGRL-WAFCC, with disulfide bridges at positions: 2-30, 4-19, 9-29, Mw 3,442 Da) was tested for targeting *Klebsiella pneumoniae* (gram-negative) and *Staphylococcus aureus* (gram-positive) thigh muscle infections. Also, tests were performed to establish antibacterial therapy of experimental bacterial infections in mice [12]. In the experimental thigh muscle model, HNP, directly labeled with technetium (^{99m}Tc-HNP), was found to accumulate for about 2% of the injected dose at sites of infection, whereas most of the injected ^{99m}Tc-HNP was rapidly removed from the circulation (half-life of 98 min) *via* renal and urinary excretion (Table **2**) [62]. Another observation was the reduced accumulation of radioactivity in sites of infection after 24 hrs which could be ascribed to a significant decrease in viable bacteria because of the microbicidal activity of ^{99m}Tc-HNP itself [12, 62]. These results demonstrate that accumulation of even tracer amounts (0.4 μg/kg) of ^{99m}Tc-HNP at sites of infection displayed a marked *in vivo* antibacterial activity in experimental infections in mice and that this activity appears to be mediated, at least in part, by increased local leukocyte accumulation. These leukocytes appeared to be a requirement for the antibacterial effect, since leukocytopenic mice did not display significant antibacterial activity after administration of ^{99m}Tc-HNP. These results were encouraging for alternative microbicidal therapy but for finding an infection seeking tracer that does not "kills its target" or that shows little or no immunomodulatory effects the search was focused on other antimicrobial compounds and less microbicidal synthetic fragments. One antimicrobial protein, lactoferrin, is known to have various domains that are responsible for binding to micro-organisms and for microbicidal activities.

Lactoferrins

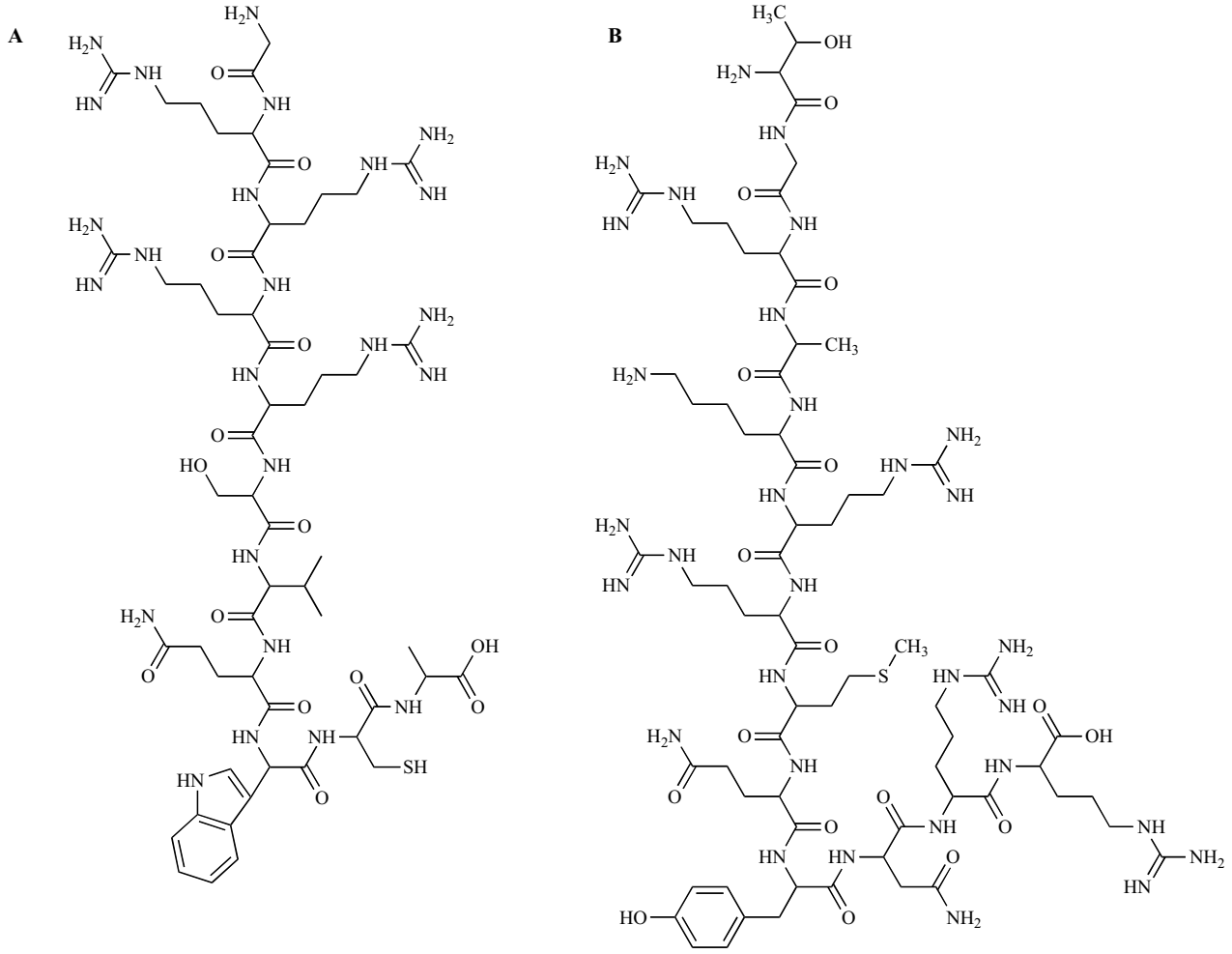
Lactoferrin (LF, 692 amino acids, Mw 77,000 Da) is a multifunctional member of the transferrin family of non-heme iron-binding glycoproteins and is found at the mucosal surface where it functions as a prominent component of the first line of host defense against infection and inflammation. The protein is also an abundant component of the specific granules of neutrophils and can be released into the serum upon neutrophil degranulation [63]. Human LF (hLF) can pass intact through the infant gut, as evidenced by its presence in the feces of breastfed infants, which is of interest in regulating mucosal immunity [64]. Oral administration of hLF in mice [65] or recombinant hLF (rhLF) in humans is effective during short-term treatment of infections or hyper-permeability-associated disorders and in the latter study lactoferrin levels up to 5 grams per person are tolerated [66, 67]. The strong antimicrobial effects of hLF have been confirmed for important bacterial and fungal pathogens [13, 14]. Therefore, the availability of recombinant LF allows clinical trails addressing the efficacy of single or combined formulations of LF with other biologically active molecules [31]. Some features regarding the performance of directly labeled ^{99m}Tc-labeled hLF are given in Table **2**. Rapid targeting of infections in mice is observed, although the binding to bacteria is poor [14, 31]. Another disadvantage of the hLF peptide is its strong microbicidal activity and sensitivity to proteolytic degradation as well as denaturation which makes the development of a kit formulation difficult. Also, the relative high abdominal uptake of ^{99m}Tc-labeled hLF hampers its use for detecting abdominal infections (Fig. **6**).

Since most natural antimicrobial peptides are cationic, it is likely that the N-terminal cationic domains of hLF play an essential role in its bactericidal activity. hLF contains two N-terminal cationic domains, i.e., RRRR (residues 2 to 5) and RKVR (residues 28 to 31). Recent data showed that the first eleven N-terminal amino acids of hLF (hLF 1-11, GRRRR-SVQWCA, Mw 1,375 Da, a linear peptide comprising the first cationic domain on hLF, Fig. **5**), is more effective against a wide variety of bacteria and fungi than the full hLF protein [13, 14, 24]. In several experimental models of systemic and focal mycoses and bacterial infections in mice, hLF 1-11 showed to be highly effective. In a model testing drug-drug interactions, it was shown that the concomitant use of conventional antibiotics and hLF 1-11 did not show any adverse effects [24, 68]. It is of interest which part of

hLF 1-11 is responsible for binding to bacteria and which part kills them. For this purpose truncated fragments of hLF 1-11 (ranging from 2-11 to 6-11) and hLF 21-31 (FQWQRNMRKVR, Mw 1,549 Da) were synthesized. They comprise the second cationic domain on hLF which is less effective than hLF 1–11 in killing of bacteria *in vitro* and in mice [14]. After direct labeling with ^{99m}Tc, the fragments were evaluated to delineate essential amino acids and their biological performance. Scintigraphic analysis of radioactivity in the various organs of mice and rabbits (Fig. **6**) that received ^{99m}Tc-labeled hLF 1-11 peptide (^{99m}Tc-hLF 1-11) showed that they were cleared from the bloodstream (half-life between 3 and 17 min) mainly *via* the liver and kidneys/urinary bladder [58, 69]. Compared to labeled hLF 1-11 truncated ^{99m}Tc-labeled hLF peptides showed lower binding to bacteria and lower accumulation in infected tissues (Table **2**). For ^{99m}Tc-hLF 21-31 peptide there was high uptake in infected tissues although the low binding to bacteria will limit its use for detecting small or hidden infections with low amounts of pathogens. Quantitative scintigraphic analysis revealed that in both (immunocompromised) mice and rabbits about 1-2% of the injected dose of the ^{99m}Tc-hLF 1-11 injected accumulated within 1 hr after administration at the site of infection. As observed with hLF, the synthetic hLF 1-11 peptide reduced the number viable bacteria as well [14]. Accordingly it can be concluded that radiolabeled hLF and hLF peptides are less suitable for infection imaging although the labeling method is used to determine the pharmacokinetics and the effectiveness of their uptake in infected tissues after various routes of administration (results submitted for publication).

Ubiquicidins

Antibacterial and immunological side effects of defensins and lactoferrin (peptides) may have limited the diagnostic use of radiolabeled AMPs so far. The search has been focused to less biological active AMPs and in this respect the ubiquicidin peptide is a potential candidate. The amphipathic α-helix human antimicrobial peptide ubiquicidin (KVHGSLARAGKVRGQTPKVAKQEKKKKKTGRAKRRMQYNRRFVNVVPTFGKKKGPNANS, UBI 1-59, Mw 6,700 Da) is a linear peptide found at low concentrations as a first line of defense inside human airway epithelial cells, activated macrophages, and in human colon mucosa [70]. In contrast to most other AMPs, UBI 1-59 is only present intracellular and is only released after severe damage during acute infection. In order to determine bacterial binding domains the structure of UBI 1-59 was analyzed through computer analy-



(Fig. 5. Contd….)

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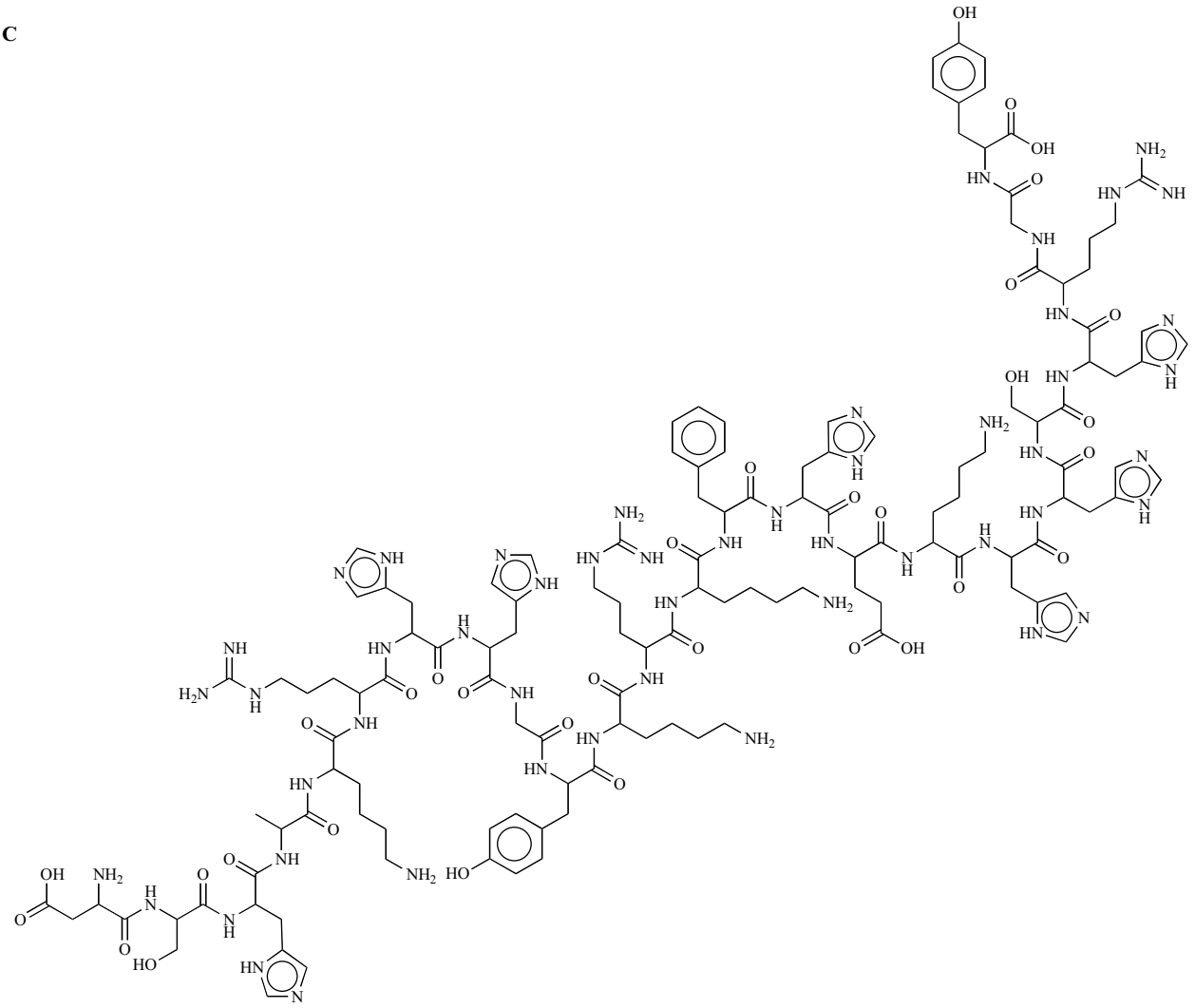

Fig. (5). (**A**) Synthetic peptide derived from the first 11 N-terminal amino acids of human lactoferrin peptide (hLF1-11), sequence = GRRRRSVQWCA. (**B**) Synthetic peptide derived from amino acids 29-41 of human ubiquicidin peptide (UBI 29-41), sequence = TGRAKRRMQYNRR. (**C**) Synthetic peptide derived of human histatin peptide (Histatin-5), sequence = DSHAKRHHGYKRKFHEKHHSHRGY.

sis and the amino acid sequence and various cationic and lipophilic derivates in the α-helix and β-sheet domains were well characterized [17] based on calculations by Garnier-Robson [71] and Kyte *et al*. [72]. A selection of fragments has been synthesized through determination of clusters consisting of cationic and/or lipophilic domains (Table **2**). Using different directly labeled ^{99m}Tc-UBI peptides numerous studies have been performed to record the pharmacology and infection detection in soft tissue and bone in mice, rats, and rabbits [54, 58, 69, 73, 74]. Recently, UBI 29-41 (TGRAKRRMQYNRR, Mw 1,693 Da, Fig. **5**) was directly labeled with ^{99m}Tc from a kit and was evaluated in humans [59, 61]. After intravenous injection ^{99m}Tc-labeled UBI peptides are rapidly removed from the circulation (half-life between 17 and 142 min). Most of the radioactivity is removed *via* the kidneys and after accumulation excreted into the urinary bladder. About 0.5-2% of the injected dose accumulates in the infected thigh muscles. Little accumulation is observed in the liver, spleen, and other tissues. No radioactivity can be observed in thyroid glands and stomach, which are the reservoirs for free or released ^{99m}Tc-activity, suggesting that the tracer is stable *in vivo*. As indicated by the target (infected thigh muscle)-to-non-target (contralateral non-infected thigh muscle) ratios (T/NT) depicted in Table **2**, ^{99m}Tc-labeled UBI peptides accumulates rapidly in the infected tissue. Examples of scintigraphic images with ^{99m}Tc-UBI 29-41 in rats showing multi-drug resistant *S. aureus* (MRSA) infected thigh muscles (before and after antimicrobial therapy) are depicted in Fig. (**7**). Fragments comprising a α-helix displayed the highest, dose-dependent antibacterial activity towards

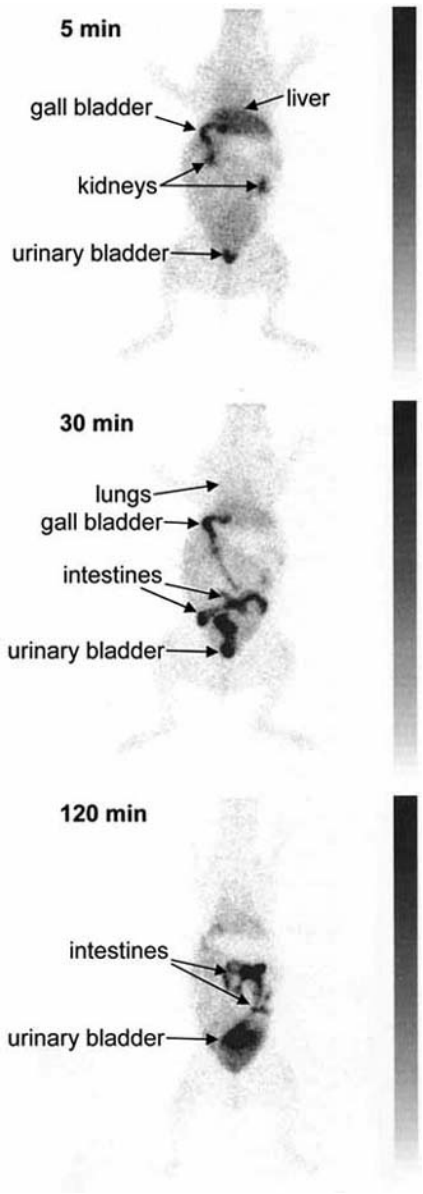

Fig. (6). Typical dynamic scintigrams of a normal rabbit at various time intervals after injection of ^{99m}Tc-labeled hLF 1–11 into an ear vein. Notice major radioactivity seen in the gall bladder followed by emptying in the intestines. Also, radioactivity in the intestines is re-absorbed into the bloodstream where after it is rapidly cleared *via* the kidneys and emptied into the urinary bladder.

MRSA. Additional experiments have shown that the strong antimicrobial activity of the peptides containing the α-helix was also effective against *S. aureus*, *Klebsiella pneumoniae*, *Escherichiae coli*, and *Candida albicans*, indicating that this domain plays an important role in the antimicrobial activity and binding of UBI towards a variety of pathogens. Using this observation a selection was made from the group of least microbicidal UBI fragments showing the highest ratio in binding to bacteria/leucocytes. This ratio was the highest for ^{99m}Tc-UBI 29-41, as determined using both *in vitro* and *in vivo* experiments [58].

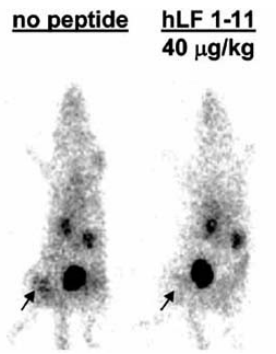

Fig. (7). Typical scintigrams of rats with antibiotic-resistant *S. aureus* infection intravenously injected with 2–7 MBq ^{99m}Tc-UBI 29-41 after treatment with 40 μg/kg hLF 1-11 or without treatment. Arrows indicate the infected thigh muscle which is only seen in untreated animals and not in rats after treatment with hLF 1-11. After the imaging experiments microbiological counting in excised thigh muscles revealed that the number of viable bacteria in hLF 1-11 treated rats was 3log reduced compared to the number of viable bacteria in untreated rats. Reprinted with permission from Nibbering *et al*. *J Nucl Med* **2004**, 45, 321-326.

To find out whether the accumulation of ^{99m}Tc-UBI 29-41 in infected tissues is indicative for the presence of the micro-organism the peptide was administered after injections with 100 times excess of non-radioactive UBI 29-41 or scrambled UBI 29-41 (Sc-UBI 29–41, KRNQRMARYRRGT, Mw 1,693 Da) as competitor. With unlabeled UBI 29-41 and Sc-UBI 29–41 as competitor the accumulation of ^{99m}Tc-UBI 29-41 in infected tissues was inhibited. However, for Sc-UBI 29-41 the effect was not significant. This indicates that besides the cationic residues the amino acid sequence is a crucial feature for binding to bacteria [54]. In order to evaluate the role of inflammatory cells in the accumulation of ^{99m}Tc-UBI 29-41 in infected tissues experiments with this tracer were carried out in leukocytopenic mice [69]. The accumulation of ^{99m}Tc-UBI 29-41 was not significantly different from that in immunocompetent mice, suggesting that the binding of ^{99m}Tc-labeled UBI 29-41 to bacteria rather than binding of the tracer to infiltrating leukocytes is the main factor in the visualization of the infection site. This observation offers new possibilities for infection diagnosis in immunosupressed patients.

Histatins

Another cationic AMP that was studied was ^{99m}Tc-labeled histatin-5 (DSHAKRHHGYKRKFHEKHHSHRGY, Mw 3,036 Da). Histatin-5 is a small histidine-rich linear AMP secreted by the parotid salivary glands [75]. It displays antifungal and antibacterial activities which have been ascribed to the presence of multiple positively charged histidine-, arginine-, and lysine residues in amphipathic helical structures [76]. Amphipathicity of histatins is essential for peptide folding and for their interaction with bacterial membranes at the water-lipid interface [77, 78]. Histatin-5 was initially used as a starting point for the design of peptides with altered antimicrobial activity and lesser susceptibility for proteolytic degradation by dimerization or substitution [79, 18]. One putative active domain of histatin-5 (Dh5, resi-

dues 11-24, KRKFHEKHHSHRGY, Mw 1,847 Da) was used as a basis for the design of directly labeled histatins ^{99m}Tc-Dhvar4 (KRLFKKLLFSLRKY, Mw 1,840 Da, with increased amphipathicity) and ^{99m}Tc-Dhvar5 (LLLFLLKK-RKKRKY, Mw 1,847 Da, with reduced amphipathicity), both showing improved binding to bacteria and enhanced microbicidal potency *in vitro* [18]. Unfortunately, compared to ^{99m}Tc-histatin-5 and ^{99m}Tc-Dh5, both ^{99m}Tc-Dhvar4 and ^{99m}Tc-Dhvar5 did not show improved performance in microbicidal activity and infection targeting (accumulation about 0.4-0.9 of the injected dose) in mice with a MRSA-infected thigh muscle (Table **2**). These findings suggest that the role of hydrophilic and hydrophobic properties of the AMPs is of lesser importance than their charge properties in membrane binding/insertion and pore forming. Biodistribution studies in mice revealed that both ^{99m}Tc-Dhvar4 and ^{99m}Tc-Dhvar5 peptides are prone to significant uptake in liver and intestines which limits their use in imaging abdominal infections.

The effect of dimerization of directly labeled ^{99m}Tc-histatins Dh5, Dhvar4, and Dhvar5 on the detection of MRSA infections mice was also evaluated. The rationale behind using homodimers of antimicrobial peptides is that multiple peptides line up together in a dose-dependent fashion so that dimerization can lead to an increase in reaction rates and thus to a significant potentiation. Therefore, constructs were synthesized in which both c-termini of the monomeric peptides are linked by a disubstituted lysine-amide acting as a flexible hydrophilic spacer that allows a large freedom of conformation. In theory, dimerization leads to an increase in reaction rates by several orders of magnitude, depending on the number of peptides involved in each individual pore, and thus to a significant potentiation. A substantial effect of dimerization of ^{99m}Tc-labeled Dh5 and Dhvar4 can lead to increased binding to bacteria and enhanced microbicidal activity *in vitro* (Table **2**), but this was not observed for killing or targeting MRSA in mice [18]. Dhvar5 showed significantly more binding to MRSA in binding studies with ^{99m}Tc-labeled peptides than Dhvar4, which can be explained by its higher charge. The dimers of ^{99m}Tc-labeled Dhvar5 showed significantly less binding than the monomeric peptide, which can be explained by shielding of the positive charge by the outward-directed hydrophobic N-terminus of the unbound monomeric part of this construct. Scintigraphic monitoring of the distribution of directly labeled ^{99m}Tc-labeled histatin AMPs in mice showed that all dimeric peptides were targeted to the sites of infection with about 0.5-0.8% of the injected dose. Despite rapid clearing from the blood circulation (half-life between 9 and 39 min) and fast localization of infections, amphipathic alterations or dimerization of these ^{99m}Tc-labeled histatin peptides does not improve drug targeting or microbicidal performance.

USE OF RADIOLABELED AMPS FOR INFECTION IMAGING

The candidate with most potential for infection imaging (direct labeled with technetium-99m UBI 29-41) was selected from an array of ^{99m}Tc-labeled AMPs on the basis of a five phase strategy. First, the *in vitro* binding of ^{99m}Tc-AMPs was determined against purified bacteria and leukocytes *in vitro* to determine the highest ratio in binding between the cell species to select specific bacteria imaging tracers [58]. Second, a peritoneal infection model was used to evaluate the peptide with respect to preferential binding of ^{99m}Tc-labeled AMPs to bacteria over infiltrating host cells [12, 58]. Third, ^{99m}Tc-labeled AMPs were injected into animals with an experimental thigh muscle infection or a sterile inflammatory process (lipopolysaccharides (LPS) or dead micro-organisms), and the uptake in these sites was compared. Fourth, the effect of dose-related accumulation of ^{99m}Tc-labeled AMPs on the number of viable pathogens at the site of infection was determined. In the fifth phase, additional investigations were undertaken regarding other characteristics of ^{99m}Tc-labeled AMPs like preferential pharmacokinetics and possible immunological side effects.

FUTURE PROSPECTS WITH RADIOLABELED AMPs

Recent data, as discussed in this review, provides evidence that biologically active domains on AMPs can be identified and tested using ^{99m}Tc-labeled AMPs. This offers possibilities for improvement of infection detection and development of a new class of antibiotics to combat infections with drug resistant pathogens.

The development of specific infection imaging agents will help the clinicians in detecting obscure infections, differentiating between infections from inflammation, and monitoring the success of antimicrobial therapy of infections with (multi-drug resistant) pathogens. The development of graft-versus-host disease (GVHD), puts transplant recipients at risk for infection for several weeks or months after engraftment. Invasive fungal diseases contribute substantially to death and illness associated with the prolonged, profound neutropenia resulting from intensive radio- and chemotherapy for hematological cancers as well as from myeloablation for allogeneic hematopoietic stem cell transplantation [80].

Further evaluation with different types of infections caused by micro-organisms such as viruses, fungi, parasites, and intracellular pathogens in humans will reveal the potential of these promising tracers. It is encouraging that for the re-discovery and development of AMPs a new class of (peptide) antibiotics has become available and a number of companies are now in phase 2 studies for testing their AMPs in humans [81]. The above diseases are widespread and if effective radiopharmaceuticals are developed for diagnostic purposes as well as for screening potential microbicidal agents, nuclear medicine will have the potential to access areas of wider impact, especially in the developing world. Findings based on radiography are nonspecific in defining acute infection and the radiologist must be aware of potential limitations of the sensitivity of radiography and computed tomography (CT). Recent developments in fusion imaging i.e. complementary imaging technologies such as high resolution SPECT-CT, PET-CT and combinations with MRI, are promising with respect to non-invasive molecular imaging of drug interaction with cellular targets [82-85].

Another development is that AMPs can be applied as carriers for (non-specific) drugs. Drug-delivery carriers need to have specific features such as low toxicity and the ability to penetrate cells or pass the blood-brain barrier. Proline-rich

cationic molecules are usually selected in the search for drug carriers for their ability to penetrate cell membranes although these cationic molecules often show high levels of toxicity [86, 87]. Otvos *et al*. showed that the molecules can easily penetrate bacterial cells as wall as human cells, and that various designed analogs are able to penetrate several types of human cells including fibroblasts [88]. The radiolabeled AMPs can be used to visualize or quantify the delivery of drugs to intracellular pathogens. However, it is of importance to determine their limitations, in particular in terms of the size limit for the cargo and whether the cargo is required to possess a specific charge. Another feature would be the potential for immunotherapy as ^{99m}Tc-labeled AMPs are able to penetrate dendritic cells easily without showing toxicity, can stimulate the immune system, and can trigger activation which can be used in vaccine development and anticancer therapy. Otvos is optimistic that this system can become the system of choice for the design of drug-delivery in eukaryotes and possibly the system of choice for dendritic cell-based immunotherapy.

CONCLUSION

^{99m}Tc-labeled cationic AMPs are studied for pathogen-specific infection detection and monitoring the efficacy of antibacterial therapy using scintigraphic techniques. Recently published data shows that directly labeled ^{99m}Tc-labeled AMPs discriminate between bacterial and fungal infections from sterile inflammation in both immunocompetent and immunocompromised animals. In this respect it was shown that the accumulation of ^{99m}Tc-labeled AMPs in infected tissues is specific due to their binding to viable bacteria, and not to interaction with host cells. This property is promising for the clinical management of patients in which this distinction is important, for example in cases with artificial implants or rejection of organ transplants. Furthermore, immunological techniques and peptide chemistry allow characterization and nomination of active domains on AMPs and the possibility to synthesize constructs with enhanced potency and altered pharmacology. The radiolabeling technique of AMPs significantly contributes to the development of a new class of tracers that can localize pathogens. The direct labeling of synthetic AMPs with ^{99m}Tc is easy and reliable and facilitates the detection of infections and monitoring antimicrobial therapy with limited side effects. One disadvantage for imaging infections is the microbicidal and membrane disrupting activities of some peptides which cause the killing of the target and decreasing target-to-nontarget ratio. However, ^{99m}Tc-labeled AMPs can still be used to study the pharmacology and to quantify the accumulation of the antimicrobial peptides at sites of infection at early intervals to determine their microbicidal activity at late intervals after counting viable pathogens. Although radiolabeled AMPs are abundantly tested in laboratory animals the number of patients in which the ^{99m}Tc-labeled AMPs are tested is still limited.

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