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Research Article

Kit with technetium-99m labelled antimicrobial peptide UBI 29-41 for specific infection detection

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Summary

The purpose of this study was to investigate radiochemical and biological characteristics of an instant kit for the preparation of ^{99m}Tc-labelled UBI 29-41 for specific detection of infections. The kit is based on ^{99m}Tc-labelling via HYNIC conjugated to the terminal amine of the peptide, producing a well-understood labelled compound.

One hour after the addition of fresh $^{99m}TcO_4^-$ to the kit ITLC and HPLC reverse-phase analysis was performed. Stability of the labelled complex was challenged and the binding to bacterial pellets was assessed. Finally, the biodistribution and accumulation in *MRSA*-infected tissues were studied using scintigraphy and *ex vivo* countings. Data were compared to a non-kit control method.

Radiochemical analysis indicated >96% labelling, stability for 24 h and the preparation was used without purification. *In vitro* studies showed 41% of radioactivity was bound to bacteria. After injection into mice with a bacterial infection the site of infection was visualized within 30 min. Kit prepared ^{99m}Tc-HYNIC-UBI 29-41 was rapidly (half-life 113 min) cleared via the kidneys and urinary bladder, essentially slower than control peptide (half-life 74 min). This slower clearance results in higher activities in blood and other tissues. Nevertheless, ^{99m}Tc-HYNIC-UBI 29-41 shows favourable radiochemical

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characteristics and deserves further evaluation in a clinical setting.

Key Words: antimicrobial peptide; HYNIC conjugation; ^{99m}Tc-labelling; kit; infection detection

Introduction

Discriminating infections from sterile inflammatory lesions is an important issue under development in nuclear medicine. Technetium-99m (^{99m}Tc) labelled cationic antimicrobial peptide UBI 29-41 binds preferentially to bacteria and *Candida albicans* with poor accumulation at sites of sterile inflammation.[1,2] Moreover, ^{99m}Tc-labelled UBI 29-41 allows monitoring of the efficacy of antimicrobial treatment in mice with an experimental infection.[3]

In this study we report on the radiochemical and biological characteristics and stability of a kit based on the preparation of ^{99m}Tc-peptide labelling via N-terminal hydrazino nicotinamide acid (HYNIC) conjugated UBI 29-41 (Figure 1). This bifunctional chelating agent was chosen as its labelling mechanism is well understood.[4,5]

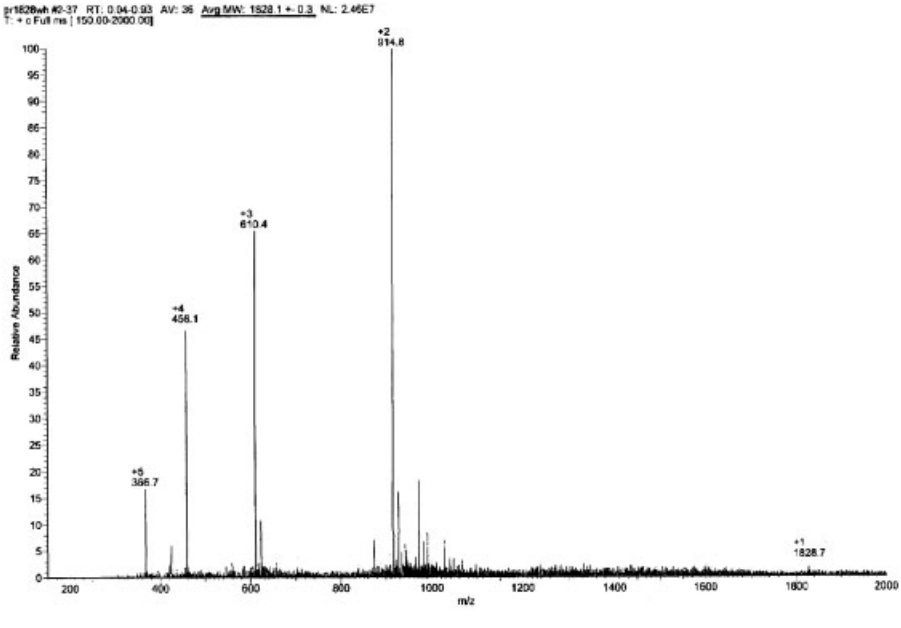

Figure 1. Mass spectrometric (MS-HPLC) analysis of HYNIC conjugated at the N-terminal threonine of UBI 29-41. Lysine and other amine groups were protected before conjugation

Results and discussion

Labelling and quality control

As determined with ITLC analysis, HYNIC-UBI 29-41was labelled with >96% yield with less than 8% release of radioactivity after 24 h of incubation in human serum at 37°C. Reverse-phase HPLC analysis of the kit showed two peaks of radioactivity with a retention time between 14 and 15 min (Figure 2). Analysis of control peptide revealed two major peaks of radioactivity with a retention time between 15 and 17 min. The preparations showed no free pertechnetate and all runs were with full recovery of radioactivity.

In vitro binding assay

In vitro testing of ^{99m}Tc-UBI 29-41 to multidrug resistant *Staphylococcus aureus* (MRSA) revealed that both the kit and control peptide showed between 40 and 41% of radioactivity bound to bacteria.

Biodistribution

Infections with *MRSA* could already be visualized within 30 min after injection of the various tracers. Typical scintigrams 1 h after injection with ^{99m}Tc-UBI 29-41 preparations are depicted in Figure 3.

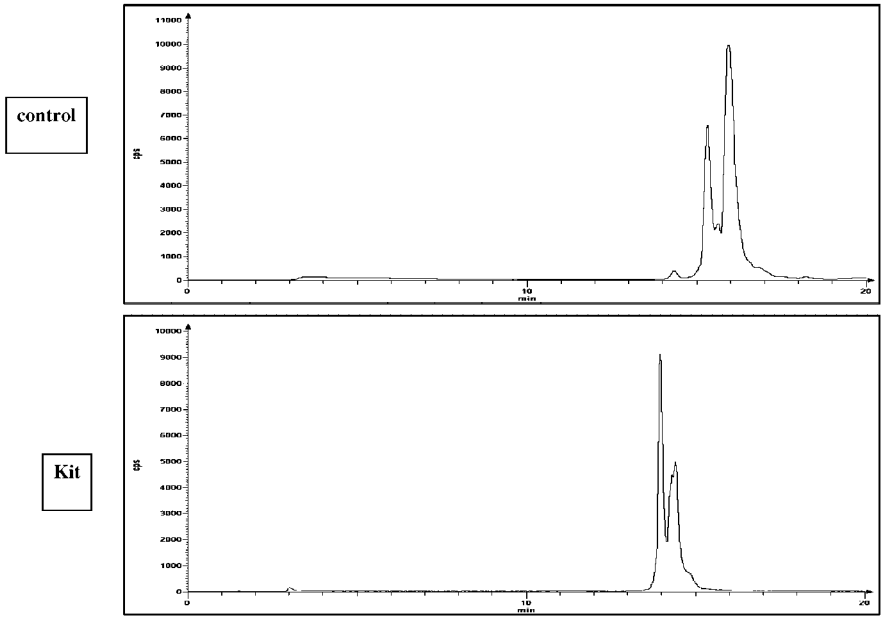

Figure 2. Typical reverse-phase HPLC profiles of ^{99m}Tc-f UBI 29-41. The radiograms of the ^{99m}Tc-labelled peptides are expressed as counts per second

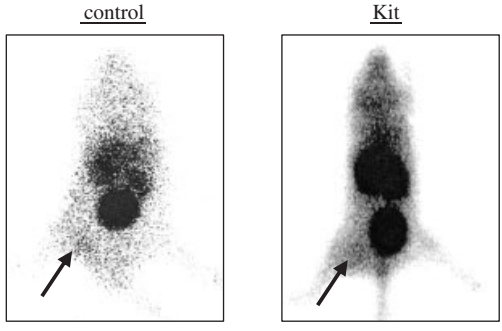

Figure 3. Typical planar scintigrams of ^{99m}Tc-UBI 29-41 at 1 h after injection into mice with a thigh muscle multi-drug resistant *S. aureus* (*MRSA*) infection. On the scintigrams the animals are shown in anterior position. The focus of infection is indicated with an arrow

Table 1. Biodistribution of ^{99m}Tc-labelled UBI 29-41 in mice with an *MRSA*-thigh muscle infection after 2 h

	^{99m}Tc-UBI 29-41			
	Control		Kit	
Tissue	(%ID/organ)	(%ID/g)	(%ID/organ)	(%ID/g)
Blood	0.9 ± 0.4	3.2 ± 1.4	10.8 ± 1.1*	15.3 ± 1.1*
Heart	0.4 ± 0.2	2.4 ± 0.7	2.5 ± 0.3*	10.1 ± 1.0*
Urine + bladder	46.8 ± 9.4	51.8 ± 10.8	7.4 ± 1.5*	37.9 ± 8.2
Kidneys	17.6 ± 4.2	76.1 ± 15.4	5.7 ± 1.0*	12.9 ± 2.8*
Liver	6.5 ± 2.9	8.8 ± 1.8	11.5 ± 2.3	15.3 ± 2.2
Spleen	1.2 ± 0.3	17.8 ± 8.2	2.2 ± 0.4*	18.0 ± 4.0
Stomach	2.2 ± 0.5	4.3 ± 1.0	11.4 ± 1.1*	16.4 ± 2.1*
Lungs	0.4 ± 0.1	4.2 ± 0.2	4.7 ± 1.3*	14.2 ± 0.5*
Thyroid	0.1 ± 0.05	1.4 ± 0.4	3.4 ± 0.1*	17.1 ± 1.8*
Intestines	13.5 ± 4.1	6.1 ± 1.5	16.9 ± 0.8	6.0 ± 0.6
Gall bladder	2.9 ± 1.4	15.8 ± 4.8	3.5 ± 0.6	19.3 ± 2.8
Infected muscle	1.02 ± 0.31	3.79 ± 1.33	6.1 ± 0.98*	10.14 ± 1.02*
Muscle	0.52 ± 0.20	1.98 ± 0.72	3.40 ± 0.98*	5.81 ± 0.73*
Infection/non-infection ratio	2.54 ± 0.31	2.08 ± 0.18	2.16 ± 0.33	1.84 ± 0.27

All data are given as the mean % (± SEM) of the injected dose/g tissue are calculated from entire excised tissues 2 h after injection of the tracer of at least four observations.
*$P < 0.01$ compared to control labelling according to Student *t*-test.

The biodistribution data are summarized in Table 1. Control ^{99m}Tc-UBI 29-41 showed major accumulation of 64% of the injected dose (%ID) in the urinary bladder and kidneys which was significantly ($P < 0.05$) higher than that of the kit (13%). For both preparations <4%ID of radioactivity was observed in stomach and thyroid gland indicating minimal release of radioactivity from the peptide in mice. The control labelling has a half-life in blood of 74 min. For

the kit we observed slower clearance (half-life 113 min) indicated with significant ($P < 0.01$) higher activity in most tissues.

Accumulation in bacterial infected thigh muscles

Target-to-non-target (T/NT) ratios calculated for various ^{99m}Tc-UBI 29-41 preparations are depicted in Figure 4. For control ^{99m}Tc-UBI 29-41 we observed the highest T/NT ratios at all intervals determined. At 2 h after injection T/NT ratios for the kit was comparable to that of control ^{99m}Tc-UBI 29-41. These findings are in accordance with our calculations from the excised tissue data as shown in Table 1. For both preparations we calculated good and significant ($P < 0.03$) correlations between the number of micro-organisms and the T/NT ratio ($R =$ between 0.64 and 0.88).

Previous experiments from our group with ^{99m}Tc-UBI 29-41 made use of a direct labelling method[3,6–8] of which the labelling mechanism has only been partly elucidated.[8,9] In view of the incomplete knowledge we considered a labelling method via the well-accepted chelator HYNIC attached to the amine terminal of UBI 29-41 during synthesis while other amine residues were protected from multiple conjugations. This approach provides a well-understood labelling mechanism that lends itself for easy kit preparation. Moreover, it avoids the risk that the attachment of such a ligand takes place at or nearby a biologically active site hampering various biological interactions.

With regard to infection detection, we observe lower T/NT ratios for the HYNIC-peptide (Figure 4), which may be due to a slower clearance of

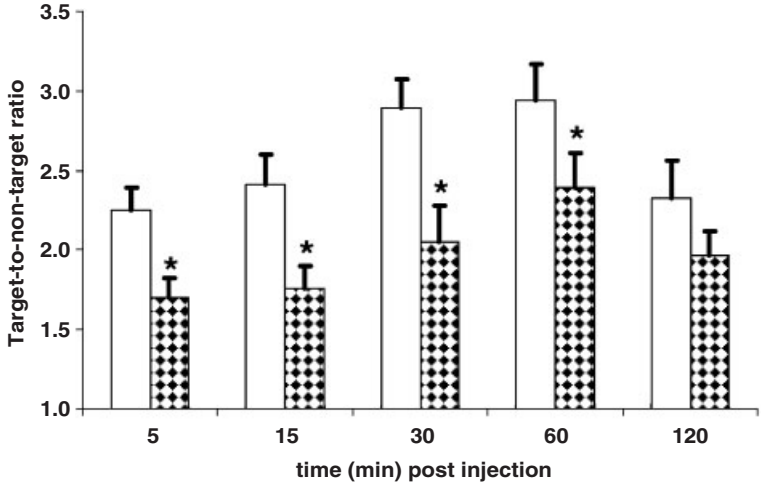

Figure 4. Accumulation of ^{99m}Tc-UBI 29-41 (open bars) and ^{99m}Tc- HYNIC-UBI 29-41 from the kit (dotted bars) in multi-drug resistant *S. aureus* (*MRSA*)-infected thigh muscles of mice. Results are mean target-to-non-target ratios ($\pm$ SEM) of at least four animals in two independent experiments

background activity (Table 1). It is conceivable that the HYNIC conjugation of the terminal amine of UBI 29-41 decreased the cationicity of the compound leading to a slower clearance by the kidneys.

Nevertheless, the radiochemical and biological features of ^{99m}Tc-UBI-HYNIC 29-41 promote the development of a kit-produced radiopharmaceutical under GMP conditions for clinical studies. The HYNIC preparation has the advantage of its well-defined chemical structure and its safety in humans.[10]

Experimental

Peptides

Antimicrobial peptide UBI 29-41 (TGRAKRRMQYNRR, 1,693 Da, LUMC. The Netherlands),[7] was synthesized as described.[11] Conjugation of HYNIC to UBI 29-41;A 6-(tert-butoxycarbonyl)-hydrazinopyridine-3-carboxylic acid (BOC-HYNIC) bifunctional ligand was synthesized as described.[4] The conjugate HYNIC-UBI 29-41 was prepared as follows: a total of 8.31 mg of BOC-HYNIC, 12.49 mg of *O*-(7-azabenzotriazolyl)-1,1,3, 3-tetra-methyluronium hexafluorophosphate (HATU, Sigma Chem. Co.) in 5.60 µl of *N*-ethyldiisopropylamine (DIPEA, Sigma Chem. Co.), and 150 µl of *N*-methyl-2-pyrolidone (NPM, Sigma Chem. Co.) were reacted for 30 min at room temperature. This solution was added to 100 mg of UBI 29-41 in 5.6 µl of DIPEA and 150 µl of NPM was added and allowed to react for 2 h at room temperature. Thereafter, to this mixture 1 ml of 5% $NaHCO_3$ (w/v) (Sigma Chem. Co.) solution and 1 ml of ethyl acetate (Sigma Chem. Co.) were added. The $NaHCO_3$ solution was extracted three times with 1 ml of ethyl acetate. The combined organic phases were washed four times with water and then evaporated to dryness. The deprotection (removing BOC group) was achieved by dissolving the dried powder into a (5/89/3/3%) H_2O/trifluoroacetic acid (TFA, Sigma Chem. Co.)/methyl phenyl sulphide (Sigma Chem. Co.)/thioanisole (TIS, Fluka Chemie GmbH Buchs, Germany) mixture and then reacted for 4 h at room temperature. After incubation, the conjugate was precipitated with ethyl ether (Sigma Chem. Co.) and centrifuged for 15 min at 500*g* at 4°C. The crude conjugate UBI-HYNIC was applied on semi-preparative reverse-phase HPLC resulting into a purity to 97%. The molecular weight of the purified conjugate was confirmed by mass spectrometry (Figure 1). The kit contains 20 µg HYNIC-UBI 29-41, 20 µg $SnCl_2$, 20 mg *N*-[*tris*(hydroxymethyl)-methyl]-glycin (tricine, Merck, Darmstadt, Germany), and 50 mg mannitol (Calbiochem®, San Diego, CA). The kit was lyophilized for 21 h at −45°C, for 10.3 h, from −45 to 40°C, and for 14.3 h at 40°C at 0.12 mbar in N_2 atmosphere and stored at 4°C.

Labelling

The kit was dissolved with 500 µl of ^{99m}Tc-pertechnetate (400–2000 MBq/ml). The mixture, with a pH of 4.0, was gently stirred at room temperature for at least 1 h. Control labelling: UBI 29-41 was directly labelled with technetium-99m as described.[7]

Quality control

ITLC. Labelling yield and stability of the ^{99m}Tc-labelled peptides was assessed by instant thin layer chromatography (ITLC) using saline or methyl ethyl ketone (MEK) as eluent.

RP-HPLC. Samples of 10–50 µl of the labelled samples were analysed on a reverse-phase HPLC column (Dionex 4.6 × 250 mm C_{18} column, Vydac, The Separations Group, Hesperia, CA) using a linear gradient as described[8] at a flow rate of 1 ml/min.

Stability of ^{99m}Tc-labelled peptides in human serum

The stability of ^{99m}Tc-labelled-UBI 29-41 was challenged by mixing equal volumes of the peptide and human serum. After incubation for 24 h at 37°C the amount of free ^{99m}Tc in the samples was determined by ITLC using MEK as eluent as described above.

Bacteria

The clinical isolate *S. aureus* 2141 (*MRSA*) was highly resistant to a variety of antibiotics.[12]

In vitro binding assay

Binding of 1 µg of ^{99m}Tc-labelled peptide to bacteria was assessed at 4°C as described.[7] Incubation for 1 h was followed by two centrifugation and washing steps to separate radioactivity associated with the bacteria from unbound activity.

^{99m}Tc-labelled UBI 29-41 in infected mice

All animal studies using specific pathogen-free, male Swiss mice were done in compliance with the Dutch laws related to the conduct of animal experiments and approved by the local Committee for Animal Experiments (protocols 99016 and 02029). As described before,[8] mice were injected in the right thigh muscle with 0.1 ml of saline containing 2×10^7 viable *MRSA*. After 18 h mice were anaesthetized and 2–5 MBq of radiolabelled peptide diluted in 0.1 ml of saline was injected into a tail vein. Serial scintigraphic imaging took place as described before.[6] Computerized image analysis and anatomically adjusted

regions of interest allowed the assessment of accumulated radioactivity over the entire infected (target; T) and contralateral (non-target; NT) muscle, and accumulation of the tracer was expressed as the ratio of the counts in the regions drawn over the target and the non-target muscles, further referred to as T/NT. *Ex vivo* countings were assessed at 2 h to determine the specific uptake in various tissues.

Statistical analysis

Differences between the data for the kit and the control method was evaluated and compared with the control method using the Student's *t*-test.

Conclusion

Our efforts to develop a useful kit preparation of ^{99m}Tc-labelled UBI 29-41 for specific infection detection have resulted in a one step instant kit. The design in which HYNIC is used as a ligand along with tricine was successful and provided consistently high radiochemical purity and biological performance.

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