Evaluation of $^{68}$Ga-DOTA-Ubiquicidin (29–41) for imaging Staphylococcus aureus (Staph A) infection and turpentine-induced inflammation in a preclinical setting

**ABSTRACT**

Synthetic antimicrobial peptide fragment, $^{99m}$Tc-Ubiquicidin 29–41, is shown to be sensitive and also specific for imaging bacterial infections. We undertook this study to explore the advantage of using a positron emission agent, $^{68}$Ga-DOTA-Ubiquicidin 29–41 ($^{68}$Ga-DOTA-UBI), for detecting Staph-A infection in an animal model, and also evaluated its ability to distinguish a turpentine-induced sterile inflammation in an animal model. Pure Ga-68 was freshly eluted from a $^{68}$Ge/$^{68}$Ga generator (IGG-100). DOTA-UBI (50 µg) was radiolabeled with pure Ga-68 (500MBq) by incubating the reaction mixture at pH 4.5 for 10 min, 95°C. Rats were infected with Staph-A at the hind leg joint of rats to form bacterial abscess. Sterile inflammation was induced in the right thigh muscle by injecting 200 µl of 100% turpentine oil. Rats were injected intravenously with 10–15 MBq of tracer, and images were acquired at different time intervals with Siemens (Biograph mCT) positron emission tomography computed tomography scanner. The early images at 6 min postinjection clearly indicated mild uptake of the agent corresponding to the infection site, which increased dramatically at 20, 30, and 60 min postinjection. The target to background ratio (T/B) increased significantly over the same time period of study (1.6, 4.2, and 6.1, respectively). There was a mild uptake of $^{68}$Ga-DOTA-UBI at the site corresponding to sterile inflammation at 6 min postinjection, which was rapidly washed off as seen at 25 and 45 min images. The images indicated fast clearance of the agent from liver and soft tissues within 6 min. Control rats showed similar biodistribution of activity. The mild uptake of $^{68}$Ga-DOTA-UBI at the corresponding Staph-A infection lesion and very fast kinetics of clearance from the blood pool and soft tissues suggested a very high clinical potential for this agent. The absence of uptake of the agent at sterile inflammation site suggests that the agent may be useful in distinguishing infection from inflammation.

**Keywords:** $^{68}$Ga-DOTA-Ubiquicidin (29–41), $^{68}$Ga-positron emission tomography, infection imaging, inflammation imaging, Staphylococcus aureus

**INTRODUCTION**

Developing an ideal agent for imaging infection has been challenging over the past four decades. A number of single photon-emission computerized tomography (SPECT) agents have emerged in the past but with varying degree of success and limitations.1,2 $^{99m}$Tc-labeled leukocytes have shown promise, but the procedure was complicated and required special skills. $^{67}$Ga-citrate was used for the past four decades for imaging infection, but it required up to 72 h waiting time before the images could be obtained. Gold standard positron emission tomography (PET) agent $^{18}$F-fluorodeoxyglucose played a significant role in infection imaging due to its very high sensitivity, but it
lacked specificity. $^{68}$Ga tracer is now readily available from a commercial $^{68}$Ge/$^{68}$Ga generator, which is cost-effective and we explored the ability of $^{68}$Ga-apo-transferrin,[3] $^{68}$Ga-Citrate-PET for the diagnostic imaging of Staph-A infection in rats and for intra-abdominal infection in patients[6] and $^{68}$Ga-DOTA-Ubiquicidin 29–41 ($^{68}$Ga-DOTA-UBI) to detect Staph-A infection lesions in an animal model.[9]

Antimicrobial peptides are low-molecular-weight proteins, which have the broad spectrum of antimicrobial activity against bacteria.[3] Ubiquicidin 29–41 (UBI 29–41) is a 12 amino acid peptide (Thr-Gly-Arg-Alu-Lys-Arg-Met-Gln-Tyr-Asn-Arg-Arg) is a synthetic cationic antimicrobial peptide fragment with a weight of 1,693 Da. $^9$9mTc-UBI 29–41 targets specifically bacterial and fungal cell wall but fails to target mammalian cells or cancer cells.[13-14] Due to such excellent properties, several studies were undertaken to show the usefulness of UBI 29–41 for imaging infection. $^{99}$mTc-UBI was shown to be a useful agent in the diagnosis of orthopedic infection.[15,16] Zijlstra et al.[17] have demonstrated the utility of $^{18}$F-Fluorine labelled 4-fluoro $^{18}$F-UBI 29–41 for imaging infection. Recent studies by Ebenhan et al.[18] showed the evaluation of $^{68}$Ga NOTA (1,4,7-triazacyclonane-triacetic acid)-UBI 29–41 PET for imaging infection. In the present study, we describe $^{68}$Ga labeling of antibacterial peptide UBI 29–41, using DOTA as the bifunctional chelator (BFC), which has high thermodynamic and kinetic stability, for detecting Staph-A infection in an animal model. We have used DOTA as the BFC for the first time to study Ga-68 labeling with UBI.[17] Subsequently, DOTA has been used as BFC for Ga-UBI by two other groups.[18,19]

MATERIALS AND METHODS

An automated radiosynthesizer was used for the production of $^{68}$Ga-DOTA-UBI. All chemicals were of pharmaceutical grade and of high purity were obtained from Merck and Sigma-Aldrich (Germany). Water for trace analysis (Trace SELECT) was purchased from Honeywell (Germany). DOTA-UBI was purchased from Auspep (Australia) with purity >95%. DOTA-UBI was supplied as freeze-dried powder in vials containing 100 µg aliquots which were stored at -80°C (+5°C) and the values were recorded. The percentage binding of $^{68}$Ga-DOTA-UBI by two other groups.[18,19]

Preparation of $^{68}$Ga-DOTA-Ubiquicidin 29–41 (29–41)

Briefly, the $^{68}$Ge/$^{68}$Ga generator was eluted with 7.0 mL of hydrochloric acid (0.1M) and the Ga-68 was bound onto a strata resin strong cation exchange and subsequently eluted with 5.0 M NaCl containing 5.5M HCl, which was then reacted with 50 µg of DOTA-UBI in 2.0 mL of water (TraceSELECT) and 400 µl of sodium acetate buffer pH 4.5 and by heating at 95°C for 10 min. After labeling, the reaction vial was cooled and passed through C-18 cartridge to remove any free Ga-68.

Determination of radiochemical purity

The radiochemical purity (RCP) was analyzed using radio-thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) methods. The percentage of RCP of $^{68}$Ga-DOTA-UBI was determined by alumina backed chromatography plate and sodium citrate (0.1 M), pH 5.5 as the solvent. Bioscan Radio-TLC Imaging instrument was used to scan the plate. The %RCP is also estimated using a Shimadzu Radio-HPLC gradient method, Kinetex C18 column (Phenomenex, Australia) (3.0 micron × 150 mm), with (A) 0.1% TFA/H2O and (B) Acetonitrile as solvents with a flow rate of 0.6 ml/min.

Stability studies

The stability of $^{68}$Ga-DOTA-UBI was evaluated at four different postlabeling time intervals at 0, 30, 60, and 3 h. Human serum stability study was performed by mixing 0.3 ml of $^{68}$Ga-DOTA-UBI solution with 1.5 ml of human serum (five times volume) and incubating at 37°C for 1 h. RCP was estimated at the end of 1 h incubation period.

In vitro binding of $^{68}$Ga-DOTA-Ubiquicidin 29–41 with Staph-A

Binding of $^{68}$Ga-DOTA-UBI to Staph A was assessed at different time intervals for up to 2 h, by incubating the labelled agent with Staph A at 37°C as follows. Briefly, in a sterile 10 mL reaction vial, 2.0 mL of $^{68}$Ga-DOTA-UBI (25MBq activity) containing 25 µg/mL DOTA-UBI was incubated with Staph A (4×10^7 CFU) in 5.0 mL saline. 1.0 mL aliquots were taken from the reaction vial at different time intervals (5, 30, 60, 90, and 120 min). The aliquots were filtered by using Millex-GV Syringe Filter Unit, 0.22 µm, PVDF, 33 mm (gamma sterilized) followed by 3 mL saline wash. The filter was pretreated with 2 mL saline before use. Activities associated with the filter and the filtrate fractions were measured by using a dose calibrator, and the values were recorded. The percentage binding of $^{68}$Ga-DOTA-UBI was calculated using the activity associated with the (filter unit × 100)/total activity (filter unit + filtrate).

The activities at different time intervals were decay corrected. A control group was set up containing radiolabeled peptide in saline (without Staph A) to determine nonspecific peptide binding.
**Inducing infection and sterile inflammation in animal model**

The experimental protocol was approved by the Western Sydney Local Health District Animal Ethics Committee (WSLHD AEC Protocol No: 9010.08.12), Westmead Hospital, Westmead NSW 2145. Male Wister rats (6–7) weeks old weighing 250–300 g were anesthetized by intra-peritoneal injection of ketamine (100 mg/ml)/xylazine (20 mg/ml) (2:1 v/v). Animals were randomly assigned into two groups. Group 1 (n = 12)-Infection was induced in the right thigh muscle by intramuscular injection of $5 \times 10^5$ CFU Staph A in 0.1 ml volume of sterile saline for injection. Three to four days after inoculation, an abscess formed. Group 2 (n = 6) sterile inflammation was induced in the right thigh muscle by intramuscular injection with 200 µl of 100% turpentine oil (Sigma Aldrich, Australia). Turpentine oil caused visible redness and swelling within 2–3 h after injection.

**Dosage and animal imaging**

Anesthetised rats in both the groups were injected intravenously in the tail vein with 10–15 MBq/0.1 mL saline of $^{68}$Ga-DOTA-UBI. The images were acquired at three different postinjection time points at 6, 30, and 60 min for Staph A infection induced rats and 6, 25, and 45 min intervals for sterile inflammation rats. Images were acquired for 5 min each with a matrix size of 200, reconstruction method: True X + TOF (Ultra HD-PET), 21 subsets using PET/computed tomography scanner (Siemens Biograph mCT). Standardized uptake value (SUV) max was calculated over different organs in rats by drawing the regions of interest by a computer-generated program, which is used routinely in patient’s studies at our department.

**RESULTS**

**Quality control**

The RCP of $^{68}$Ga-DOTA-UBI was >99%. $^{68}$Ga-DOTA-UBI stayed at the origin [Figure 1a], while free Ga-68 moved with the solvent front [Figure 1b]. The RCP of $^{68}$Ga-DOTA-UBI was > 98% at 0, 30, 60 min, and 3 h after radiolabeling without any significant changes. The stability was not tested beyond 3 h, as the T1/2 of $^{68}$Ga is only 68 min. The retention time by HPLC for free Ga-68 was 2 min and for the labelled product 7.2 min [Figure 1c]. The radiolabeling yield was >99%. $^{68}$Ga-DOTA-UBI complex was stable in human serum as shown by RCP >99% when studied for up to 1 h at 37°C.

**Biodistribution of $^{68}$Ga-DOTA-Ubiquicidin 29–41 in normal rats**

Biodistribution of $^{68}$Ga-DOTA-UBI was shown in normal rats at 6, 30, and 60 min postinjection period [Figure 2]. The images clearly showed that there was significant uptake of the agent in the kidneys and the bladder. The activities associated with the other soft tissues were very low and were cleared rapidly as shown at 6 min postinjection image. There was very little background activity in the soft tissues at 30 and 60 min postinjection images.

**Estimation of standardized uptake values at different organs in a normal rat**

Using a normal rat, uptake of the agent at various organs was estimated at different time intervals using a computer program, and the values were expressed as SUV [Figure 3]. The activities associated with the heart, muscle, bone, and liver were shown to be very low over the time period studied (SUV < 3). On the other hand, the activities associated with the kidneys have increased to SUV = 15 at 30 min period, and then began to wash off. There was significant activity associated with the bladder up to 50 min postinjection (SUV = 30) which then declined, commensurate with the kidney activity as expected.

**In vitro binding studies**

Results of in vitro binding studies clearly showed that $^{68}$Ga-DOTA-UBI was able to bind avidly with Staph-A up to...
50% of the injected dose (ID) within 30 min. There was a mild decrease with time, but the percentage binding was retained at 48% of ID even at 120 min of incubation [Figure 4].

**68Ga-DOTA-Ubiquicidin 29–41 for imaging Staph-A infection in an animal model**

The results clearly showed that there was a mild uptake of 68Ga-DOTA-UBI activity at the area corresponding to bacterial lesion within 6 min postinjection in rats [Figure 5]. The intensity of the uptake has increased significantly at 30 and 60 min postinjection period. Very high uptake of the agent in the kidneys and bladder was comparable to the biodistribution of the agent in normal rats. The activity associated with other soft tissues was very low indicating fast clearance of the agent from soft tissues. This is consistent with rapid accumulation activity in the kidneys and bladder.

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**Figure 2:** 68Ga-DOTA-Ubiquicidin 29–41 (10 MBq/0.1mL saline) was injected into healthy normal rats, and the images were acquired at 6, 30, and 60 min postinjection. The images clearly show an avid accumulation of the agent by the kidneys and bladder. There is a mild cardiac blood pool activity at but all the other organs show very little activity even at 6 min postinjection.

**Figure 3:** Standardized uptake value max was calculated at each time point for different organs in healthy rats, and the values were plotted in the graph as shown.

**Figure 4:** In vitro binding of 68Ga-DOTA-Ubiquicidin 29–41 with Staph A was performed as described in the method section. The percentage binding of the tracer by Staph A was plotted against time as shown.

**Figure 5:** 68Ga-DOTA-Ubiquicidin 29–41 (10 MBq/0.1mL saline) was injected into a Staph A infected rat, and images were acquired at different time intervals (6, 30, and 60 min postinjection). Infection lesions are shown by the arrows. Mild uptake of the agent was seen at the infected site within 6 min of post injection, which becomes very intense at 30 and 60 min postinjection.
68Ga-DOTA-Ubiquicidin 29–41 for imaging sterile inflammation in an animal model

There was a mild uptake of 68Ga-DOTA-UBI seen at the site corresponding to sterile inflammation at 6 min post injection, which was rapidly washed off as seen at 25 and 45 min images [Figure 6]. The uptake of the agent in the kidneys and bladder was comparable to biodistribution of the agent in normal rats including fast clearance of the agent from other soft tissues. A measurable uptake of the agent was also observed in the liver at 6 min post injection, which decreased significantly over 45 min time.

DISCUSSION

In recent years, several preclinical studies were reported using 99mTc-UBI 29–41 to study bacterial and fungal infection in animal models. Clinical trials report by Akhtar et al. with 99mTc-UBI 29–41 also added further support that 99mTc-UBI is a highly sensitive and specific agent for detecting infective foci in bone and soft tissues of humans. However, its clinical utility is compromised due to poor image quality of 99mTc, which is intrinsic to SPECT agents. The present study was conducted to study the utility of 68Ga-DOTA-UBI for bacterial infection imaging, instead of 99mTc, to take the advantage of high resolution and sensitivity associated with 68Ga PET tracer. We have used DOTA as the BFC (bifunctional chelator) to label the UBI peptide fragment with 68Ga, as it is believed to have higher biological, thermodynamic, and kinetic stability in vivo.

The other purpose of our study was to investigate if this agent was capable of differentiating sterile inflammation from bacterial infection. Studies by Ebenhan et al. described 68Ga-labelled-UBI with NOTA as the BFC, and they have demonstrated its utility in imaging infection and its ability to differentiate infection from inflammation. Our results showed that 68Ga-DOTA-UBI could be prepared by a simple method with high yield (>95%) and high RCP (>99%) within 10 min. In our experimental design, an optimal pH of 4.5 was used for radiolabeling of the peptide with 68Ga, which is comparable to reports in the literature.

The overall labeling efficiency of 68Ga-DOTA-UBI was >99%, and therefore, no further C-18 purification was required. Using sodium acetate (2.5 M) as buffer, NOTA-conjugated peptides were labeled at 50°C with the yields between 40 and 50% labeled product; however, optimal labeling efficiencies (>99%) were achieved at 85°C. In our studies, the stability of the purified, labeled compound was positively tested, without any significant change of RCP for up to 6 h, to warrant its prospective use in preclinical studies. The agent was stable in human serum when studied for up to 1 h at 37°C.

Our in vitro results clearly indicated very high binding, which is nearly 50% of the added activity of 68Ga-DOTA-UBI to bacteria, which is consistent with reports in the literature as >45% binding of 99mTc NOTA-UBI 29–41 (Lys [Abz]) to the Staph A cells was reported by Ebenhan et al. and 34.6 ± 3.0% and 40–50% binding found for 99mTc-UBI 29–41. Welling et al. studied a 99mTc-UBI 29–41 fragment with a scrambled amino acid sequence and reported significantly decreased bacterial binding, thus supporting our in vitro findings. Available literature on the in vitro studies suggested that binding of 68Ga-DOTA-UBI to bacteria could be the result of its high thermodynamic stability, selectivity, and stereospecificity.

Our in vivo results clearly indicated an avid uptake of the agent at the infection lesions within 6 min post injection, which is faster than any other studies reported in the literature. The avidity of uptake increased dramatically at 30 and 60 min post injection. Bio-kinetics studies by Meléndez-Alafort et al. in pediatric patients with bone infection explained that the short uptake time of UBI 29–41 at sites of infection could be due to the antimicrobial peptide UBI 29–41 interacts electrostatically with the membrane lipids of the bacteria. After entering the cell, the radiopharmaceutical could be bound to a cytoplasmatic specific site on a target bacterial protein causing a fast cell death with the subsequent bacterial removal.

Our studies indicated fast clearance of the agent from liver and soft tissues within 6 min post-injection and the delayed images clearly showed low activity in the blood pool and
soft-tissues. A very high activity was associated with the kidneys and urinary bladder, as they are the excretory mechanism for the tracer. Our findings are consistent with recent human dosimetry measures\cite{28,29} in four healthy volunteers (2 women and 2 men) using $^{68}$Ga-DOTA-UBI, which showed that the tracer was rapidly cleared from the body by urinary excretion (kidney and bladder). They also suggested that the dose to the urinary bladder wall can be reduced by frequent bladder voiding. Control rats showed similar biodistribution of activity. Sterile inflammation induced rats showed a significant uptake of $^{68}$Ga-DOTA-UBI in the liver at 6 min post injection, which may be due to high injected activity. Liver activity was rapidly washed off as seen at 25 and 45 min images. The uptake of the agent in the kidneys and bladder was comparable to the biodistribution of the agent in normal rats.

CONCLUSIONS

$^{68}$Ga-DOTA-UBI can be prepared using an automated radio synthesizer within 15 min. In vitro studies showed that the tracer bound the bacteria, Staph-A, with very high affinity. The in vivo studies showed mild uptake of $^{68}$Ga-DOTA-UBI at Staph-A infection lesion within 6 min, which increased significantly at 30 and 60 min post-injection periods. Clearance from the blood pool and soft tissues showed very fast kinetics. $^{68}$Ga-DOTA-UBI was not accumulating in sites corresponding to sterile inflammation. Therefore, $^{68}$Ga-DOTA-UBI PET has a very high clinical potential for imaging infection and probably differentiate infection from inflammation.

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Conflicts of interest

There are no conflicts of interest.

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