

Comparison of ^{99m}Tc -alafosfalin and ^{67}Ga -citrate in a mouse model of bacterial infection

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Abstract

BACKGROUND: The antibiotic-peptide ^{99m}Tc -alafosfalin was assessed as an infection imaging agent in *Staphylococcus aureus* infected mice by comparison with ^{99m}Ga -citrate, and also examined for influence of septic status on tracer biodistribution by comparison with normal mice.

MATERIAL AND METHODS: Intramuscular doses of *S. aureus* were administered into the right thigh muscle of mice and the infection was allowed to develop for 20 hours. In separate experiments, ^{99m}Tc -alafosfalin and ^{67}Ga -citrate were subsequently administered and allowed to localise. Quantitative organ distribution studies were performed in conjunction with scintigraphic images at 1 and 4 hours post injection. An additional biodistribution with ^{99m}Tc -alafosfalin in normal mice was also performed.

RESULTS: ^{99m}Tc -alafosfalin was predominantly renal excreted, with low liver, intestine and bone uptake. There was no difference in the uptake of these organs when infected mice were compared with normal mice. ^{99m}Tc -alafosfalin activity in the intestine at 1 and 4 hours was substantially less than ^{67}Ga -citrate. For ^{99m}Tc -alafosfalin, infected/non-infected thigh ratios of 2.8/1.0 and 3.6/1.0 were determined at 1 and 4 hours post injection respectively. ^{67}Ga -citrate gave ratios of 1.6/1.0 and 3.7/1.0 at the corresponding time points.

CONCLUSIONS: ^{99m}Tc -alafosfalin uptake was more rapid than ^{67}Ga -citrate, yet diffuse at the infectious sites in mice. The small

and juvenile mouse model resulted in uptake of the phosphonic acid tracer by active bone growth areas which may be a disadvantage. This ^{99m}Tc -antibiotic peptide has potential as an infection imaging agent, and will be investigated further in another rodent infection model in the future.

Key words: technetium-99m-alafosfalin, antibiotic-peptide, infection imaging

Introduction

^{111}In -labelled leucocytes are the gold standard radiopharmaceuticals for detecting focal infection and inflammation [1]. ^{111}In -oxine or ^{111}In -tropolonate are the lipophilic agents used for binding the radionuclide to leucocytes which are isolated from a sample of patient blood. ^{99m}Tc -HMPAO labelled leucocytes have also found an extensive clinical role for this indication [2], resulting in certain advantages over ^{111}In , including improved image resolution and reduced overall radiation burden to the patient. ^{99m}Tc -stannous fluoride colloid-leucocytes have been used in Australia over the last fifteen years, primarily for the clinical diagnosis of inflammatory bowel disease [3, 4]. This radiolabelling mechanism is based on phagocytic engulfment by neutrophils, monocytes and eosinophils, in conjunction with surface binding to lymphocytes [5]. Other agents have been successfully employed to label leucocytes in vivo [6], such as ^{99m}Tc and $^{123/131}\text{I}$ -iodine labelled antibodies or antibody fragments. The drawback with cell labelling techniques however, is that patient blood is manipulated ex vivo, in complex, time consuming procedures where needle-stick injuries and cross infection are possible.

Over the last decade, an increasing number of new infection imaging agents prepared from kits have appeared in the literature, evidence of a need to simplify the current cell labelling methodology by employing a single radioactive patient dose. ^{99m}Tc -ciprofloxacin (Infecton) is a radiotracer used in the United Kingdom, that may distinguish between bacterial and sterile inflammation in patients [7, 8]. When compared with ^{99m}Tc -HMPAO labelled white cells, ^{99m}Tc -ciprofloxacin was found to be more sensitive in the diagnosis of infectious spondylitis but lower in specificity for detecting musculoskeletal infection [9]. This ^{99m}Tc -antibiotic however, is not prepared from an instant kit formulation [10].

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Other commercially available cold kit products containing agents of biological origin include BW250/183 and Leukoscan [11], or the experimental agent LeuTech [12]. In the search for a radiopharmaceutical that is easily and rapidly prepared for infection imaging, a cold kit formulation of the synthetic dipeptide alafosfalin was developed [13]. This particular antibiotic interferes with normal cell wall synthesis of both gram negative and gram positive bacteria by intracellular inhibition of alanine racemase [14]. The aim of this study was to validate ^{99m}Tc -alafosfalin as an infection imaging agent in *Staphylococcus aureus* infected and non-infected mice, and compare localisation to the established non-cell labelling agent ^{67}Ga -citrate.

Material and methods

Alafosfalin cold kits were prepared according to a literature procedure [13]. Sodium [^{99m}Tc]-pertechnetate was obtained from a wet bed $^{99}\text{Mo}/^{99m}\text{Tc}$ -generator (TC9M1; Australian Radioisotopes; Sydney; Australia). ^{67}Ga -citrate used for the mouse biodistribution study was obtained commercially (Australian Radioisotopes; Sydney; Australia). Experiments were performed in triplicate unless stated otherwise.

Radiolabelling and quality control

An alafosfalin cold kit was reconstituted with sodium ^{99m}Tc -pertechnetate (10–200 MBq) in saline (0.9%; 2.0 mL) and allowed to stand for 10 minutes prior to use. Radiochemical purity (% RCP) of ^{99m}Tc -alafosfalin was determined by paper electrophoresis in barbitone buffer run at 400 V/30 minutes [15]. ^{99m}Tc -alafosfalin with > 95% radiochemical purity was used.

Quantitative studies in mice

Experiments performed with the mice complied with "The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes NHMRC" and according to a protocol of the Animal Ethics Committee of the Institute of Medical and Veterinary Science.

Mouse infection model. Cultures of *Staphylococcus aureus* ATCC 25293 (*S. aureus*) were prepared in CSF broth and diluted with normal saline to give 1×10^8 colony-forming units/mL. Mice (Balb/C; female; 18–20 g) were injected with a dose of *S. aureus* (0.1 mL; 1×10^7 cfu) into the right thigh muscle and the infection was allowed to develop for 20 hours.

Radiotracers in infected mice. Six of the *S. aureus* infected mice were injected into the tail vein with ^{99m}Tc -alafosfalin (25 μL ; 120 KBq). Groups of three mice were later sacrificed using Halothane asphyxiation at 1 and 4 hours post radiotracer injection (pi) for each study. Left and right thigh muscle plus other organs of interest were excised, blotted dry, weighed and counted with a gamma counter (Packard Auto-Gamma 5650; Hewlett Packard) over a ^{99m}Tc window (70–210 keV). Blood was also obtained from the descending aorta in the abdominal cavity, weighed and counted. The urine, carcass and organs were related to a standard (dose equivalent), and the % injected dose/organ (% id) was calculated as a fraction of that standard. Carcasses were counted separately in a large volume counter (Biosentry; AEI-EKCO, Australia) linked to a multichannel analyser (Model 3100; Canberra Industries Inc; USA). Urine and carcass values are reported as

% id, and all other organs are reported as % id/gram organ, calculated by dividing % id by the respective organ mass. The ratio of infected (I) muscle to non-infected (NI) muscle was calculated by dividing the % id/g of the right thigh muscle by that of the control left thigh muscle respectively. This procedure was repeated in separate experiments with an equivalent dose of ^{67}Ga -citrate (30 μL ; ~ 1 MBq).

^{99m}Tc -alafosfalin in normal mice. As a comparison to the mouse infection model, biodistribution of ^{99m}Tc -alafosfalin was also examined in non-infected (normal) mice at 1 and 4 hours. At 4 hours, the small and large intestine were each dissected and counted. The large intestine (99 mm length) was sectioned in two pieces, to isolate the caecum (24 mm), and the small intestine was sectioned into three similarly sized pieces (71, 86 and 105 mm).

Statistical analysis

Statistical analyses were performed with t-tests to determine p values for each radiotracer regarding the % id/gram uptake or I/NI ratios as (i) within comparisons for infected mice, or (ii) between comparisons for infected versus normal mice. Statistical significance was defined as a p value less than 0.05. Results are expressed as mean \pm standard error.

Qualitative studies in mice

Scintigraphic images. Three infected mice were injected with ^{99m}Tc -alafosfalin (0.1 mL; 1–2 MBq) and sacrificed at 1 and 4 hours post injection as above. Planar static images of each mouse were acquired in an anterior position for ten minutes on the collimator of a gamma camera (Starcam 300M; GE) with their legs out-stretched. A semi-quantitative analysis was performed on the 1 and 4 hour images using regions of interest to derive counts in infected and non-infected thighs. Region counts excluded bone activity, were background-corrected, and then used to calculate I/NI ratios.

Results

Quantitative studies in mice

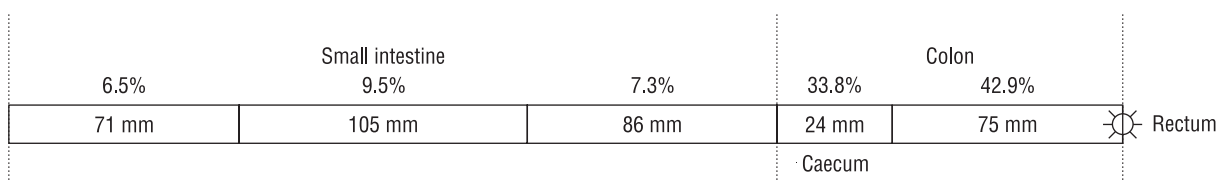
Results of the physiological distribution of ^{99m}Tc -alafosfalin in *S. aureus* infected and non-infected mice at 1 and 4 hours are shown in Table 1. Non-infected (normal) mice were found to give almost the same organ distributions of ^{99m}Tc -alafosfalin to infected mice at the localisation times of 1 and 4 hours. There were no significant differences between infected mice versus normal mice for % id/g organ values of the liver ($p = 0.113$), intestines ($p = 0.097$), kidneys ($p = 0.191$) at 1 hour pi and liver ($p = 0.174$), intestines ($p = 0.103$), kidneys ($p = 0.210$) at 4 hours pi. There was a significant difference between rodent groups of blood uptake at 1 hour ($p = 0.022$) and 4 hours ($p = 0.017$), which was attributed to the high variability associated with low mass blood samples for the *S. aureus* infected mice group.

Of the gut activity (0.41% id/g) at four hours, 23% was found in the small intestine and 77% in the large intestine (Fig. 1). A major portion of the total colon activity was located in the caecum (44%), the first section after the small intestine. ^{99m}Tc -alafosfalin is mainly renal excreted, yielding 2.4–3.0% id/g kidneys and greater than 70% urine activity passed at 4 hours post injection.

In the mouse infection model, ^{99m}Tc -alafosfalin was found to significantly accumulate more in the infected thigh of mice than

Table 1. Distribution of two radiotracers in *S. aureus* infected and normal mice

Organ	% Id/g organ (mean \pm SE) at time (hours) pi of radiotracer					
	^{99m}Tc -alafosfalin (normal)		^{99m}Tc -alafosfalin (infected)		^{67}Ga -citrate (infected)	
	1	4	1	4	1	4
Blood	3.55 \pm 0.41	1.72 \pm 0.15	5.83 \pm 0.22	3.86 \pm 0.28	28.82 \pm 0.95	21.24 \pm 1.22
Liver	0.60 \pm 0.06	0.53 \pm 0.06	0.44 \pm 0.02	0.40 \pm 0.02	10.42 \pm 1.54	10.17 \pm 0.45
Intestines	0.45 \pm 0.10	0.41 \pm 0.08	0.20 \pm 0.00	0.21 \pm 0.01	3.72 \pm 0.07	8.36 \pm 0.10
Stomach	0.42 \pm 0.08	0.36 \pm 0.03	0.29 \pm 0.02	0.40 \pm 0.01	1.67 \pm 0.08	2.13 \pm 0.23
Kidneys	4.46 \pm 0.78	3.00 \pm 0.49	3.34 \pm 0.05	2.37 \pm 0.07	6.57 \pm 0.19	7.74 \pm 0.62
R. thigh (I)	–	–	1.57 \pm 0.05	1.46 \pm 0.09	3.07 \pm 0.18	4.73 \pm 0.74
L. thigh (NI)	–	–	0.59 \pm 0.07	0.41 \pm 0.03	1.92 \pm 0.03	1.27 \pm 0.10
Carcass	32.2 \pm 1.3	25.1 \pm 1.2	31.9 \pm 1.3	25.0 \pm 0.2	72.7 \pm 2.4	61.2 \pm 1.1
Urine	63.7 \pm 2.0	72.0 \pm 1.7	63.1 \pm 1.5	70.0 \pm 0.6	0.5 \pm 0.2	0.8 \pm 0.3
Ratio (I/NI)	–	–	2.77 \pm 0.29	3.61 \pm 0.29	1.60 \pm 0.09	3.72 \pm 0.48

**Figure 1.** ^{99m}Tc -alafosfalin distribution in mouse intestines at 4 hours pi.

the control left thigh after 1 hour ($p = 0.005$) and 4 hours ($p = 0.005$) localisation. The I/NI thigh ratios increased significantly from 2.8 to 3.6 during the same period ($p = 0.025$). This trend was also observed with uptake of the non-specific agent ^{67}Ga -citrate at 1 hour ($p = 0.016$) and 4 hours ($p = 0.028$), as well as a significant increase in I/NI ratios from 1.6 to 3.7 ($p = 0.027$). At 1 hour post radiotracer administration there was significantly better localisation of ^{99m}Tc -alafosfalin compared to ^{67}Ga -citrate (ratios, $p = 0.044$) at the infection site. However, no difference was observed at 4 hours (ratios, $p = 0.457$) suggesting that although ^{67}Ga -citrate has less initial accumulation, subsequent uptake increases up to 4 hours.

Scintigraphic imaging studies

Scintigrams obtained of ^{99m}Tc -alafosfalin in infected mice at 1 and 4 hours are shown in Figure 2. Both scans demonstrated higher activity in infected tissue compared to non-infected tissue. There was an accumulation of activity in the kidneys and bones, particularly in areas of active bone growth including the knees, skull and shoulders. Activity in the knees was in close proximity to the infection sites, providing additional counts to that area. The ratio of counts in regions of interest for infected to non-infected thighs were 1.6 at 1 hour and 2.7 at 4 hours.

Discussion

The physiological distribution of ^{99m}Tc -alafosfalin in normal mice was found to be essentially identical to that of *Staphylococcus aureus* infected mice. These results suggest that a localised thigh

muscle infection does not alter the biodistribution of the tracer in internal organs in mice. In particular, there was no significant difference in tracer uptake for liver, intestines, stomach or kidneys at 1 and 4 hours ($p > 0.05$).

In mice both ^{99m}Tc -alafosfalin and ^{67}Ga -citrate resulted in higher uptake in the infected thighs over the control thighs. The ratio of ^{99m}Tc -alafosfalin at 4 hours uptake was as good as ^{67}Ga -citrate (Table 1). As evidenced from a I/NI ratio of 2.8 at 1 hour, ^{99m}Tc -alafosfalin accumulated at the infectious site faster than ^{67}Ga -citrate initially. However, ^{99m}Tc -alafosfalin ratios increased by 30% from 1 to 4 hours, whereas ^{67}Ga -citrate more than doubled, suggesting the former is near its maximal uptake and the latter is still accumulating at the infection site [16]. This fits in with the current practice where gallium scans are usually reported after 24 hours. This tracer does have a major advantage over ^{67}Ga -citrate as an imaging agent — that being the far lower activity of non-target organs. Thus, early imaging of a ^{99m}Tc -agent would be preferred over ^{67}Ga -citrate, because ^{67}Ga -citrate requires a delay of 24–48 hours before a result is obtained. The non-specific organ uptake of ^{67}Ga -citrate is likely to result in a higher radiation burden than ^{99m}Tc -alafosfalin, and the decay characteristics of isotopic ^{67}Ga are less favourable for image acquisition than ^{99m}Tc .

Blood, liver, intestines, stomach and kidney activities are all lower for ^{99m}Tc -alafosfalin than ^{67}Ga -citrate, and their uptake either decreases or remains unchanged between 1 and 4 hours post injection. The high blood uptake and prolonged retention of ^{67}Ga -citrate is due to the transchelation of ^{67}Ga metal to transferrin and lactoferrin. The prolonged bowel activity is a major limitation of this agent for localising abdominal abscesses [17]. In comparison, the intestinal activity of ^{99m}Tc -alafosfalin was very low relative to the injected

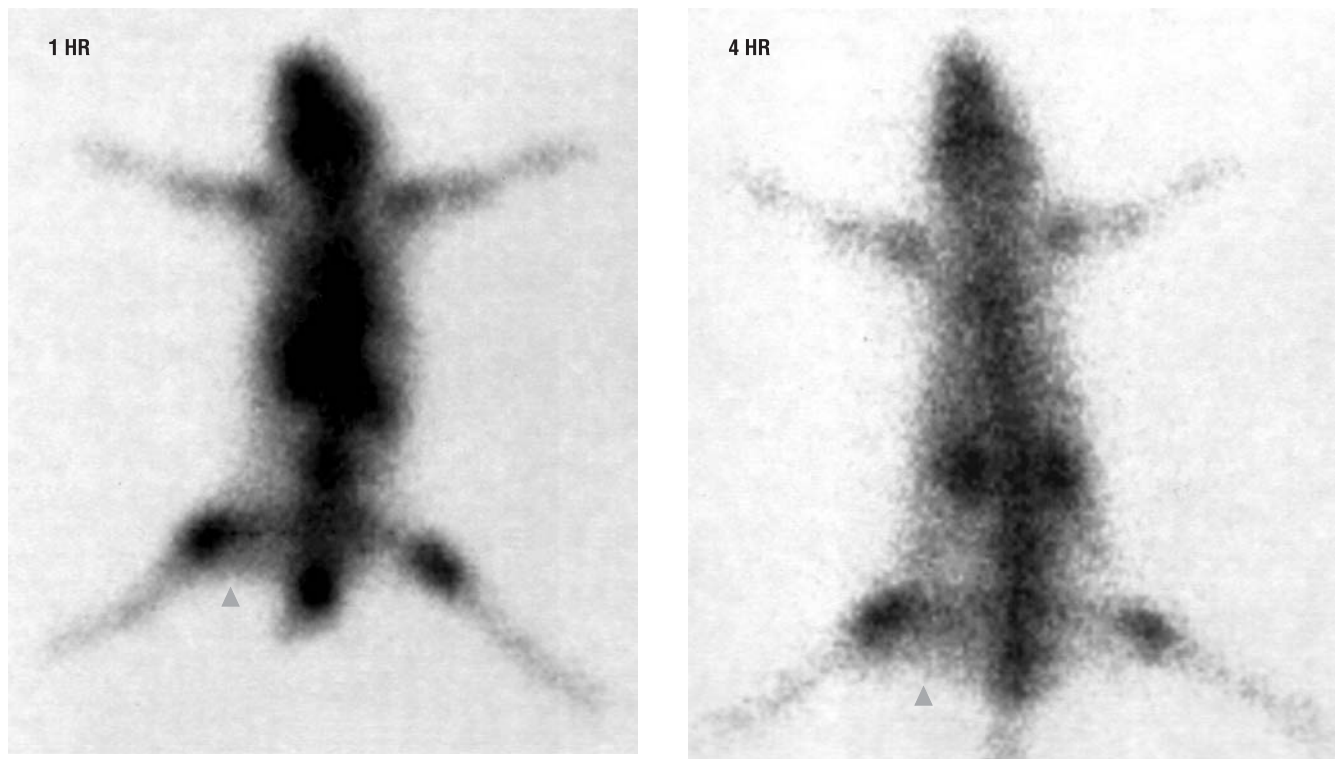


Figure 2. Whole body images taken at 1 and 4 hours pi of ^{99m}Tc -alfafosfalin in *S. aureus* infected mice.

dose, and this was concentrated in the caecum and distal colon.

Although 70% of the injected ^{99m}Tc -alfafosfalin dose is excreted in the urine after 4 hours, there is sufficient tracer uptake at the infectious sites. Furthermore, low blood activity of ^{99m}Tc -alfafosfalin contributed to a favourable infection to non-infection ratio. From the images, ^{99m}Tc -alfafosfalin uptake was relatively diffuse at the site of infection. During biodistribution, infectious lesions were observed to be focused in the thigh muscles, which suggests the dispersed uptake on the images reflects low binding of ^{99m}Tc -alfafosfalin to bacterial cells. Perhaps sufficient cold alfafosfalin antibiotic in the dose elicits a therapeutic effect at the infected thighs. The mechanism of ^{99m}Tc -alfafosfalin uptake in *S. aureus* infection is not clearly understood, but endogenous antibiotic peptides are involved in the inflammatory response [18]. Although the regions of interest excluded bone activity, image I/N ratios were underestimated compared to those obtained from the biodistributions, emphasising the importance of the quantitative test.

Conclusions

^{99m}Tc -alfafosfalin accumulates at sites of infection earlier in mice than ^{67}Ga -citrate. Uptake of the ^{99m}Tc -agent was diffuse rather than focused. The juvenile mouse model used was somewhat small for accurate validation of ^{99m}Tc -alfafosfalin, because uptake of the phosphonic acid tracer by active bone growth areas influences the target-to-background ratio. This ^{99m}Tc -antibiotic peptide does appear to have potential as an infection imaging agent, and will be further investigated in a larger, mature rodent model in the future.

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