Technetium-99m labelled fluconazole and antimicrobial peptides for imaging of *Candida albicans* and *Aspergillus fumigatus* infections

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Abstract. The aim of this study was to investigate whether technetium-99m labelled fluconazole can distinguish fungal from bacterial infections. Fluconazole was labelled with ^{99m}Tc and radiochemical analysis showed less than 5% impurities. The labelling solution was injected into animals with experimental infections. For comparison, we used two peptides for infection detection, i.e. UBI 29–41 and hLF 1–11, and human IgG, all labelled with ^{99m}Tc. Mice were infected with *Candida albicans* or injected with heat-killed *C. albicans* or lipopolysaccharides to induce sterile inflammation. Also, mice were infected with *Staphylococcus aureus* or *Klebsiella pneumoniae*. Next, accumulation of ^{99m}Tc-fluconazole and ^{99m}Tc-labelled peptides/IgG at affected sites was determined scintigraphically. ^{99m}Tc-fluconazole detected *C. albicans* infections (T/NT ratio=3.6±0.47) without visualising bacterial infections (T/NT ratio=1.3±0.04) or sterile inflammatory processes (heat-killed *C. albicans*: T/NT ratio=1.3±0.2; lipopolysaccharide: T/NT ratio=1.4±0.1). *C. albicans* infections were already seen within the first hour after injection of ^{99m}Tc-fluconazole (T/NT ratio= 3.1±0.2). A good correlation (R^2=0.864; P<0.05) between T/NT ratios for this tracer and the number of viable *C. albicans* was found. Although ^{99m}Tc-UBI 29–41 and ^{99m}Tc-hLF 1–11 were able to distinguish *C. albicans* infections from sterile inflammatory processes in mice, these ^{99m}Tc-labelled peptides did not distinguish these fungal infections from bacterial infections. It is concluded that ^{99m}Tc-fluconazole distinguishes infections with *C. albicans* from bacterial infections and sterile inflammations.

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Introduction

Anticancer therapy, transplantation and AIDS give rise to infections with fungi, such as *Candida albicans* and *Aspergillus fumigatus*. The former is a major cause of mucosal and systemic infections [1, 2]. *Aspergillus fumigatus* is a ubiquitous mold, which may cause invasive aspergillosis in transplant recipients. In this context it is clinically relevant to develop diagnostic tools that can discriminate fungal infections from bacterial infections and sterile inflammatory processes. A previous study reported on fungus imaging with fluorine-18 fluconazole and positron emission tomography (PET) [3]. Although *Candida*-infected tissues were rapidly visualised with this radiolabelled antifungal agent, this approach suffered from rather poor accumulation at sites of infection and high amounts of radioactivity were deposited in the liver. We hypothesised that technetium-99m-labelled fluconazole might have different radiopharmacological characteristics. This article reports on our in vitro tests and animal experiments with ^{99m}Tc-labelled fluconazole for improved detection of fungal infections. As comparative agents we included ^{99m}Tc-labelled cationic peptides [4].

Materials and methods

Fluconazole and peptides. Fluconazole [306 Da, 2, 4-difluoro-α, α[1]-bis(1*H*-1,2,4-triazol-1-ylmethyl) benzyl alcohol ($C_{13}H_{12}F_2N_6O$)] was purchased from Pfizer (Pfizer Inc., New York, N.Y.). Linear peptides derived from ubiquicidin (UBI 29–41) and from human lactoferrin (hLF 1–11) were synthesised as previously described [5]. Human polyclonal immunoglobulin G (IgG; Central Laboratory of the Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands) was included as a control [6].

Labelling procedure. Fluconazole was labelled with ^{99m}Tc as described for peptides [4, 6] with minor modifications. In a labelling vial were mixed 10 µl of a fluconazole solution (2 mg/ml), 4 µl of a solution containing 950 mg/ml $SnCl_2 \cdot 2H_2O$ and 2 mg/ml sodium pyrophosphate·$10H_2O$ and 4 µl of a solution of KBH_4 in 10 mg/ml 0.1 *M* NaOH. After addition of 0.1 ml of ^{99m}Tc-sodium pertechnetate solution (200 MBq/ml of saline), the mixture was gently stirred at room temperature for 120 min and then diluted with phosphate-buffered saline (PBS, pH 7.5) to a final volume of 1 ml. Peptides and IgG were labelled with ^{99m}Tc as described previously [4, 6]. In our experiments we used 0.1 ml of these labelling solutions, referred to hereafter as ^{99m}Tc-fluconazole, ^{99m}Tc-labelled peptide and ^{99m}Tc-IgG.

HPLC analysis. We analysed the reaction mixture after labelling of fluconazole by anion exchange high-performance liquid chromatography (HPLC). Samples were applied on a MONO-Q HR 5/5 column attached to a chromatography apparatus equipped with an on-line UV detector set at 220 nm and an NaI (Tl) crystal gamma detection system. A linear gradient with two different eluents, i.e. 20 m*M* 2-amino-2 hydroxymethyl-1,3-propanediol, pH 10.4 (A) and 1 *M* NaCl in the same buffer (B), at a flow rate of 1 ml/min was used. The gradient was performed as follows: 100% A for 5 min, 0%–100% B in 5 min and 100% B for 10 min.

ITLC analysis and Sep-Pak analysis. Labelling yield and stability of ^{99m}Tc-fluconazole were determined using instant thin-layer chromatography (ITLC) with saline or methyl ethyl ketone as the eluent. After 2 min, the strips were mounted on the detector of a gamma camera and three fractions were quantified. Stability of ^{99m}Tc-fluconazole was determined after incubation in human serum for 24 h at 37°C. Next, amounts of free/released pertechnetate and ^{99m}Tc-fluconazole were assessed by ITLC as described above. Labelling solutions were subjected to Sep-Pak Light C_{18} cartridges and trapped colloidal radioactivity was determined as described previously [7, 8].

Micro-organisms. Fluconazole-resistant *C. albicans* Y01–19 (*C. albicans*; minimal inhibitory concentration >256 µg fluconazole/ml) was purchased from Pfizer. *C. albicans* was cultured overnight in Sabouraud broth and sub-cultured for 2.5 h at 37°C. *Aspergillus fumigatus* (*A. fumigatus*) was a clinical isolate and was cultured on potato dextrose agar for 4 days at 30°C. The suspension of mycelial fragments and conidia was filtered through sterile gauze to remove the mycelia and the number was counted in a haemocytometer in order to prepare the desired inoculum. *Staphylococcus aureus* 25923 (*S. aureus*) and *Klebsiella pneumoniae* 43816 (*K. pneumoniae*) were obtained from the American Type Culture Collection. Overnight cultures of bacteria were prepared in brain heart infusion broth in a shaking waterbath at 37°C.

In vitro binding of ^{99m}Tc-fluconazole to micro-organisms and human cells. Binding of ^{99m}Tc-fluconazole to micro-organisms and to (activated) human leucocytes was assessed at 4°C as described elsewhere [6]. 0.1 ml of the solution containing 1 nmol of ^{99m}Tc-fluconazole was transferred to an Eppendorf vial, containing approximately 2×10^7 colony forming units (CFU) of *C. albicans*, *A. fumigatus* or bacteria in 0.9 ml of 50% (v/v) of 0.01 *M* acetic acid in PBS supplemented with 0.01% (v/v) Tween-80 (PBST buffer). The mixtures were incubated for 1 h at 4°C and the radioactivity in the pellet after centrifugation was determined in a dose calibrator. Binding of ^{99m}Tc-fluconazole to 2×10^7 human leucocytes was determined under similar conditions, except that the PBST buffer was supplemented with 50 units/ml of heparin. The radioactivity associated with *C. albicans*, *A. fumigatus*, bacteria and (activated) leucocytes is expressed as percentage of added ^{99m}Tc activity bound to 2×10^7 cells.

Infections with C. albicans, A. fumigatus and bacteria in mice. Specific pathogen-free, male Swiss mice and leucocytopenic mice (for *A. fumigatus* infections) were used as described previously [8]. For infections with *C. albicans* or bacteria 2×10^6 CFU and for *A. fumigatus* 2×10^4 conidia in 0.1 ml of saline were injected into thigh muscles of mice. Sterile inflammation was induced by intramuscular injection of 0.1 ml of saline containing either 50 ng lipopolysaccharide or 2×10^8 heat-killed *C. albicans*. Scintigraphy, scintigraphic analysis and ex vivo countings including microbiological assessment of the number of *C. albicans* or bacteria were performed as described previously [4, 6].

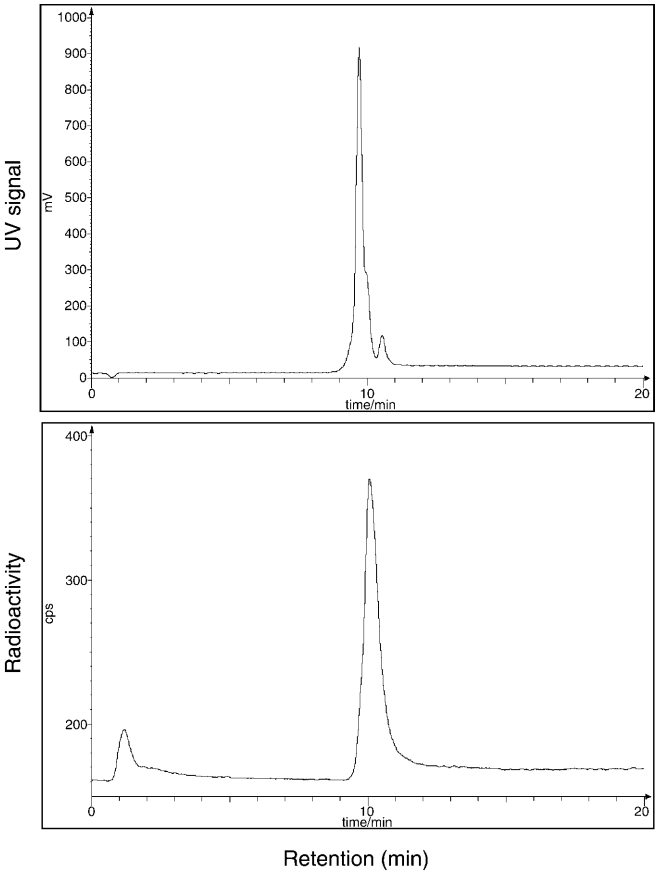

Fig. 1. Typical profile of ^{99m}Tc-fluconazole on a MONO-Q anion exchange chromatography column. *Upper part:* UV (220 nm) monitoring after labelling. *Bottom part:* ^{99m}Tc-radioactivity monitoring

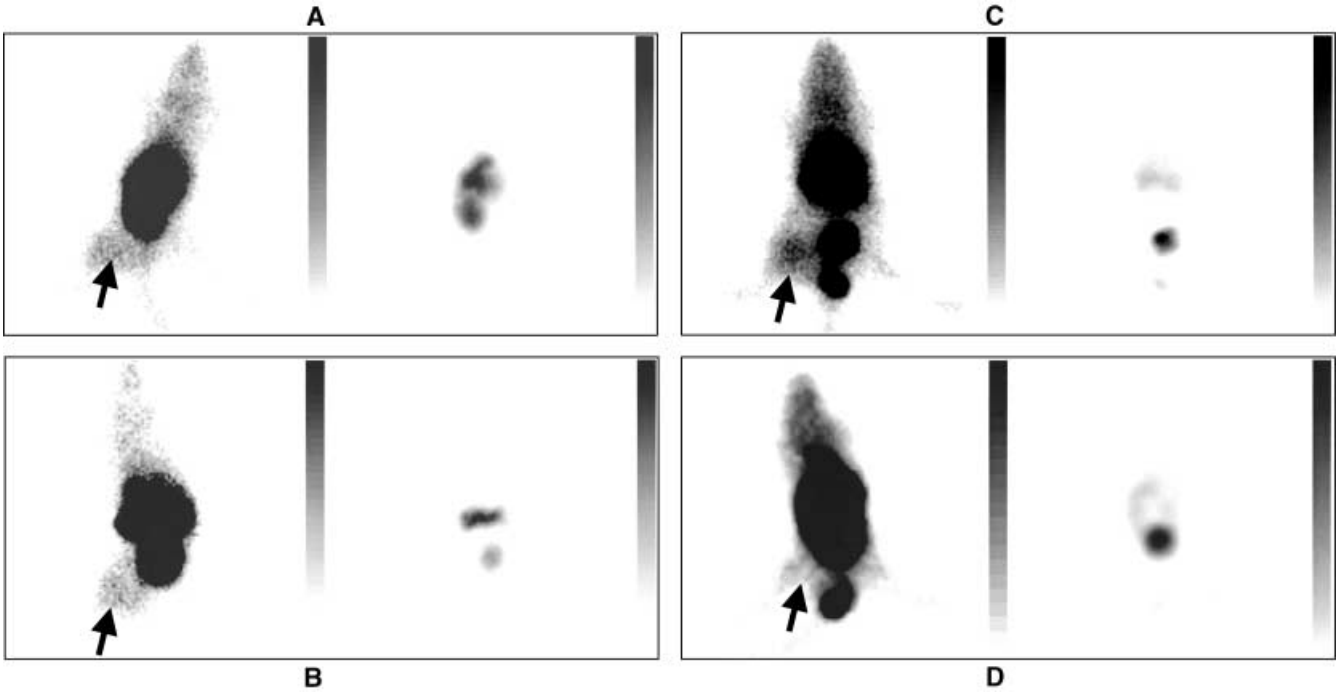

Fig. 2. Biodistribution of fluconazole (**A**), hLF 1–11 (**B**) and UBI 29–41 (**C**), all labelled with ^{99m}Tc, in mice with a *C. albicans*-infected thigh muscle, and ^{99m}Tc-fluconazole in lipopolysaccharide inflamed thigh muscles (**D**). *In each image: Right:* scintigraphic imaging of the biodistribution of the tracers in the entire animal. *Left:* scintigrams of the same animal with higher contrast visualising the thigh muscle infection/inflammation indicated by an *arrow* at 1 h after injection of the tracers. All images show unprocessed scintigrams

Results

Quality control

HPLC analysis showed a major peak of radioactivity (Fig. 1), representing about 90% of the total radioactivity and ascribed to ^{99m}Tc-fluconazole, coinciding with unlabelled fluconazole (between 9 and 10 min). Free pertechnetate and colloids represented less than 5% of the total activity in the labelling solution. Incubation of the labelling solutions in human serum for 24 h at 37°C showed minimal release of radioactivity (<5%) as determined by ITLC.

In vitro binding assay

In vitro binding of ^{99m}Tc-fluconazole to *C. albicans*, *A. fumigatus*, bacteria and (activated) leucocytes revealed that it preferentially binds to *C. albicans* (38%±3%, n=18). These values were significantly higher (P<0.05) than those for *A. fumigatus* (18%±2%, n=19) and (activated) human cells (12%±1%, n=15). The lowest values were found for ^{99m}Tc-fluconazole binding to *S. aureus* (3%±0.4%, n=12) and *K. pneumoniae* (6%±0.1%, n=8).

Detection of infections and sterile inflammatory processes in mice

Typical scintigraphic images of the accumulation of ^{99m}Tc-labelled compounds in thigh muscles infected with viable *C. albicans* are depicted in Fig. 2. Target-to-non-target (T/NT) ratios for the different compounds are shown in Fig. 3. A good correlation (R^2=0.864, P<0.05; n=12) was found between T/NT ratios at 2 h after injection of ^{99m}Tc-fluconazole and the number of viable *C. albicans* present in the infected thigh muscle (Fig. 4). Accumulation of ^{99m}Tc-fluconazole, ^{99m}Tc-hLF 1–11, ^{99m}Tc-UBI 29–41 and ^{99m}Tc-IgG at sites of *C. albicans* infection at 4 h after injection of the tracer, as assessed by autopsy, amounted to 2.1±1.1%ID/g, 2.4±0.7%ID/g, 2.5±1.0%ID/g and 3.2±1.2%ID/g, respectively (n=4). At 4 h post injection ^{99m}Tc-fluconazole accumulated poorly in thigh muscles with a bacterial infection (T/NT ratio=1.3±0.04; n=6), as compared to accumulation at sites of fungal infections (P<0.05). In contrast, ^{99m}Tc-UBI 29–41, ^{99m}Tc-hLF 1–11 and ^{99m}Tc-IgG detected both *C. albicans* and bacterial thigh muscle infections, confirming previous data [4, 6]. ^{99m}Tc-fluconazole and ^{99m}Tc-labelled peptides did not detect sterile inflammations (Fig. 3). ^{99m}Tc-IgG detected both heat-killed *C. albicans* and sterile inflammations in thigh muscles.

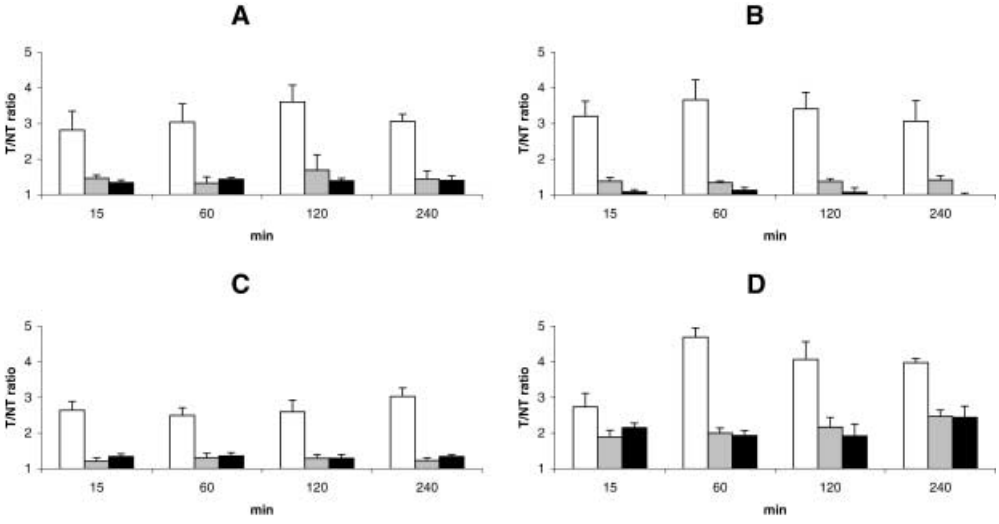

Fig. 3. Time-dependent accumulation of ^{99m}Tc-fluconazole (**A**), ^{99m}Tc-hLF 1–11 (**B**), ^{99m}Tc-UBI 29–41 (**C**) and ^{99m}Tc-IgG (**D**) in thigh muscles infected with *C. albicans* (*open bars*) or inflamed with heat-killed *C. albicans* (*hatched bars*) or lipopolysaccharide (*LPS, closed bars*). Each symbol represents the mean (±SEM) target-to-non-target (*T/NT*) ratio for at least four animals in two independent experiments. Significant (P<0.05) T/NT ratios for *C. albicans*-infected thigh muscle at 1 h and 2 h after injection of the ^{99m}Tc-labelled tracers were observed for hLF 1–11 and fluconazole. ^{99m}Tc-UBI 29–41 also significantly (P<0.05) visualised *C. albicans* thigh muscle infection; however, the T/NT ratio was less than those for the previously mentioned compounds

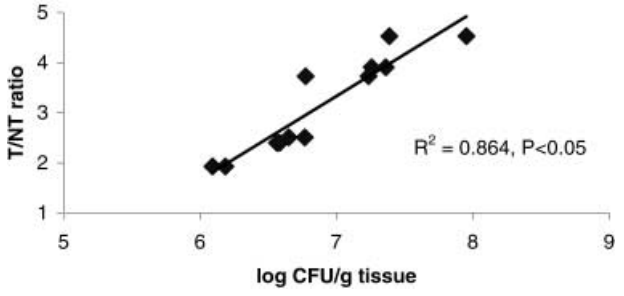

Fig. 4. Relation between the number of viable *C. albicans* per gram thigh muscle and the accumulation of ^{99m}Tc-fluconazole, expressed as the target-to-non-target (*T/NT*) ratio, in infected tissues

Detection of infections and sterile inflammatory processes in leucocytopenic mice

In leucocytopenic mice the scintigraphic results showed that ^{99m}Tc-labelled peptides, but not ^{99m}Tc-fluconazole, accumulated in *A. fumigatus*-infected thigh muscle. The time-dependent increase in the T/NT ratios of the different compounds is shown in Fig. 5. Furthermore, accumulation of ^{99m}Tc-fluconazole, ^{99m}Tc-hLF 1–11, ^{99m}Tc-UBI 29–41 and ^{99m}Tc-IgG at sites of *A. fumigatus* infection at 4 h after injection of the tracer, as assessed by autopsy, amounted to 0.6±0.2%ID/g, 1.5±0.3%ID/g, 1.3±0.5%ID/g and 1.3±0.7%ID/g, respectively (n=4). No accumulation

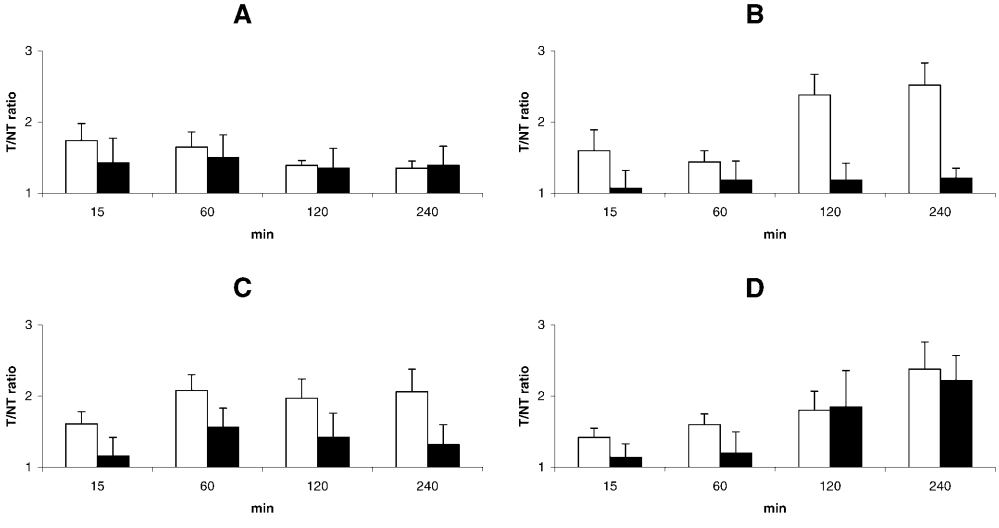

Fig. 5. Time-dependent accumulation of ^{99m}Tc-fluconazole (**A**), ^{99m}Tc-hLF 1–11 (**B**), ^{99m}Tc-UBI 29–41 (**C**) and ^{99m}Tc-IgG (**D**) in thigh muscles infected with *A. fumigatus* (*open bars*) or inflamed with lipopolysaccharide (*LPS, closed bars*) in leucocytopenic mice. Significantly (P<0.05) higher T/NT ratios at 2 h after injection of ^{99m}Tc-hLF 1–11 and ^{99m}Tc-UBI 29–41 were observed in *A. fumigatus*-infected muscles compared with sterile inflammations. Each symbol represents the mean (±SEM) T/NT ratio for at least four animals in two independent experiments

Table 1. Biodistribution of ^{99m}Tc-labelled compounds in mice infected with *C. albicans*

^{99m}Tc compound	Injected radioactivity (% injected dose)								
	Bladder			Kidneys			Liver		
	15 min	60 min	240 min	15 min	60 min	240 min	15 min	60 min	240 min
Fluconazole	29±3	34±2	29±7	24±2	22±7	22±4	19±2	10±2	8±7
hLF 1–11	12±2	18±3	27±3	15±3	15±2	19±2	24±2	26±2	38±2
UBI 29–41	23±3	32±5	17±3	19±2	22±2	12±2	17±2	14±2	10±1
IgG	17±3	47±2	7±3	14±7	20±2	18±2	17±2	14±2	10±1

Values are the mean±SD of at least four observations

of fluconazole, hLF 1–11 or UBI 29–41, all labelled with ^{99m}Tc, was seen in sterile inflammations.

Biodistribution

The biodistribution data for the different ^{99m}Tc-labelled compounds in immunocompetent mice are summarised in Table 1. In *C. albicans*-infected mice, ^{99m}Tc-fluconazole and ^{99m}Tc-UBI 29–41 were removed from the circulation mainly via kidneys/bladder and to a smaller extent by the liver. ^{99m}Tc-hLF 1–11 showed relatively high accumulation in liver and intestines. The biodistribution of ^{99m}Tc-IgG was comparable to that of ^{99m}Tc-fluconazole. Similar data were found in *A. fumigatus*-infected leucocytopenic mice (data not shown).

Discussion

The main conclusion from our investigations is that ^{99m}Tc-fluconazole is an excellent marker for *C. albicans* infections. This conclusion is based on the following findings. First, in mice ^{99m}Tc-fluconazole detects infections with *C. albicans* but not bacterial infections or sterile inflammatory processes, which is in agreement with in vitro binding results. Second, the good correlation between ^{99m}Tc-fluconazole accumulation and the numbers of viable *C. albicans* indicates that ^{99m}Tc-fluconazole is suitable for therapy monitoring. Third, in contrast to ^{18}F-fluconazole, ^{99m}Tc-fluconazole is removed from the circulation mainly via the kidneys/bladder with little accumulation in the liver. Moreover, ^{99m}Tc-fluconazole seems a selective tracer for *C. albicans* since it is not suited to detect *A. fumigatus* infections, which is in agreement with our in vitro data and likely due to alterations in cytochrome P450 of *A. fumigatus* [9]. Although the biological activity is evident, the chemical nature of this radioagent has not yet been elucidated. At present, studies are being performed with Re and ^{99m}Tc to unravel the chemical structure of the labelled compound.

Our investigations have revealed that ^{99m}Tc-UBI 29–41 and ^{99m}Tc-hLF 1–11 are able to distinguish *C. albicans* infections from sterile inflammations in immunocompetent mice and are also able to discriminate between *A. fumigatus* infections and sterile inflammation in leucocytopenic mice. However, neither peptide allowed discrimination between fungal infections and bacterial infections in either immunocompetent or leucocytopenic mice. Nevertheless, the results show that ^{99m}Tc-fluconazole can discriminate *C. albicans* infections from *A. fumigatus* and bacterial infections and from sterile inflammations, which may have a real clinical impact [10].

In conclusion, our scintigraphic results obtained in mice demonstrate that ^{99m}Tc-fluconazole is able to distinguish *C. albicans* infections from bacterial infections and sterile inflammations and that it may be suitable in monitoring the efficacy of antifungal therapy during an infection with *C. albicans*.

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