Toxicity assessment of ^{99m}technetium-labeled human beta-defensin-3 in CD1 mice

Liberatore Mauro[1] MD,
Anagnostou Christos[2] MD,
Scaccianoce Sergio[3] BSc,
Frantellizzi Viviana[1] MD,
Drudi Francesco Maria[1] MD,
D'Elia Piera[1] BSc, PhD,
Barteri Mario[4] Bschem,
Pala Alessandro[1] BSchem

1. Department of Radiological, Oncological and Anatomo-Pathological Sciences, "Sapienza" University of Rom, Italy
2. Department of Nuclear medicine, PET-CT Center, "Santa Maria" Hospital, Terni, Italy
3. Department of Human Physiology and Pharmacology
4. Department of Chemistry, "Sapienza" University of Rome, Rome, Italy.

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Correspondence address:
Christos Anagnostou, MD,
Santa Maria Hospital,
Viale Tristano di Joannuccio, 1,
05100 Terni, Italy.
Tel: 0039 0744 205495,
Fax: 0039 0744 205496
chr.anagnostou@yahoo.it

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Abstract

Objective: Human beta-defensin-3 (HBD-3) is an antimicrobial peptide which is up-regulated during inflammation. Based on the previously demonstrated capacity of technetium-99m (^{99m}Tc) labelled HBD-3 of distinguishing infection from inflammation in rats, we have decided to collect information on the potential toxicity of the tracer in view of its possible use for imaging in humans. **Materials and Methods:** Recombinant HBD-3 underwent labeling with ^{99m}Tc. The CD1 mice were selected as standard rodent species. Ten mice, 5 male and 5 female, were subjected to physical examination and housed in a dedicated room in 5 per cage. After 9 days pre-test period, all mice were weighted for dose adjustment and received intravenously 6mcg/mouse of ^{99m}Tc-HBD-3. Mortality was recorded daily, while body weight was registered once a week. Clinical observation of animals was performed daily for sickness symptoms due to the drug treatment. At day 19 a second dose of 6mcg/mouse ^{99m}Tc-HBD-3, was administered. Twenty-four hours after the second dose (day 20) the animals were euthanized. A piece of liver, kidneys, heart and lungs was collected for histopathological analysis. **Results:** Our results showed that the labelled-HBD-3 dose did not induce significant toxicity in mice. Of course these parameters were not sufficient to authorize use in humans. This non-toxic dose of HBD-3 when translated from animals to humans resulted in an equivalent dose of approximately 25 times higher than that needed for imaging. **Conclusion:** Our non toxicity data of using ^{99m}Tc-beta-defensin-3 in mice offer a further indication in favour of the clinical use of this radiopharmaceutical in all cases where discrimination between infection and inflammation is needed.

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Introduction

In the last 20 years a number of radiopharmaceuticals have been proposed in order to localise "in vivo" inflammatory and infectious processes by using conventional nuclear medicine methods. On the basis of the physiological characteristics and uptake mechanism, these compounds can be classified in various groups. One of these groups is characterised by a non-specific uptake mechanism that relies on some physio-pathological changes that occur at sites of infection/inflammation such as enhanced vascular permeability, enhanced blood flow and enhanced transudation of plasma proteins. Gallium-67 (^{67}Ga)-citrate, labelled human immunoglobulin G (HIG) and labelled liposome belong to this group.

Another group is based on the enhanced influx of granulocytes, typical of inflammatory processes. Several compounds, characterised by different uptake mechanisms, belong to this group, such as labelled granulocytes and anti-granulocyte labelled antibodies (anti-NCA-95 Ig, anti-NCA-90 Fab', anti SSEA-1 Ig M), both utilising enhanced influx of neutrophils at the site of lesion.

Another group includes labelled chemotactic peptides (f-Met-Leu-Phe), cytokines (IL-1, IL-8, PF-4) and complement factors (C5a, C5adR) that act via receptor binding.

Furthermore, enhanced influx of lymphocytes can be detected by the receptor binding of labelled cytokine IL-2. Increased metabolic requirements in infection/inflammation site can be depicted by enhanced uptake of labelled fluorine-18-fluorodeoxyglucose (^{18}F-FDG), which represents another group of tracers. Labelled cyprofloxacin and anti-microbial peptides were shown be able to detect the presence of micro-organisms because of their affinity for these cells.

The above imaging methods have been exhaustively described in a recent comprehensive review article [1]. Unfortunately, only part of them is commercially available (labelled granulocytes, anti-granulocyte antibodies, ^{67}Ga-citrate, labelled HIG and, more important, none of them has proved to be suitable for distinguishing between infection and inflammation.

There is growing evidence of the ability of certain antimicrobial peptides to differentiate between inflammation and infection, provided their microbiological activity still remains after labelling. This activity is supposed to act trough specific binding to bacterial cell membrane [2, 3]. In particular, human beta-defensin-3 (HBD-3) is a 4-kDa peptide and its antimicrobial activity depends on its ability to induce lesions in the lipid composition of the membranes of gram-positive and-negative bacteria [4]. As several antimicrobial peptides, however, beta-defensin-3 may exhibit adverse effects such as hemolytic activity [5] although not all authors agree to this [6].

Recently, a labeling method of HBD-3 with technetium-99m (^{99m}Tc) has been described by our group and the resulting tracer has been shown to retain antibacterial activity and also the capacity of discriminating between infection and aseptic inflammatory processes in adult rats [7]. Indeed, the development of tracers capable of this reliable differentiation, which is often highly relevant to clinical management, has received considerable attention [8-12].In this paper we studied the possible toxic effects that may be induced by the intravenous (i.v.) injection of ^{99m}Tc-labeled HBD-3, in mice.

Materials and Methods

Radiolabelling of HBD-3

We used 600mcL recombinant HBD-3 (GenScript) and 30ncM in 0.1M phosphate buffer, pH 8 after sulfhydryl addition which was labeled with 200mcL of ^{99m}Tc (600MBq) in physiological saline previously reduced to an oxidation state lower than 7 with a large molar excess of Sn^{+2} (SnCl2.2H20 Sigma). Unreacted ^{99m}Tc was removed by gel-filtration through a Sepadex G-10 column equilibrated and eluted in physiological saline. The labeling yield of the overall reaction procedure was approximately 70% and the specific activity at the range of 7-8MBq/mcg .

Animal studies

All animal experiments were approved by the institutional animal care committee. The CD1 mice were selected as standard rodent species. Ten mice, 5 male and 5 female, 11-12 weeks of age (Harlan Italy, Correzzana, Italy) were subjected to physical examination shortly after arrival and housed in a dedicated room, 5 per cage. Each cage was identified by a tag and each animal marked with picric acid solution. Temperature and relative humidity were set at 22±2°C and 50%±10% respectively. Artificial lighting provided a 24 hours cycle of 12 hours light/12 hours dark (light 7a.m.-7p.m.). Food pellets of 12mm, (Global Diet 2018 certificate, Mucedola srl, Settimo Milanese, Italy) and water were available ad libitum. The animal care and husbandry were in compliance with international policies [13, 14]. After a 9 days pre-test period (quarantine), the experimental period was as follows: at day 1, all mice were weighted for dose adjustment and travenously injected with ^{99m}Tc-hBD-3 (6 mcg/mouse, injection volume=200mcL). This dose was arbitrarily extrapolated from the dose, of 3mcg, which was previously shown to be able to bind to the infection site when i.v injected in rats [7]. Normalization of this dose to body surface area according to the suggestions of Reagan-Shaw et al. (2008) [15] resulted in a value of 72mcg/m^2, while similar calculations allowed to convert the 6mcg dose to a 720mcg/m^2 dose per mouse; therefore the amount of HBD-3 administered per mouse, and per normalized for body surface-area, was 10 times higher than the dose that was effective in targeting infection in rats.

Mortality was recorded daily, while body weight was registered once a week. Clinical observation of animals was performed daily for sickness symptoms due to treatment. Changes in their appearance (piloerection, kyphosis, disheveled fur) their behavior (altered grooming or nesting) and activity (altered exploring) were monitored and recorded daily. At day 19 a second dose of 6mcg/mouse ^{99m}Tc-HBD-3, was administered. Twenty-four hours after the second dose (day 20) the animals were euthanized under deep anaesthesia induced with isofurane by CO_2 inhalation. A specimen of liver, kidneys, heart and lungs was collected for histopathological analysis. Tissues were fixed in buffered-formaline (10%), paraffin embedded, sectioned in 3 microns thick sections and stained with hematoxilin-eosin.

Results

After ^{99}mTc-HBD-3 i.v injections we noticed no mortality. The administration of the compound was generally well tolerated except that the male mice seemed slightly hypokinetic 4 days after the first injection. Seventeen days after the first injection, one female mouse had alopecia on the head.

Table I reports body weight changes along the twenty days of study. It can be noticed that in male mice, the body weight increased throughout the experimental period, while in female mice the body weight decreased at the day the second dose was injected as compared to day 13.

At sacrifice time, no toxic effects of ^{99m}Tc-HBD-3 were macroscopically observed. At the microscopic examination, in 2 mice (one male and one female) single lung foreign body granulomas organized around amorphous particles birefringent under polarized light were observed (Figure 1). No lesions were observed in all other organs examined. As a result, no lesion attributable to treatment with ^{99m}Tc-HBD-3 was evident.

Discussion

The main purpose of the present study was to investigate the toxicity of ^{99m}Tc-labeled HBD-3 in CD1 mice of both sexes in general and specifically in the liver, kidneys, heart and lungs. Technetium-99m is the radioisotope most commonly

Table I. *Body weight changes along the study. In male mice, the body weight increases all along the experimental period. In female mice, the body weight decreases at day 19 compared to day 13.*

		Group A			
		Day 1	Day 7	Day 13	Day 19
Animal	Sex				
1	Female	24,51	23,85	26,6	24,8
2	»	24,1	24,66	25,77	23,72
3	»	27,31	25,92	29	27,16
4	»	24,12	23,7	25,76	24,94
5	»	24,34	24,31	26,76	23,82
Mean		24.876	24.488	26.778	24.888
S.D.		1.3710	0.8859	1.3249	1.3853
		Group B			
6	Male	30,64	32,74	26,34	37,86
7	»	29,61	32,83	37,84	40,21
8	»	30,68	33,73	37,56	37,97
9	»	30,79	33,4	37,45	39,06
10	»	29,65	32,45	35,04	37,08
Mean		30,274	33,03	36,846	38,436
S.D.		0,590618	0,521392	1,159776	1,216976

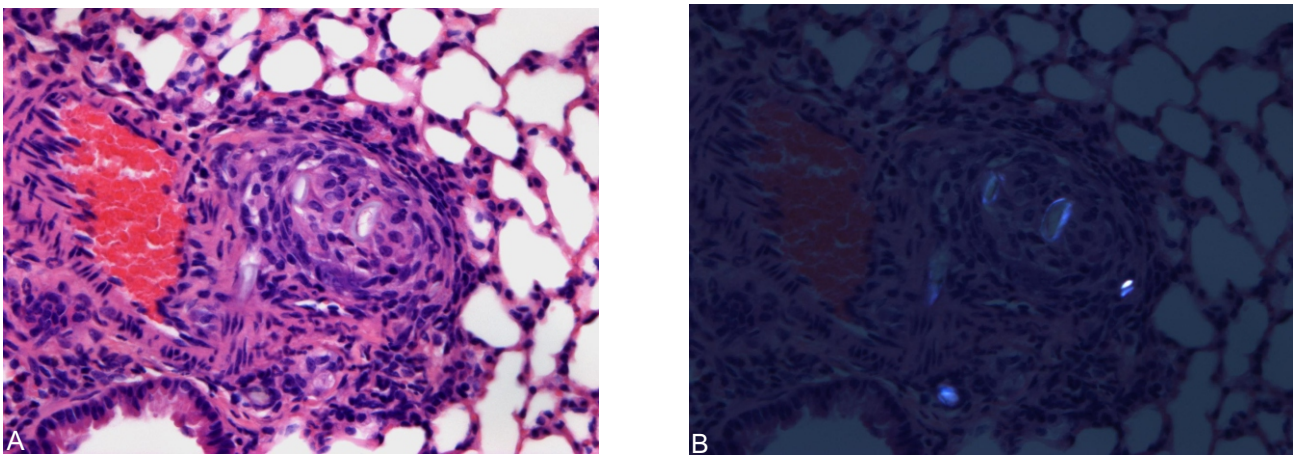

Figure 1A. Hematoxylin-eosin staining of a paraffin embedded lung tissue section. Notice that granuloma is organized around foreign bodies. **Figure 1B**, shows the birifringence due to the same foreign particles as in (A) when the tissue section was observed under polarized light

used in clinical nuclear medicine and is obtained by beta-decay from a ^{99}Mo generator and has been used as non toxic. Human defensins have strong antimicrobial and immunomodulatory properties [16]. Human BD-3, a 5kDa antimicrobial cationic (alkaline) peptide, is an important component of the innate immune system [17], constitutively expressed and up-regulated by inflammatory and infectious stimuli at the transcriptional level [18]. Human BD-3 has been reported to exhibit little, if any, cytotoxic activity against eukaryotic cells in that, when used at concentration as high as 500mcg/mL in normal saline induced hemolysis in only <0.5% of human erythrocytes [19]. The same authors also reported that human BD-3 at a concentration of 10mcg/mL had no effect on the viability of human monocyte THP-1 cells. More recent experiments in three different human epithelial carcinoma cell lines at concentrations as high as 25mcM demonstrated that wild-type human BD-3 and its synthetic analogs did not induce relevant cytotoxic effects [20].

To our knowledge, there is no information available on the "in vivo" toxicity of ^{99m}Tc-human BD-3 nor of the unlabelled peptide. In this study we fulfilled general obligations for describing toxicity characteristics of tracers potentially endowed with diagnostic properties. As for the possibility of toxic effects of human BD-3 associated to its intriguing immunomodulatory characteristics, there is evidence demonstrating the agonistic activity of human BD-3 for toll-like receptors (TLR) 1 and 2 of monocytes and myeloid dendritic cells resulting in the induction of pro-inflammatory cytokines and in the enhancement of adaptive immune response [21]. On the contrary, an anti-inflammatory role for HBD-3 has been postulated both "in vivo" and "in vitro" based on its capacity of inhibiting the accumulation of TNF-alpha and IL-6 proteins in lipopolysaccharide-stimulated bone marrow derived macrophages [22]. The mechanism of the anti-inflammatory activity of human BD-3 was suggested to be due to the targeting of the TLR signalling pathway resulting in transcriptional repression of pro-inflammatory genes [23].

Whatever the nature of interaction of human BD-3 at the dose levels used with cells of the immune system, some consequences of this targeting could be hypothesized. In the pro-inflammatory mechanism, an exaggerated inflammation from TLR agonist might drive tissue damage to cause a range of pathological states. Alternatively, the TLR antagonist functioning of the immunomodulatory peptide would lead to the amelioration of inflammation induced tissue damage, but with a potential for decreased local immunity and poorer control of microbial infections [24].

We studied the toxicity of the same molecule for which we have previously reported its utility in discriminating the presence of bacteria in a rodent model of mixed inflammation/infection [7]. In this paper we assessed the potential toxicity of the peptide, by i.v injecting in mice a dose of ^{99m}TcHBD-3, one order of magnitude greater than that which is effective for in vivo imaging of targeted infections in rodents [5]. Mice of both sexes after i.v administration of ^{99m}TcHBD-3 were daily observed and weighted for 18 days. In the male, but not in the female mice, a delayed (4 days) and transient light hypokinetic effect was observed. At present, we can only speculate with regard to this effect. In fact, it is theoretically possible that the interaction of the HBD-3 with lipid membrane can lead to the formation of ion-channels on the neuro-muscular junctions, thus inducing muscular failure, with a mechanism similar to that of others [25]. However, it has been demonstrated that the HBD-3 could only induce lesions in those membranes resembling the lipid composition of the outer membrane of sensitive bacterial strains [4]. Histopathological examination of kidneys, liver, heart and lungs was performed. Macroscopically detectable lesions were not observed in any of the tissues selected, but 2 out of 10 treated mice had microscopically detectable lung foreign body granulomas. The presence of these granulomas could not be attributed to the ^{99m}Tc-labeled peptide, since the peptide was previously freed from insoluble contaminants by gel chromatography through Sephadex G-10 (see materials and methods). It is more probable that these foreign bodies take origin from keratin fragments which accidentally entered blood stream at the time of injection.

Tests of functional activity of the liver and kidneys in basal conditions were not carried out for financial reasons. However the normal anatomo-pathological findings suggested that these organs were normal when the test started.

We can suggest, that under the applied experimental conditions, the two repeated i.v injections of 6mcg HBD-3 (total 12mcg), did not induce any significant toxicity.

Having faith in the previously mentioned suggestions for dose translation from mouse to human, we have calculated the human dose, based on body surface area, equivalent to 6mcg/mouse: this equivalent dose was 19.5mcg/kg and corresponded to 1362mcg for a 70kg person. Given the specific activity of the tracer, such a potentially non-toxic dose is associated with a high ^{99m}Tc activity of 10215MBq. Indeed such a dose of a similar tracer injected, would not be allowed, due to the photon-associated damage of ^{99m}Tc. From the clinical point of view, it is reasonable to assume that a radioactivity of about 370MBq would be sufficient for diagnostic use in humans. This corresponds to 50mcg HBD-3 and represents <4% of the dose supposed to be non-toxic. We are aware of the limits when extrapolating a mouse dose to man, since different species are susceptible to different pathogens, and the disease pattern induced by each pathogen will be model and species dependent [26]. In addition, it would be necessary to repeat the experiments of toxicity in a greater number of mice and in other species, taking into account additional parameters of toxicity.

In conclusion, we believe that toxicity data presented in this study further support the clinical use of ^{99m}Tc-HBD-3 for imaging in all cases when differential diagnosis between infection and inflammation is needed.

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The authors declare that they have no conflicts of interest

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