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Synthesis and biological evaluation of a novel ^{99m}Tc labeled 2-nitroimidazole derivative as a potential agent for imaging tumor hypoxia

Yoann Joyard [a,b], Vadim Le Joncour [c], Hélène Castel [c], Chérif Bounana Diouf [a], Laurent Bischoff [b], Cyril Papamicaël [b,*], Vincent Levacher [b], Pierre Vera [a], Pierre Bohn [a,*]

[a] Department of Nuclear Medicine, Henri Becquerel Center and Rouen University Hospital and QuantIF-LITIS (EA4108), 1 rue d'Amiens, 76038 Rouen Cedex, France
[b] UMR CNRS COBRA 6014, 1 rue Tesnière, 76130 Mont-Saint-Aignan, France
[c] Inserm U982, Laboratory of Neuronal and Neuroendocrine Communication and Differentiation, University of Rouen, Mont-Saint-Aignan, France

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ABSTRACT

Tumor hypoxia plays a major role in reducing the efficacy of therapeutic modalities like chemotherapy and radiation therapy in combating cancer. In order to target hypoxic tissues, a tripeptide ligand having a 2-nitroimidazole moiety, as a bioreductive species, was synthesized. The latter was radiolabeled with ^{99m}Tc for imaging hypoxic regions of tumors and was characterized by means of its rhenium analogue. The biodistribution and scintigraphic image of the corresponding ^{99m}Tc-complex showed accumulation in tumor and these results suggest that it could be a marker for imaging tumor hypoxia.

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Hypoxia is characterized by an imbalance between the rate of cellular oxygen consumption and oxygen supply in cells. Hypoxic tissues are a hallmark of advanced solid tumors. The distribution of hypoxic tissue in tumors is heterogeneous and variable over time. It has been shown at the beginning of the twentieth century that hypoxia plays a role in increased radio resistance.[1] Indeed, oxygen has a crucial role in the response to radiotherapy. Being a potent radiosensitizer, it enhances the effect of ionizing radiation leading to a lethal effect on tumor cells by breaking the double-stranded DNA. More recently it has been shown that hypoxia was a predictor for drug resistance.[2] The decrease in the response to radiotherapy of hypoxic cells then leads to an increased risk of metastasis and poor prognosis for the patient.[3] It is therefore of major interest to have a precise knowledge of the level of oxygen in the tissues in order to understand the formation mechanism of this hypoxic state, and develop strategies to address this imbalance. This requires quantifying the level of oxygenation of tissues with good spatial and temporal resolution.

We can distinguish two classes of markers of hypoxia: the nitroimidazole ligands and non-nitroimidazoles ligands. Nitroimidazole derivatives are known for their ability to be selectively retained in hypoxic tissues. Nakamura has first demonstrated in 1955 that, the 2-nitroimidazole showed activity against particular types of infections associated with a deficiency of oxygen.[4] Therefore nitroimidazole analogues were synthesized and proved to be effective against bacteria and protozoa that proliferate in hypoxic cells.[5] This particular behavior of nitroimidazole derivatives in oxygen-deficient environment led to their study in the detection of hypoxia.[6,7]

Most clinically used tracers are ^{18}F-labeled tracers in order to perform a PET imaging but this radiochemistry needs a cyclotron and a GMP laboratory. Thus, owing to limited availability, high cost of production and short half-life of cyclotron isotopes, a ^{99m}Tc-based agent could be a better alternative, especially as all nuclear medicine centers possess gamma cameras whereas only few has one PET-CT camera. So, many radio pharmaceuticals[8–10] (Fig. 1) having a nitroimidazole moiety and radiolabeled with technetium-99m for SPECT imaging of hypoxic tissues were developed. However, due to their high background levels in normal tissues, quantification of hypoxia may be difficult.[11] So, there is a crucial need to develop new technetium-99m candidates.

* Corresponding authors. Tel.: +33 (0)2 355 224 84; fax: +33 (0)2 355 229 62 (C.P.); tel.: +33 (0)2 320 829 23; fax: +33 (0)2 320 825 50 (P.B.).
E-mail addresses: cyril.papamicael@insa-rouen.fr (C. Papamicaël), pierre.-bohn@chb.unicancer.fr (P. Bohn).

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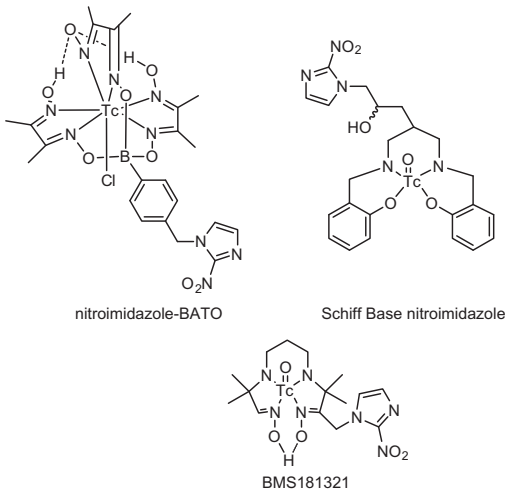
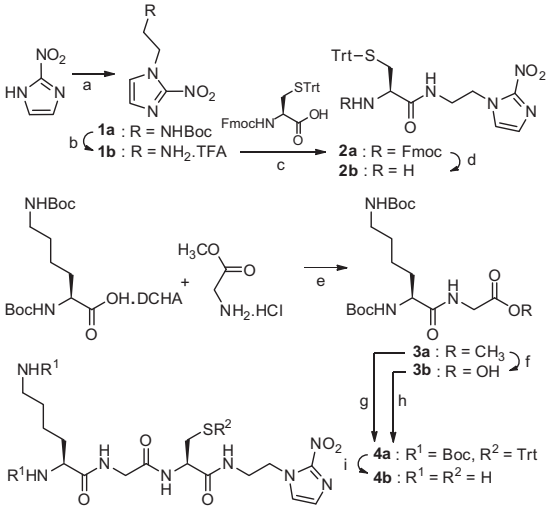


Figure 1. Structures of selected technetium-containing nitroimidazoles tested for imaging.

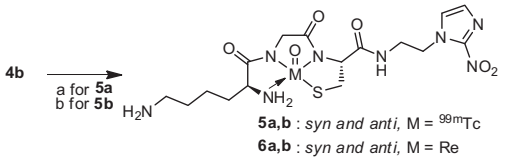
There are many examples in the literature[12] reporting the use of peptides acting as chelating agent of ^{99m}Tc (N_xS_{4-x}). But to date there are only few examples of hypoxia radiotracers using such ligands to complex ^{99m}Tc. Indeed, only Ballinger and co-workers.[13] report the use of sequences glycine–serine–cysteine and glycine–cysteine–glycine to chelate ^{99m}Tc.

In this study, a cysteine–glycine–lysine sequence with a bioreducible moiety, 2-nitroimidazole, was synthesized and labeled with ^{99m}Tc. We chose to use a 2-nitroimidazole moiety as a vector since it has a better response to hypoxia than 4- and 5-nitroimidazoles isomers.[14] The latter was then evaluated as a tumor hypoxia marker.

The chelating agent **4b** was synthesized through a multi-step reaction using 2-nitroimidazole as a starting material. The synthesis procedure is outlined in Scheme 1. On the one hand, according to a procedure reported in the literature by Hay et al.,[15] 2-nitroimidazole was reacted with *tert*-butyl 2-bromoethylcarbamate to give compound **1a**. Then, the Boc protecting group was cleaved with trifluoroacetic acid to lead to the expected derivative **1b**. The so-obtained amine was reacted with commercially available Fmoc-L-Cys(Trt)-OH using propylphosphonic anhydride T3P® to afford the desired protected nitroimidazole derivative **2a**. The subsequent Fmoc-deprotection was achieved by classical reaction with piperidine, leading to amine **2b**. On the other hand, a peptidic coupling reaction with available glycine methyl ester hydrochloride and Boc-L-Lys(Boc)-OH, DCHA gave the protected dipeptide **3a**. The ester group of **3a** was also converted into an acid moiety to give **3b**. Then, the required protected tripeptide **4a** was prepared from ester **3a** following a classical hydrolysis with LiOH and coupling with **2b** in the presence of the cationic coupling agent HBTU. Moreover, it should be noted that the protected tripeptide **4a** was also synthesized starting from **3b** and T3P®. Finally, the cleavage of both NH-Boc and *S*-Trityl protecting groups was conducted by means of TFA in the presence of triethylsilane and phenol as scavengers to give the desired tripeptide **4b**. Optimization of the radiolabeling procedure of **4b** was then conducted (Scheme 2, Table 1). So, peptide **4b** (10 μg) was radiolabeled with sodium tartrate (20 mg in 0.5 mL, pH 7), $^{99m}TcO_4^-$ (740 MBq) and tin(II) chloride (40 μg in 0.1 mL). The resulting solution was analyzed by radio-HPLC. The reaction was first studied at 25 °C and a 95% radiochemical yield was obtained when the reaction time was changed from 10 to 60 min (Table 1, entries 2 and 3). We then decided to perform the reaction at 100 °C (Table 1, entry 4). These conditions have enabled us to considerably reduce the reaction time to 10 min (Table 1, entry 4). Using this labeling procedure, the complex was obtained with a radiochemical purity of 95% as a *syn* and *anti*-diastereoisomers mixture **5a**,**b** (Fig. 2). It has been demonstrated that the presence of two radiometric peaks is due to the resolution of diasteroisomers resulting from the chiral centers on the peptide backbone and the chiral technetium.[12b,16] It is difficult to determine directly the formation of the ^{99m}Tc-complex because of the extremely small amount of the compound present. However, the formation of ^{99m}Tc-complex **5a**,**b** could be indirectly determined by comparison with **6a**,**b** which can be easily prepared. Re and Tc belongs to the same group of the periodic table and are similar in size. In our case, the analogue rhenium complex **6a**,**b** was



Scheme 1. Reagents and conditions: (a) *tert*-Butyl 2-bromoethylcarbamate, K_2CO_3, DMF, 110 °C, 5 h (57%); (b) TFA, 25 °C, 10 min (99%); (c) T3P®, DIEA, CH_2Cl_2, 25 °C, 12 h (86%); (d) piperidine (20% mol), DMF, 25 °C, 16 h (53%); (e) T3P®, DIEA, CH_2Cl_2, 25 °C, 12 h (86%); (f) LiOH, H_2O, 25 °C, 12 h (85%); (g) LiOH, H_2O, DMF, CH_2Cl_2 then **2b**, HBTU, DIEA, DMF, 25 °C, 12 h (43%); (h) **2b**, T3P®, DIEA, CH_2Cl_2, 25 °C, 12 h (53%); (i) TFA, H_2O, TES, phenol, 0 °C, 1 h (52%).



Scheme 2. Reagents and conditions. (a) $SnCl_2$, $^{99m}TcO_4^-$, sodium tartrate pH 7.0, 100 °C, 10 min (radiochemical yield 95%); (b) $ReOCl_3(PPh_3)_2$, NaOAc, MeOH, 70 °C, 4 h.

Table 1
Labeling conditions of ^{99m}Tc-complex **5a**,**b** with sodium pertechnetate and tin(II) chloride

Entry	**4b** (μg)	Sodium tartrate (mg)	Final pH	Temperature (°C)	Time (min)	RCY (%)
1	100	—	5	25	10	76
2	10	20	5	25	10	89
3	10	20	5	25	60	95
4	10	20	5	100	10	95
5	10	20	9[a]	100	10	38
6	10	20	4[b]	100	10	38

[a] Phosphate buffer 0.2 M pH 9, 0.5 mL.
[b] Citric acid buffer, 20 mM pH 4, 0.5 mL.

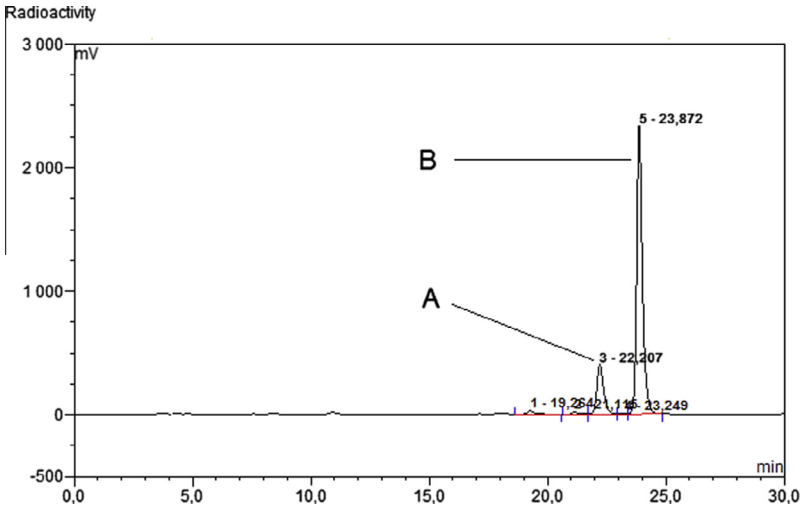


Figure 2. Representative radio-HPLC pattern of *syn* and *anti*-diasteroisomers **5a**,**b**. Peaks A and B are thought to be *syn* and anti-forms of the Tc=O group.

prepared by substitution of ligand **4b** on the rhenium precursor $ReOCl_3(PPh_3)_2$ (Scheme 2).

The HPLC analysis, UV detection, of the reaction mixture showed two main peaks with similar retention times of the corresponding technetium-99m complex **5a**,**b**.

Besides, the LC-MS analysis of the reaction mixture exhibited two identical molecular ions at *m*/*z* 645.1 ($[M+H]^+$), which confirm the presence of *syn* and *anti*-diastereoisomers. In addition, the molecular ion value gives a further indication concerning the nature of the bond with the metal and the amine function of the lysine. Indeed, in this case, the metal undergoes a dative bond with amine function instead of a bond with the corresponding amide ion[12a]

Complex **5a**,**b** was in vitro evaluated. The human glioblastoma cell line was cultured,[17] and used as a model of brain cancer. Glioblastoma multiforme is the most common and most aggressive malignant primary brain tumor in humans. Treatments are surgery, chemotherapy with temozolomide or bevacizumab and radiation therapy.[18] Glioblastoma is well-known to form tumors with hypoxic zones, which are resistant to standard radiation therapy.[19] The use of hypoxic tracers could help to customize treatments[20] with a real-time adjustment of the therapeutic beams (Image-guided radiation therapy).[21]

In hypoxic conditions, after 24 h, an increased expression of hypoxia-inducible factor (HIF1) was observed (Fig. 3) as a sign of cellular stress in T98 cells. However, at this time, no significant uptake of tracer **5a**,**b** occurred. (Fig. 4) After 48 h in the same hypoxic conditions, expression of HIF1 increased by a factor of 2.5 (Fig. 3) and subsequently the tracer retention became highly significant (Fig. 4). Nitroimidazole moiety is only reduced in cells with long-lasting hypoxia, and reduction of this compound leads in covalent bonding to macromolecules and subsequently in tracer retention. Reduction of nitroimidazole only occurs in cells deprived of oxygen with an acidic and reductive cytoplasm. After 24 h in hypoxic conditions, T98 cells were stressed but their cytoplasms were not sufficiently reductive to retain compound **5a**,**b**. After 48 h, cell cytoplasm has reached optimal conditions to reduce the nitroimidazole moiety. Compound **5a**,**b** is thus a potential tracer of severe hypoxia.

Four nude mice[25] were xenografted with T98 cells in their left hind legs. 3 weeks after xenografting, the tumors have reached an average diameter of 10 mm. Compound **5a**,**b** was injected in tail vein (10 MBq). Two hours later, animals were sacrificed and the percent of injected dose per gram of tissue was calculated (Table 2). The tumor-to-muscle ratio was 2.70 ± 0.23 in these conditions.

This result is better than the reported uptake of [^{18}F]-FMISO in CaNT tumor, but lower than [^{99m}Tc]-HL91 and [^{64}Cu]-ATSM (Table 3).[26] This comparison should be interpreted carefully since this is not the same cell line used. To the best of our knowledge,

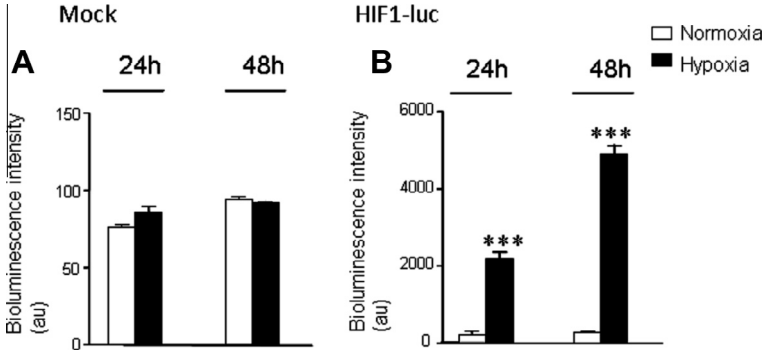


Figure 3. Effect of hypoxia on HIF1 production and luciferase activity. T98 glioblastoma cell line transfected[22] with no cDNA (Mock, **A**) and with cDNA encoding the HIF1 luciferase reporter vector (HIF1-Luc, **B**) were cultured in normoxic (20% O_2) or hypoxic (1% O_2) conditions, 24 h or 48 h post-transfection. The luciferase activity was expressed as arbitrary bioluminescence units (au). In T98 not transfected with the HIF1-Luc cDNA vector, both normoxic and hypoxic conditions failed to evoke luciferase production. In T98-expressing luciferase under the HIF1-responsive element, 24 h and 48 h of hypoxic conditions drastically stimulate luciferase activity, suggesting enhanced production of HIF1.[23] Bars represent means ± SEM. of a representative experiment with 10 replicates in each group. Statistical significance $^{***}P$ <0.001; hypoxia versus normoxia in B (one-way Anova followed by a Bonferroni post-test).

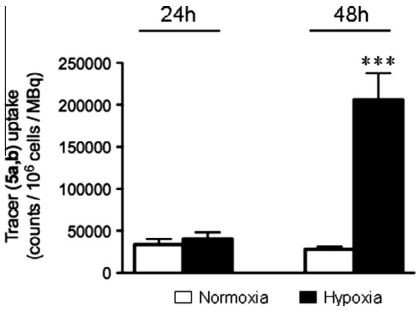


Figure 4. Effect of chronic hypoxia on tracer **5a**,**b** uptake.T98 glioblastoma cell lines were cultured in normoxic (20% O_2) or hypoxic (1% O_2) conditions during 24 or 48 h. After 24 h, no significant difference in uptake of tracer **5a**,**b** was observed in cells regardless of culture conditions. After 48 h, an uptake and retention of tracer **5a**,**b** was only observed in cells cultured in hypoxic conditions, suggesting that the nitroimidazole could be reduced in the cytoplasm of cells deprived of oxygen.[24] Bars represent means ± SEM of a representative experiment with 8 replicates in each group. Statistical significance $^{***}P$ <0.001; hypoxia versus normoxia after 48 h (one-way Anova followed by a Bonferroni post-test).

Table 2
Biodistribution (%ID/g, mean ± SD) in mice (n = 4) bearing a T98 tumor (glioblastoma cell line) 2 h following the injection of ^{99m}Tc labeled **5a**,**b**

Organ	**5a**,**b** uptake (%ID/g)
Kidney	3.73 ± 0.59
Liver	2.50 ± 0.50
Large intestine	0.20 ± 0.03
Muscle	0.11 ± 0.02
T98 tumor	0.29 ± 0.06
T/M ratio	2.70 ± 0.23

comparison between [^{99m}Tc]-HL91, [^{18F}]-FMISO and [^{64}Cu]-ATSM uptakes in T98 tumor is not described in the literature.

To evaluate the interest of compound **5a**,**b** as a potential agent for imaging tumor hypoxia, T98 glioblastoma cell line was xenografted in the right hind limb of nude mouse and after four weeks the tumor diameter was 10 mm. Scintigraphic image was obtained with 10 MBq of compound **5a**,**b** 60 min after injection into the tail vein. An important uptake was observed in kidneys and intestinal tract as a sign of tracer excretion. A heterogeneous tracer uptake was observed in the tumor (an interesting tumor-to-muscle ratio of 10 was determined) as a potential uptake in hypoxic areas (Fig. 5). Besides, scintigraphic images were also obtained after 2 and 3 h and we observed a constant tumor-to-muscle ratio.

In conclusion, the tripeptide **4b** can be labeled with technetium-99m to give the complex **5a**,**b** with a radiochemical purity of 95%. A specific uptake of the latter compound **5a**,**b** in hypoxic tumor cells was observed in vitro. A first biodistribution assay showed a tumor-to-muscle ratio of 2.70 ± 0.23 in vivo. To complete this study, a scintigraphic image showed a specific tracer uptake in hypoxic areas. These results suggest that the complex **5a**,**b** might be an useful tracer for hypoxic tumor imaging.

Table 3
Reported T/M ratio for hypoxia imaging agents in nude mice (isoflurane + room air; adenocarcinoma NT; 2 h post-injection)[26]

Imaging agent	T/M ratio
[^{99m}Tc]-HL91	4.6 ± 0.7
[^{18}F]-FMISO	2.2 ± 0.4
[^{64}Cu]-ATSM	4.0 ± 0.3

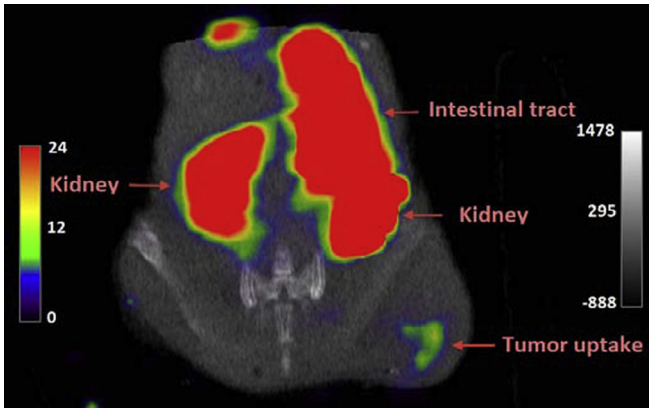


Figure 5. Scintigraphic image obtained with 10 MBq of complex **5a**,**b** 60 min after injection into the tail vein.

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17. *Cell culture*. The human glioblastoma cell line T98 was obtained from the American Type Culture Collection (ATCC, LGC Standards, Molsheim, France). The T98 cells were maintained in HAM-F12 (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FBS, Lonza, Bâle, Suisse), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (Gibco, Fischer Scientific, Illkirch, France) at 37 °C in a humidified 5% CO_2 incubator. Culture media were replaced every 3 days.
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22. For transfection, T98 cells were plated in 24-well plates (Costar, 10×10^3 cells/well) for 24 h before transfection in HAM-F12 (for T98 cells), supplemented with 10% FBS. The pHIF1-pTL-Luc (HIF1 Luciferase reporter vector, Panomics, Milano, Italy) is designed to measure transcriptional activity of hypoxia inducible factor-1 (HIF-1). HIF-1 binds to the hypoxia responsive element and activates genes involved in angiogenesis, glucose metabolism, cell proliferation, survival and invasion/metastasis. The transient transfection of the Plasmid pHIF1-Luc (0.2 μg/well) or without vector (Mock) was performed using FuGene 6 (1.8 μl/well, Promega) following the manufacturer's protocol. The response of these cells to hypoxia was obtained after 24 or 48 h incubation in the presence of a gas mixture containing 1% O_2, 5% CO_2 and balanced N_2 (Incu Safe Incubator, VWR, Fontenay-sous-Bois, France).
23. *Luciferase assay*. All cell extracts were prepared and analyzed using the Luciferase Assay System (Promega, Madison, WI). Briefly, 24-well plates were washed twice with cold 1× phosphate-buffered saline (PBS) and 100 μl of 1× lysis buffer was then added to the cells. Samples were lysed and collected and 20 μl aliquots were assayed using luciferase assay reagent. Luminescence was measured using a Victor V plate reader (PerkinElmer, Courtaboeuf, France). Background absorbance from samples not transfected was systematically measured.
24. *Tracer uptake assay*. T98 cells were incubated with **5a,b** (2–3 MBq) for 1 h then washed twice with cold PBS. Cells radioactivity was measured by a Wallac Wizard 1470 Gamma Counter. Results were expressed as decay-corrected counts in 10^6 cells/MBq.
25. Animals were maintained in a facility approved by the French ministry of agriculture in accordance with current regulations and standards and registered under the number B76-450-05. 'Principles of laboratory animal care' (NIH publication No. 86-23, revised 1985) were strictly followed. Animals were housed four per cage and fed ad libitum. The experiments have been approved by an ethical committee and registered under the number N/02-06-12/12/06-15.
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