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New Potential *In Situ* Anticancer Agent Derived from [^{188}Re]rhenium Nitro-Imidazole Ligand Loaded 5th Generation Poly-L-Lysine Dendrimer for Treatment of Transplanted Human Liver Carcinoma in Nude Mice

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver tumor and one of the fifth most common tumors worldwide. In this article we report the results of *in vivo* studies of potential anticancer agent "[^{188}Re] rhenium-ImDendrim" derived from [^{188}Re]rhenium-nitro-imidazole-methyl -1,2,3-triazol-methyl-di-(2-pycolyl)amine as radioactive ligand loaded 5th generation poly-L-lysine denrimer (172,3 kDa, 20 nM).

Methods: 5.0 × 10^6 cells were subcutaneously injected into mice. Once tumor established, 4 mice lots were treated with a single dose of the test item (37, 74, 92.5 and 111 MBq of [^{188}Re]rhenium-ImDendrim, respectively) compared to control lots (free [^{188}Re]rhenium and non-radioactive ImDendrim). By the end of the study in six weeks post-test compound administration, the tumors were collected for histological analysis.

Results: The treatment was well tolerated. In fact, [^{188}Re]rhenium-ImDendrim shows high significant anti-tumor property in this experimental cancer model even with the lowest dose of 37 MBq compared to control groups. These results were further confirmed by histological analysis. Large tumor mass only observed in tumor's sections from mice in the control groups, were disappeared in favour of collagen tissue in treated groups. In conclusion, this novel potential radiopharmaceutical agent has giving promising experimental results by showing an anti-tumoral activity in this experimental of liver cancer model in mice under the tested conditions.

Keywords: Hepatocellular carcinoma (HCC); Dendrimer; *In situ* anticancer agent; Nanomedicine

Introduction

Cancers are among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012 [1].

Hepatocellular carcinoma (HCC) is the most common primary liver tumor and one of the fifth most common cancers worldwide representing 7.9% of all malignancies [2]. In northern Europe the incidence is less than 5 per 100,000 people. In areas of high incidence such as China and South East Asia HCC is reported to be about 20 per 100,000 people [3].

Until now, the curative resection is the most effective therapy for HCC. However, the percent of patients able to be resected is reported to be between 10 and 50% due to impaired liver function and delayed diagnosis [4-6]. Furthermore, recurrence rates remain high following tumour resection with a dismal 8.9% five-year recurrence-free survival [7]. General chemotherapy and radiotherapy offer somewhat unsatisfactory responsiveness. Consequently, there is a clinical need for advances in loco-regional liver treatments in patients with HCC.

Currently, local therapies for HCC include transcatheter hepatic arterial infusion chemotherapy (TACE transarterial chemoembolization) and radiofrequency ablation; both treatments with the intent of providing local control while preserving essential vascular structures, liver parenchyma and adjacent organs [8-15]:

In the one hand, TACE (Transarterial Chemoembolization) is the standard of care for patients with intermediate HCC as described by the Barcelona Clinic Liver Cancer group. TACE involves the injection of a chemotherapeutic agent and then a further agent that embolizes the artery feeding the tumor thus attempting tumor necrosis via reduction in blood supply and a cytotoxic effect. TACE, although a palliative treatment option, has been shown to offer improved survival in patients with unresectable HCC when compared to supportive treatment alone [16].

In the other hand, Radiofrequency/microwave ablation is a potential treatment option for HCC, especially in patients who are not fit enough to tolerate resection or transplant. It involves passing a needle directly into the tumor through the skin, or directly at open or laparoscopic surgery. Radiofrequency or microwave energy is administered to heat the tumor and kill tumor cells. It is not a curative procedure but provides improvement in long term outcomes in patients who would not be able to tolerate curative procedures [2].

Otherwise the radioembolization is a local transarterial approach that capitalizes on tumor hypervascularity to deliver high doses of radiation therapy while preserving normal parenchyma [17-20]. This treatment has been used in HCC, colorectal cancers (CRC), neuroendocrine tumors (NE) and a variety of other primary cancers [21-29].

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The radioembolization device consists of 20-40 μ particles (beads), where the ^{90}Ytrium is integrated in a glass or resin matrix. [^{90}Y]Yttrium is beta emitter with a physical half-life of 64 hours and means tissue penetration is about of 2.5 mm [29,30].

The micron-sized beads loaded with [^{90}Y] Yttrium (20-40 μM) are placed in contact with the tumor but there are not diffusible within the tumor. Thereby, in the presence of a large tumor volume, deep areas of the tumor remain inaccessible for the treatment.

An alternative method involves with lipiodol as vehicle of β emitters such as [^{131}I]Iodine or [^{188}Re]rhenium and could be promptly absorbed by hyper vascularized tumor after injection [31,32].

Recently, direct intratumoral injection of nonremovable radioactive material has been proposed for treatment of hepatic tumors such sulfide [^{188}Re]rhenium colloidal or dendrimers loaded [^{188}Re]rhenium complexes [33-35].

In this context, we have developed a potential *in situ* anticancer treatment of hepatic tumors using [^{188}Re]rhenium-nitro-imidazole derived ligand loaded 5th generation poly-L-lysine dendrimer [35]. This method has at least three advantages as a powerful therapeutic tool:

a) A spherical polycationic supramolecule dendrimer vector whose diameter is adaptable to embolize neo-vascularization of tumor stroma. In addition, the dendrimer injected directly into or near the tumor, remains in the site of injection and then could be considered as nano delivery system [36,37];

b) [^{188}Re]Rhenium, an isotope with a 16.9 h half-life, has a maximum beta energy compared to that of [^{90}Y]Yttrium and gamma emission of 155-KeV (15%) photon that is suitable for imaging [38]. This β emitter complexed with a nitro-imidazole ligand vector that is preferentially taken up by the hypoxic cells, resulting in the radiotoxic effect optimization [39];

c) Providing new targeting anticancer agents *in situ* for treating primary and/or metastatic tumors [35].

In this paper we report the preparation and the results of *in vivo* preclinical studies of new potential *in situ* therapeutic agent "[^{188}Re] rhenium-ImDendrim" derived from [^{188}Re]rhenium-nitro-imidazole-methyl-1,2,3-triazol-methyl-di-(2-pycolyl)amine as radioactive ligand loaded 5th generation poly-L-lysine denrimer (172,3kDa, 20nM).

Methods

The experiment agent "ImDendrim" used in this study is consisting of 5th generation *poly-L-lysine* dendrimer (from Colcom, France) mixed with *nitro-imidazole-methyl-1,2,3-triazol-methyl-di-(2-pycolyl) amine* (Figure 1). All chemicals used in the synthesis of nitro-imidazole-

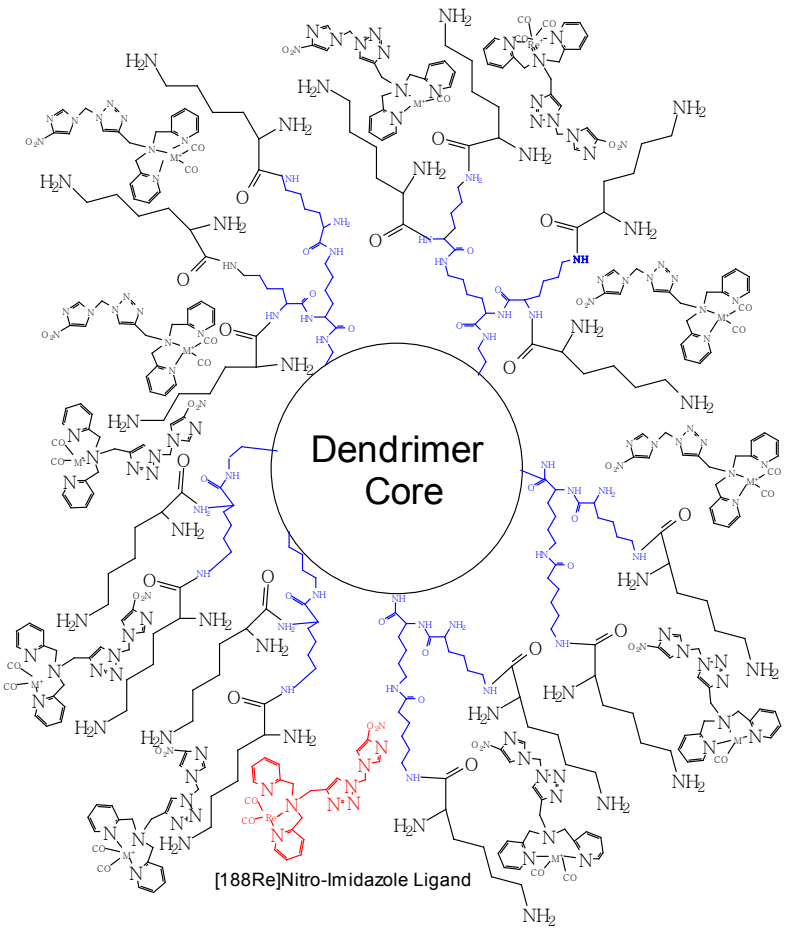

Figure 1: Structure of [^{188}Re]rhenium-ImDendrim.

[^{188}Re]rhenium-ImDendrim is composed by [^{188}Re]rhenium nitro-imidazole-methyl-1,2,3-triazol-methyl-di-(2-pycolyl)amine as radioactive nitro-imidazole derived ligand loaded 5th poly-L-lysine Dendrimer

methyl-1,2,3-triazol-methyl-di-(2-pycolyl)amine were purchased from Sigma-Aldrich, France. [^{188}Re]rhenium ($^{188}ReO_4$ Na) was eluted from an $^{188}W/^{188}Re$ Generator (Institute for Radioelements, Belgium).

Synthesis of nitro-imidazole-methyl -1,2,3-triazol-methyl-di-(2-pycolyl)amine (nitroimidazole derived ligand)

The synthesis of ligand procedure is summarized in Figure 2. The chloro-alkyl-nitro-imidazole compound (2) was obtained by adding the amine function on the chloro methyl imidazole (1). The azido-methyl-nitroimidazol compound (3) was obtained by substitution of the chlorine atom by an azide group. The final compound nitro-imidazole-methyl -1,2,3-triazol-methyl-di-(2-pycolyl)amine (5) was obtained by dissolving propargyl di-(2-picolyl)amine (4) with the azido-methyl-nitruroimidazol compound (3) in a solvent mixture dioxane/water at 100°C in the presence of copper sulfate and sodium ascorbate.

Protocol

Paraformaldehyde (14.6 g, 0.49 mol) was added to chloro methyl imidazole (50 g, 0.44 mol) in 500 ml of chloroform and the reaction was kept under stirring for 48 hours at room temperature. Then, thionyl chloride (57.9 g, 0.49 mol) in dry dichloromethane was added drop wise under an atmosphere of argon. The white precipitate of azido-methyl-nitro-imidazole was filtered, washed with dichloromethane and dried.

Propargyl di-(2-picolyl)amine (8.5 g, 36 mmol) was added to azido-methyl-nitroimidazol (6 g, 36 mmol) in a solvent mixture dioxane/water at 100°C in the presence of copper sulfate(1.7 g, 6.8 mol) and sodium ascorbate (2.9 g, 15 mmol) and the reaction was kept for 4 h at 90°C. The suspension was filtered and dried. Then obtained nitro-imidazole-methyl-1,2,3-triazol-methyl-di-(2-pycolyl)amine compound was redissolved in 450 ml of tetrahydromethylfuran and the organic phase was washed by 350 ml of ammonium solution buffered at pH12. After tetrahydromethylfuran solvent removal, the resulted brown oil was dried at high vacuum (8 g, yield≈56%). ^{1}H NMR (D_2O, 200 MHz, δ/ppm relative to TMS): H_2: 8.3 ± 0.2 ppm (d), $H_{a,a'}$: 8.23 ± 0.2 ppm (d), H_3: 7.99 ± 0.2 ppm (s), H_1: 7.97 ± 0.2 ppm (d), $H_{c,c'}$: 7.62 ± 0.2 ppm (t); $H_{d,d'}$: 7.39 ± 0.2 ppm (d); $H_{b,b'}$: 7.11, ± 0.2 ppm (t), NCH_2N: 6.57 ± 0.2 ppm (s).

Preparation of nitro imidazole derived ligand/dendrimer samples for freeze-drying

50 mg of polylysine dendrimer were dissolved in 4 ml of sterile saline purged with N_2 gas. 2 ml of ethanol solution of nitro-imidazole derived ligand (15 mg/ml) was drop wise added under vigorous stirring to obtain a homogenous solution. The volume was completed to 10 ml using N_2 purged saline. The solution was dispensed in 1ml quantities into sterile penicillin vials and fitted with sterile rubber closures. The vials were transferred to the freeze-dryer and the process continues for 24 h. The vials were closed under dry sterile nitrogen gas and stored at 6–8°C.

Labelling with [^{188}Re]Rhenium

In a typical radiolabelling procedure, 350 µL of perrhenate saline solution (1,850 MBq of [^{188}Re] ReO_4, NaCl), 100 µL of hydrochloric acid at 2 N and 250 µL of 2-(N-morpholino)-ethane-sulfonic acid hydrate (MES) at 0.5 N were added into the vial containing borane ammonia complex (3.5 mg) and the mixture was purged with CO gas and heated at 60°C for 20 min. The lyophilized nitro-imidazole derived ligand/dendrimer already dissolved in 500 µl of saline were added to the previous vial and then heated at 60°C for 1 h. The radiochemical purity of Imdendrim was controlled by TLC coupled with Gamma radioactivity scanner (Raytest, Germany).

In Vivo Tests

The *in vivo* experimental protocols were carried out in accordance with the strict French ethical requirements relating to animal testing.

Cell culture and *in vivo* grafting

The human HCC tumor cell line, HepG2 (obtained from ATCC), was routinely cultured at 37°C, 5% CO_2, containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate (Sigma-Aldrich). The tumor cells required passaging to ensure their viability and reach the required amount of cells. The log-growing tumor cells were trypsinized, counted and used for these studies. Then 5.0×10^6 cells/200 µL were subcutaneously injected into mice.

Subject/experimental animals

60 Athymic nude male mice aged 4-6 weeks (from Harlan Laboratories, France) were used for this study. The animals were housed in a climate-controlled room with a 12/12 h light cycle. The subjects had free access to food and water during housing.

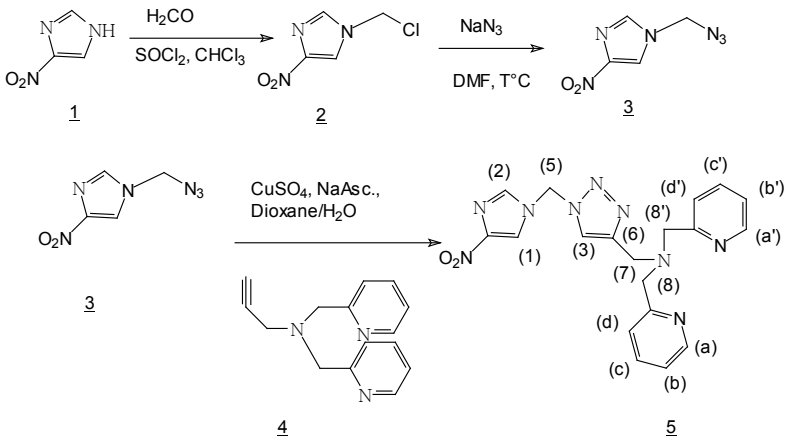

Figure 2: Synthesis of nitro-imidazole derived Ligand*.

Where: *Nitroimidazole derived Ligand : Nitro-imidazole-methyl -1,2,3-triazol-methyl-di-(2-pycolyl)amine

Tumor inhibiting study

Sixty nude mice bearing HepG2 xenograft tumors were divided into six groups. Four groups were treated with a 0.1 ml of [^{188}Re] rhenium-ImDendrim at doses of 37, 74, 92.5 and 111 MBq by a single intratumoral injection. Two groups served as the controls and were injected with free [^{188}Re]rhenium and none radioactive ImDendrim respectively. The tumors were measured every 2 or 3 days with vernier calipers in two dimensions.

The mean tumor volumes (MTV and MTV+SEM) will be by using the following formula:

Tumor volume=(Width^2. Length)/2

In vivo SPECT/CT imaging

After 3 and 24 h post *in situ* administration of [^{188}Re]rhenium-ImDendrim, 3 mice from each group were anesthetized using 80 mg/kg body weight for Ketamine and 8 mg/kg body weight for Xylazine injected intraperitoneally and successively placed in hybrid SPECT/CT scanner for *in vivo* imaging (eXplore speCZT, GE Healthcare, USA). Anatomical CT imaging was performed initially followed by SPECT imaging. Thus to being reconstructed into a single image, the CT data was used for attenuation correction of SPECT images. SPECT/CT image analysis was performed using MicroView software (GE Healthcare Inc.) [40].

Clinical Evaluation

Daily clinical examination of all animals was carried out including: behaviour, signs of suffering (hunching, convulsions, weakening, difficulty for moving or feeding, etc.). Determination of body weight twice a week for each mouse, a body weight curve was designed (Mean ± SD).

Histological Study

At the end of experiment (six weeks post injection), all animals were anesthetized and then sacrificed according to ethical guidelines in France. Then, the tumors were immediately collected and six representative tumor sections of each group were fixed in 4% formalin and paraffin-embedded for histological analysis.

Statistics

Data were expressed as means ± standard deviation (SD). Statistical significance of mean values in two groups or treatment effects were analyzed with Student's t-test or one-way ANOVA using Microsoft Excel program. A p-value less than 0.05 are considered as significant.

Results

The experiment [^{188}Re]rhenium-ImDendrim ([^{188}Re]rhenium-nitro-imidazole-methyl-1,2,3-triazol-methyl-di-(2-pycolyl)amine loaded poly-L-lysine dendrimer) injected *in situ* into mice has a high radiochemical purity yield estimated at 99.5%.

Under this experimental condition, no clinical signs and no mortality were observed in any treated groups.

Mean body weight was the same in all groups at the injection of treatment. Weight loss was more marked in treated groups as compared to untreated groups. The difference became significant after 15 days and remained significant thereafter for all the measurements shown in Figure 3. Clinically, All treated mice remained in complete remission and in apparent good health for 6weeks, until end of experiment.

Antitumor effect

At the time of the injection, tumor volumes were similar in the six groups. As observed in Figure 4, all tumors in treated groups continued to grow for first week, reaching a maximum size of 235 mm^3 ± 104 mm^3. By day 14, the differences between the tumor volumes in the treated and control groups were very significant ($p<0.002$). In contrast, there is no significant difference between the control Groups, the average volumes of tumors injected with free [^{188}Re]rhenium and non-radioactive

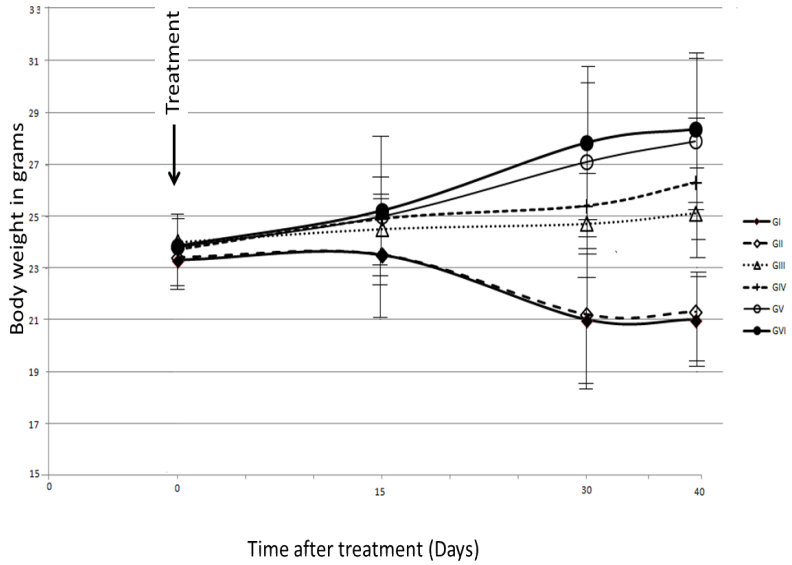

Figure 3: Mice body weight (BW) of HepG2 liver tumor bearing mice.

Where: Group 1 (GI): Non-radioactive ImDendrim; Group 2 (GII): 37 MBq of free [^{188}Re]rhenium per mouse; Group 3 (GIII): 37 MBq of [^{188}Re]rhenium-ImDendrim per mouse; Group 4 (IV): 74 MBq of [^{188}Re]rhenium-ImDendrim per mouse; Group 5 (GV): 92.5 MBq of [^{188}Re]rhenium-ImDendrim per mouse; Group 6 (GVI): 111 MBq of [^{188}Re]rhenium-ImDendrim per mouse

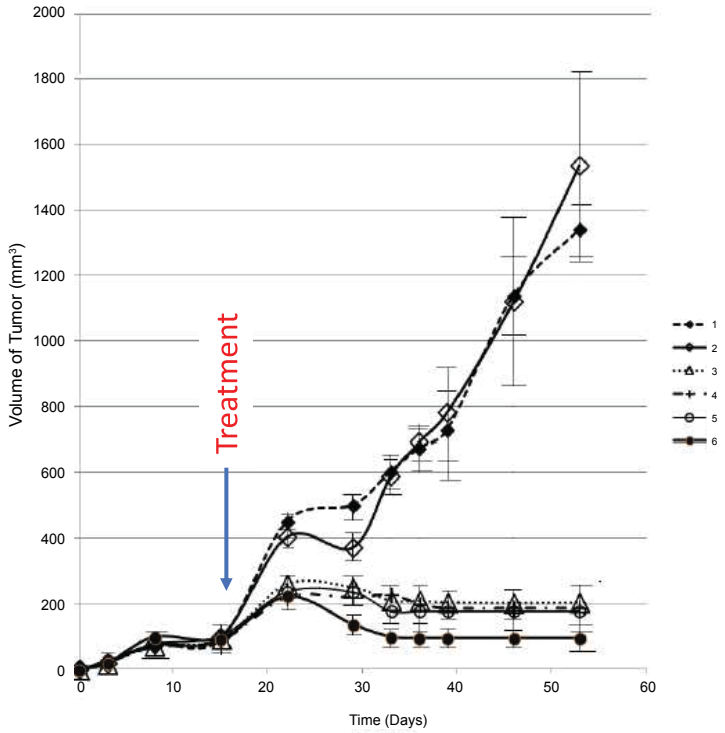

Figure 4: Tumor volume measurements of HepG2 liver tumor bearing mice.

Where: Group 1: Non-radioactive ImDendrim; Group 2: 37 MBq of free [^{188}Re]rhenium per mouse; Group 3: 37 MBq of [^{188}Re]rhenium-ImDendrim per mouse; Group 4: 74 MBq of [^{188}Re]rhenium-ImDendrim per mouse; Group 5: 92.5 MBq of [^{188}Re]rhenium-ImDendrim per mouse; Group 6: 111 MBq of [^{188}Re]rhenium-ImDendrim per mouse.

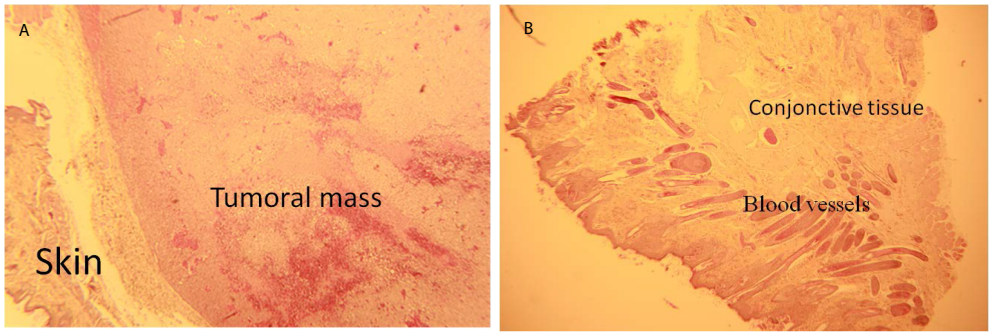

Figure 5: Comparative Histological analyses on tumors in control groups test (a) and treated groups (b) The large tumor mass observed in control group (5a) was replaced in treated mice by scar and collagen tissue (5b).

ImDendrim were 1,600 ± 90 and 1,341 ± 473 mm^3. In comparison, the volumes injected with 37, 74, 92.5 and 111 MBq [^{188}Re]rhenium ImDendrim were 204 ± 55, 187 ± 74 and 178 ± 47 and 96 ± 40 mm^3, respectively. These differences are further illustrated in Figure 4. In all mice, tumor remission was complete, with no evidence of relapse that confirmed by histological analyses. The large tumor mass observed in control group was replaced in treated mice by scar and collagen tissue which is apparent in Figures 5a and 5b. There is no significant difference observed between the treated groups.

In vivo SPECT/CT imaging

24 h postinjection of [^{188}Re]rhenium-ImDendrim, the quasi total administered radioactivity was retained locally in the injection site. As shown in Figure 6, time-course study showed no significant diffusion outside the injection area and notably no significant uptake in different organs including lung, heart, liver kidney and brain.

Discussion

In situ injection of no removable radioactive material using dendrimers is a promising alternative method for tumor treatment because it can deliver high doses of radiation to target sites and prevent normal tissues and no target organs from radiation while delivering radiopharmaceutical to target sites [33-35].

In this context, the purpose of this investigation was to evaluate the therapeutic effectiveness of [^{188}Re]rhenium-ImDendrim consisting of [^{188}Re]rhenium *nitro-imidazole-methyl-1,2,3-triazol-methyl-di-(2-pycolyl)amine as radioactive ligand loaded 5th generation poly-L-lysine*

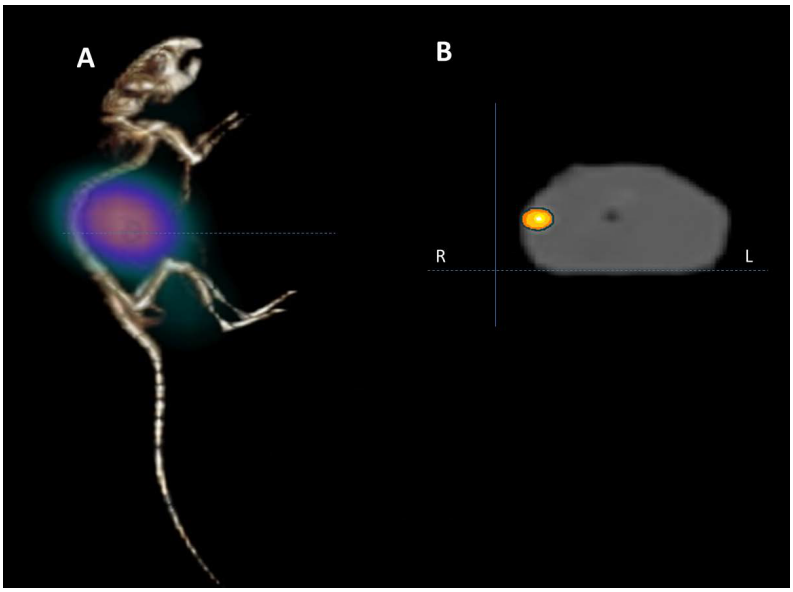

Figure 6: SPECT/CT images of tumor-bearing mice after *in situ* injection of [^{188}Rhenium]-rhenium-Imdendrim.
Mice were injected with 37 MBq of [^{188}Re]rhenium-Imdendrim and scanned with SPECT/CT at 3 h after injection. The Quasi total [^{188}Re]rhenium-ImDendrim is retained in tumor volume after its *in situ* administration in mice and no significant spread toward other organs was visualized in the sagittal plane (A) and transversal plane (B)

dendrimer on mice bearing HCC HepG2 tumors. The charged cationic *poly-L-lysine* dendrimer complex showed high retention in the site of injection and no local or general toxicity after single or iterative *in situ* injection [41].

Our previous *in vivo* studies on radioactive 5th generation poly-L-lysine dendrimer labelled with [^{188}Re]rhenium or [^{99m}Tc] Technetium injected intravenously at 100 mg/L in the rats, showed no acute or sub-acute toxicity and the pulmonary uptake was transient with its constant clearance l=0.57 h^{-1} (half-life=1.2 h) and important constant uptake in liver (4.74% of injected dose/g) [42].

In the present experiment, [^{188}Re]rhenium-ImDendrim was well tolerated while it was intra tumor injected as single dose varying from 37 to 111 MBq on the mice bearing tumors.

Indeed, no clinical signs and no mortality were observed in any treated groups. Concerning mice body weight evolution (Figure 3), all treated groups by 2 weeks post injection of [^{188}Re]rhenium-ImDendrim had significant higher body weight than the control groups in connection with health improvement of the treated mice. These results suggest that the used doses under current experimental conditions did not induce notable toxicity in mice.

According to the obtained results, *in vivo* SPECT/CT imaging study, quasi total [^{188}Re]rhenium-ImDendrim is retained in tumor volume after its *in situ* administration in mice and no significant spread toward other organs was observed (Figure 6). Such local retention of radioactive [^{188}Re]rhenium-Imdendrim could dramatically reduce its side-effect. In addition, the [^{188}Re]rhenium beta particles has the average penetration in tissue of 3.8 mm (maximum 11 mm) and results in a more homogeneous tumor distribution of the radiation dose [33,34].

[^{188}Re]rhenium-ImDendrim showed high significant anti-tumor property in this experimental cancer model even with the lowest dose of 37MBq of activity per mouse, in comparison of injection of non-radioactive ImDendrim or free [^{188}Re]rhenium which do not have any anti-tumor effect. Without poly-L-lysine dendrimer as nano carrier, the free complex of [^{188}Re]rhenium or [^{99m}Tc] Technetium nitro-imidazole derived ligand (Nitro-imidazole-methyl-1,2,3-triazol-methyl-di-(2-pycolyl)amine) has a low tumor bioavailability due to its large distribution volume estimated at 167 L.kg^{-1} (unpublished data). The charged poly-L-lysine groups of dendrimer electro-statically attract the [^{188}Re]rhenium -nitro-imidazole derived ligand and limit its diffusion rate. The rate diffusion seems to be under the influence of the *in situ* environment since the released [^{188}Re]rhenium-nitro-imidazole derived ligand could be specifically attracted by the hypoxic tumoral cells [39]. In this condition, The tumor bioavailability of this [^{188}Re]rhenium-nitro-imidazole derived ligand mixed with poly-L-lysine dendrimer could be enhanced in presence of hypoxic condition specially observed in case of large tumor volume.

At 6th week post treatment in all mice, tumor remission was complete, with no evidence of relapse that confirmed by histological analyses.

In conclusion, this novel potential therapeutic agent has giving promising experimental results by showing an anti-tumor activity in this experimental liver cancer model in mice under the tested conditions. Further works concerning the optimization of injected radioactive ImDendrim dose in terms of the tumor volume are in progress.

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