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Novel ^{188}Re multi-functional bone-seeking compounds: Synthesis, biological and radiotoxic effects in metastatic breast cancer cells

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ABSTRACT

Introduction: Radiolabeled bisphosphonates (BPs) have been used for bone imaging and delivery of β$^-$ emitting radionuclides for bone pain palliation. As a β$^-$ emitter, ^{188}Re has been considered particularly promising for bone metastases therapy. Aimed at finding innovative bone-seeking agents for systemic radiotherapy of bone metastases, we describe herein novel organometallic compounds of the type *fac*-[^{188}Re(CO)$_3$(k^3-L)], (L = BP-containing chelator), their *in vitro* and *in vivo* stability, and their cellular damage in MDAMB231 cells, a metastatic breast cancer cell line.

Methods: After synthesis and characterization of the novel organometallic compounds of the type *fac*-[^{188}Re(CO)$_3$(k^3-L)] their radiochemical purity and *in vitro* stability was assessed by HPLC. *In vivo* stability and pharmacokinetic profile were evaluated in mice and the radiocytotoxic activity and DNA damage were assessed by MTT assay and by the cytokinesis-block micronucleus (CBMN) assay, respectively.

Results: Among all complexes, **^{188}Re3** was obtained with high radiochemical purity (>95%) and high specific activity and presented high *in vitro* and *in vivo* stability. Biodistribution studies of **^{188}Re3** in Balb/c mice showed fast blood clearance, high bone uptake (16.1 ± 3.3% IA/g organ, 1 h p.i.) and high bone-to-blood and bone-to-muscle radioactivity ratios, indicating that it is able to deliver radiation to bone in a very selective way. The radiocytotoxic effect elicited by **^{188}Re3** in the MDAMB231 cells was dependent on its concentration, and was higher than that induced by identical concentrations of **[^{188}ReO$_4$]$^-$**. Additionally, **^{188}Re3** elicited morphological changes in the cells and induced DNA damage by the increased number of MN observed.

Conclusion: Altogether, our results demonstrate that **^{188}Re3** could be considered an attractive candidate for further preclinical evaluation for systemic radionuclide therapy of bone metastases considering its ability to deliver radiation to bone in a very selective way and to induce radiation damage.

1. Introduction

Several malignant solid tumors and multiple myeloma have high tendency to metastasize to bone, leading to intractable and debilitating pain, which severely impairs the quality of life of patients. Bone metastases, more frequent in patients with advanced breast, prostate or lung cancer, weaken the structural integrity of the bone and lead to increased skeletal morbidity, bone pain, pathological fractures, spinal cord compression or vertebral collapse and hypercalcemia of malignancy [1–3].

Bone pain is usually the earliest and most common symptom associated with the development of bone metastases [4]. Currently, in the bone metastatic setting, the combination of different types of therapeutic agents is used to both target the host microenvironment and to reduce tumor burden, and increase survival times after bone metastases are detected [5]. Hence, efforts should be made to relieve pain and maintain the quality of life among these patients.

The available pain-relieving approaches include external beam radiotherapy (EBRT), opioid-based analgesia and bisphosphonates, among others [6]. EBRT provides successful palliation of painful bone metastases in patients with a limited number of localized metastatic sites [6]. However, for those presenting widespread bone metastases with multifocal sites of pain, EBRT is not the best option since harmful radiation of essential organs would be a problem.

Bisphosphonates (BPs) are degradation-resistant structural analogs of pyrophosphates that bound avidly to bone, binding strongly to the hydroxyapatite (HA) crystals, and are taken up by osteoclasts, resulting in inhibition of osteoclast-mediated bone resorption. Moreover, BPs exert a variety of direct and indirect anticancer activities that affect both tumor cells and the surrounding microenvironment, and that stimulate immune reactions [3,7]. For patients with diffuse symptomatic skeletal metastases systemic radiotherapy using radiolabeled BPs based on β$^-$ or α emitting radionuclides is desirable since they target the bone matrix in areas of increased bone turnover characteristic of metastization sites. Consequently, this therapeutic approach enables selective delivery of β$^-$ or α particles to areas of amplified activity targeting simultaneously multiple metastases leading to a pain therapeutic/palliative effect.

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For systemic radiotherapy of bone metastases several radionuclides have been explored such as ^{89}Sr ($T_{1/2}$ = 50.5 d, $E_{\beta max}$ = 1.46 MeV), ^{153}Sm ($T_{1/2}$ = 1.95 d, $E_{\beta max}$ = 0.8 MeV), ^{186}Re ($T_{1/2}$ = 3.7 d, $E_{\beta max}$ = 1.07 MeV, 92.5%), ^{188}Re ($T_{1/2}$ = 16.98 h, $E_{\beta max}$ = 2.12 MeV, 71%) and ^{223}Ra ($T_{1/2}$ = 11.4 d, $E_{\beta average}$ = 5.78 MeV) [8–14]. $^{89}SrCl_2$ and ^{153}Sm-EDTMP are approved radiopharmaceuticals for the treatment of pain from all osteoblastic bone metastases. $^{89}SrCl_2$ is directly incorporated into the bone as a calcium analog, whereas ^{153}Sm-EDTMP targets the hydroxyapatite bone matrix. ^{153}Sm also emits γ-rays (103 keV, 29%), which allow imaging by single photon emission computed tomography (SPECT). The bisphosphonate HEDP has been radiolabeled with ^{186}Re and ^{188}Re and is under clinical evaluation for bone pain treatment. The energetic β^- particles of ^{188}Re have a maximum penetration in tissue of 10–11 mm, which is a suitable option for large tumors [15,16]. It also emits γ-rays (155 keV, 15%) appropriate for SPECT that can be explored for dosimetry purposes, assess biological distribution and monitor therapeutic efficacy [17]. Its short physical half-life (16.98 h), fitting the pharmacokinetics of most bone-seeking agents, allows the use of high doses and minimizing the problem of radioactive waste handling and storage. In addition, ^{188}Re can be readily obtained as a "no carrier added" radioisotope from an in-house ^{188}W/^{188}Re generator, in contrast to ^{186}Re which is produced in nuclear reactors through neutron capture of stable ^{185}Re and thus is obtained as carrier-added [18].

$^{223}RaCl_2$ is a novel bone-seeking calcium mimetic α-emitting radiopharmaceutical that accumulates in regions of active bone turnover. ^{223}Ra has a complex decay scheme in which 4 α particles are generated during each decay, resulting in high linear energy transfer (LET) that induces lethal damage to tumor cells [19,20]. A phase III clinical trial on prostate cancer patients showed decrease in skeletal related events and, for the first time, an increase in overall survival was observed with systemic radiotherapy. These outcomes resulted in FDA and EMA approval in 2013.

In spite of the relevant clinical success of $^{223}RaCl_2$, there is still room for β^--emitters, namely for bone relief in patients with castration-resistant prostate cancer who are not eligible for $^{223}RaCl_2$ due to the presence of visceral metastases, and in patients with osteoblastic metastases from primaries other than prostate [21]. However, which radionuclide provides the best pain palliation with the lowest associated side-effects remains to be answered and requires further studies [22]. In theory, ^{89}Sr due to its longer half-life is expected to cause longer duration in pain response but few data confirm that assumption and this benefit must be weighed against the increased risk of myelosuppression [22]. Therefore, the use of bone-seeking radiopharmaceuticals with relatively short half-life β^- emitters (*e.g.*, ^{153}Sm, $^{186/188}$Re) is recommended aiming to reduce hematologic toxicity and allow subsequent therapies or repeated injections [22]. Another aspect regarding the selection of the radionuclide is related to its production and cost per dose. Indeed, there is a growing interest on generator-produced radionuclides instead of nuclear reactor or accelerator based. That is the case of ^{188}Re that can be routinely available in any nuclear medicine center through a generator, providing many doses and thus reducing costs significantly [18].

In our previous studies, which aimed the design of novel ^{99m}Tc(I)-labeled bisphosphonates with improved bone-seeking properties, we have synthesized and biologically evaluated a set of ^{99m}Tc(I) complexes **Tc1-Tc5** (Fig. 1) with various molecular weights, overall charge, hydrophilicity and different positions of BP attachment in order to get insight of structure/activity relationship [23–25]. In contrast to ^{99m}Tc-MDP and ^{188}Re-HEDP, where the BP unit acts simultaneously as a targeting group and a radionuclide-binding ligand, in our complexes we have separated the targeting BP unit from the bifunctional chelator to improve their efficacy. Some of the complexes displayed high bone uptake, comparable to the gold standard ^{99m}Tc-MDP, allowing imaging and the simultaneous delivery of bisphosphonates to bone tissue in a high specific and selective way [23]. The promising biological profile of **Tc2-Tc4** (Fig. 1) encouraged further research toward the synthesis and biological evaluation of their ^{188}Re congeners for bone metastases therapy. Herein, we describe the synthesis, characterization and biological evaluation of their ^{188}Re congeners (**^{188}Re2-^{188}Re4**) in order to evaluate their potential as new bone-seeking agents for systemic radiotherapy of bone metastases.

The ^{188}Re complexes suited for this purpose are expected to carry toxic doses of radiation to bone metastases due to its high bone affinity. However, there are few data available concerning the radiotoxic and genotoxic effects after treatment with β^--emitting radiopharmaceuticals. Moreover, the mechanisms underlying cell death are also poorly understood. So far, to the best of our knowledge, only some *in vitro* experiments with $[^{188}ReO_4]^-$ were performed using micronuclei (MN) induction as radiation biomarkers and apoptosis as a measure to

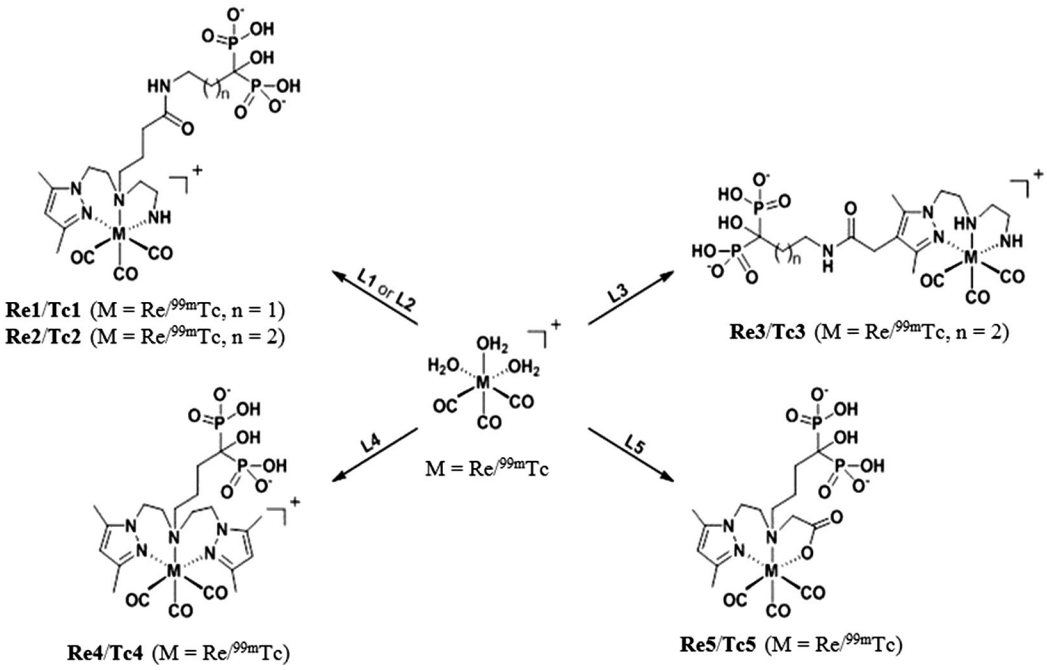

Fig. 1. $M(CO)_3$ complexes containing pendant bisphosphonate units (M = ^{99m}Tc, Re).

ascertain the cellular sensitivity to the beta emission of ^{188}Re. Results from this study showed that the radiocytotoxic effect and the frequency of MN were dependent on the different radioresistance of the tumor cells tested. These observations suggested that ^{188}Re emissions could be successfully explored *in vitro* and *in vivo* after conjugation with a suitable vector [26,27]. In this way, the genotoxic effect of ^{186}Re-bisphosphonate (^{186}Re-HEDP) was evaluated after *in vivo* administration to healthy humans [28]. These results indicated that ^{186}Re-HEDP induces genotoxicity in lymphocytes, especially T-cells that regulate bone metastases and tumor growth in bone, suggesting that this could be the mechanism by which this radiopharmaceutical reduces the pain of patients.

Based on those preliminary observations we explored the cellular sensitivity of the highly metastatic breast cancer cells MDAMB231 as tumor model to study the radiotoxic and genotoxic effects of the most promising ^{188}Re complexes described herein (**^{188}Re2-^{188}Re4**).

2. Materials and methods

2.1. General

Unless otherwise stated, all chemicals and solvents were of reagent grade and were used without purification. The compounds 2-(1-(2-(tert-butoxycarbonyl(2-(tert- butoxycarbonylamino)ethyl)amino) ethyl)-3,5-dimethyl-1H-pyrazol-4-yl)acetic acid [29], (4-amino-1-hydroxybutylidene)bisphosphonic acid trisodium salt tetrahydrate (alendronate, ALN) [30], 1-(2-bromoethyl)-3,5-dimethyl-1H-pyrazole [31] and *tert*-butyl 2-bromoethylcarbamate [32] were prepared according to published methods. The starting material *fac*-[Re$(H_2O)_3(CO)_3$]Br was synthesized by the literature method [33].

Carrier-free Na[^{188}ReO_4] was freshly eluted from a commercial ^{188}W/^{188}Re generator (ITG), using 0.9% saline solution. The radionuclide purity of ^{188}Re was analyzed by gamma spectroscopy with a high-purity germanium (HPGe) detector and the radiochemical purity of Na[^{188}ReO_4] was more than 95% by paper chromatography developed with 0.9% NaCl.

HPLC analysis of the Re and ^{188}Re complexes was performed on a Perkin–Elmer LC pump 200 coupled to a LC 290 tunable UV-Vis detector and to a Berthold LB-507A radiometric detector, using an analytic Macherey-Nagel C18 reversed-phase column (Nucleosil 100–10, 250 × 4 mm) with a flow rate of 1 mL/min. HPLC solvents consisted of 0.05 M TEAP (triethylammonium phosphate solution) buffer, pH 2.2 (solvent A) and methanol (solvent B), using the following gradient: t = 0–3 min, 0% eluent B; 3–3.1 min, 0–25% eluent B; 3.1–9 min, 25% eluent B; 9–9.1 min, 25–34% eluent B; 9.1–20 min, 34–100% eluent B; 20–24 min, 100% eluent B; 24–26 min, 100–0% eluent B; 26–30 min, 0% eluent B.

2.2. Radiosynthesis

2.2.1. Preparation of [^{188}Re$(CO)_3(H_2O)_3$]$^+$

The radioactive precursor *fac*-[^{188}Re$(CO)_3(H_2O)_3$]$^+$ was prepared using a slightly modified method described in the literature [34]. Briefly, 5 mg of $BH_3.NH_3$ and 3 mg of $K_2[H_3BCO_2]$ were placed in a 10 mL glass vial. The vial was sealed with an aluminum capped rubber stopper and flushed with nitrogen for 5–10 min using a needle to balance the pressure. Finally, 1 mL of carrier-free Na[^{188}ReO_4] previously mixed with 7 μL of H_3PO_4 (85%) was added to the vial and incubated at 60–75 °C for 15–20 min. A 20 mL syringe was used to keep the balance of H_2 gas formed during the reaction. Both colloidal and free ^{188}Re could be removed using a small Sep-Pak column (Plus QMA; Waters Co.) or by HPLC.

2.2.2. General procedure for the synthesis of fac-[^{188}Re$(CO)_3(k^3$-L)] (L = L2 − L4): ^{188}Re2-^{188}Re4

In a nitrogen-purged glass vial, 100 μL of a 10^{-3} M aqueous solution of the appropriate ligand (**L2-L4**) was added to 900 μL of a solution of the precursor *fac*-[^{188}Re$(CO)_3(H_2O)_3$]$^+$ previously prepared. Both purified and non-purified precursors were used. The reaction mixture was then heated to 100 °C for 30–45 min, cooled on an ice bath and then purified by Sep-Pak C18 using saline solution and ethanol as eluents. The radiochemical purity was evaluated by HPLC and also by ITLC-SG using as mobile phase methanol/12 M HCl (99/1, v/v) and methylethylketone.

2.3. In vitro studies

2.3.1. In vitro stability

The ^{188}Re-labeled complexes (**^{188}Re3-^{188}Re4**, 100 μl, ≈10 MBq) were added to 1 mL of PBS pH = 7.4 or in cell culture medium (DMEM). The mixture was incubated at 37 °C and the radiochemical purity was evaluated by ITLC-SG and HPLC at different time points (2, 4, 24 and 48 h).

2.3.2. Hydroxyapatite (HA) binding

The HA-binding studies of **^{188}Re3-^{188}Re4** were performed based on a modified reported procedure [35]. Briefly, 50 μL of each complex (~27.8 μCi/25 μL) was incubated at 37 °C with 15 mg of solid HA and 500 μL of phosphate buffered saline (PBS) solution (pH 7.2). Samples were collected at different time points for (1, 2, 4 and 17 h) and the liquid and solid phases were separated by centrifugation (7500 rpm/5 min). The solid phase was washed twice with 500 μL of PBS pH 7.2. The activity in the liquid and solid phases was determined using an ionization chamber.

2.4. Biodistribution studies

The biodistribution of the complexes was evaluated in groups of 4–5 adult Balb/c female mice (Charles River) weighing approximately 15 g each. The animals were injected intravenously with 100 μL (3–10 MBq) of each preparation via the tail vein and were maintained on normal diet ad libitum. All animal studies were conducted in accordance with the highest standards of care, as outlined in European law. Mice were killed by cervical dislocation at 1 and 4 h after injection. The injected radioactive dose and the radioactivity remaining in the animal at sacrifice were measured with a dose calibrator (Capintec, CRC25R). The difference between the radioactivity in the injected and the sacrificed animal was assumed to be due to total excretion from whole animal body. Blood samples were taken by cardiac puncture when the animals were killed. Tissue samples of the main organs were removed, weighed and counted using a gamma counter (Berthold, LB211). Biodistribution results were expressed as the percentage of the injected activity (I.A.) per gram of tissue. Statistical analysis of the biodistribution data (*t*-test) was done with GraphPad Prism and the level of significance was set as 0.05.

2.4.1. In vivo stability

The stability of the complexes was assessed in urine and murine serum by RP-HPLC analysis under the analytical conditions described above to test the original radiolabeled complexes. The samples were taken at the sacrifice time 1 h post injection. The urine collected was filtered through a Millex GV filter (0.22 μm) before RP-HPLC analysis. Blood collected from the mice was centrifuged at 3000 rpm for 15 min and the serum was separated. The serum was treated with cold ethanol in a 1:2 (v/v) ratio to precipitate the proteins. After centrifugation at 3000 rpm for 15 min at 4 °C, the supernatant was collected, filtered through a Millex GV filter (0.22 μm) and analysed by RP-HPLC and ITLC-SG.

2.5. Cellular uptake assays

The MDAMB231 cells were seeded in 24-well plates at a density of 2.5×10^5 cells in 0.5 mL medium (DMEM + Glutamax I supplemented

with 10% FBS and 1% antibiotics) in a humidified 5% CO_2 atmosphere at 37 °C and allowed to attach overnight. After that, the medium was removed and the cells were incubated for 0.5 h to 24 h with ~5 × 10^5 cpm (~30 kBq) of the radioactive complex in 0.5 mL medium at 37 °C. Incubation was ended by discarding the medium and washing the cells with a fresh one followed by a two steps washing procedure with cold PBS. Subsequently, the cells were lysed with 1 N NaOH during 10 min incubation at 37 °C to evaluate the cellular associated radioactivity at different time points over the 24 h incubation period. The radioactivity in the medium, PBS and cell lysates were separately collected and counted in a γ-counter. Cellular uptake data were based on three determinations for each time point and were expressed as a percentage of total applied radioactivity (mean ± SD).

2.6. Radiocytotoxicity assays

MDAMB231 cells were seeded in 96-well plates at a density of ~10^3 cells in 0.2 mL of DMEM supplemented with 10% FBS and 1% antibiotics and allowed to attach overnight in a humidified 5% CO_2 atmosphere at 37 °C. Next day, the medium was replaced by 0.2 mL of a dilution series of the radioactive compounds in medium (0.05–14 MBq) and cells were incubated for more 34 h. At the end of incubation period, the radioactive compounds were removed and the cells were incubated with 0.2 mL of a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) solution (0.5 mg/mL in PBS) for additional 3–4 h at 37 °C, 5% CO_2. The purple formazan crystals formed inside the cells were then dissolved in 0.2 mL of DMSO by thorough shaking, and the absorbance was read at 570 nm, using a plate spectrophotometer (Power Wave Xs, BioTek). The radiocytotoxic effect of the compounds was expressed as the percentage of viable cells related to the cells present in the wells to which only medium was added.

2.7. Micronuclei assays

MDAMB231 cells were seeded in 25 mL culture flasks at a density of ~10^6 cells in 5 mL of complete DMEM and allowed to attach overnight in the incubator at 37 °C. The medium was replaced by the radioactive compound in medium (26 MBq/5 mL) and cells were incubated for another 34 h. After incubation, a series of wash steps in medium was followed before the micronuclei assays.

The number of micronuclei (MN) was determined by the cytokinesis blocked micronuclei assay. Briefly, 2 mg/mL cytochalasin-B was added to the medium 20 h after irradiation to arrest cytokinesis and cells were cultured for more 24 h. After that, cells were subjected to a hypotonic treatment (RPMI medium, water and FBS); cells were harvested, centrifuged (800 rpm, 10 min) and then re-suspended twice in the wash solution (RPMI medium, water and FBS) and again centrifuged (800 rpm, 8 min). The cells were fixed during 20 min in cold methanol:acetic acid (3:1). Slides were prepared and stained in a solution of 4% Giemsa dye in phosphate buffer (pH 6.8) for 8 min. The slides were coded and scored under a light microscope at 400× magnification. MN were identified according to a previously described method [36]. The frequency of binucleated (BN) cells containing one or more MN was also scored.

3. Results and discussion

3.1. Radiosynthesis of complexes of the type fac-[^{188}Re(CO)$_3$(k^3-L)] (^{188}Re2, L = L2; ^{188}Re3, L = L3; ^{188}Re4, L = L4)

The organometallic precursor *fac*-[^{188}Re(CO)$_3$(H_2O)$_3$]$^+$ used for the radiosynthesis of complexes **^{188}Re2**-**^{188}Re4** was prepared by direct reduction of sodium perrhenate ([^{188}ReO_4]$^-$), eluted from a ^{188}W/^{188}Re generator (ITG) with 0.9% saline solution (Fig. 2), under optimized reaction conditions. Owing to its high reducing properties and stability under neutral/acidic conditions the water-soluble aminoborane $BH_3 \cdot NH_3$ was selected as reducing agent and potassium boranocarbonate ($K_2[H_3BCO_2]$) was used as both reducing agent and solid source of CO, according to a reported procedure [34]. The amount of boranes and acid used was carefully balanced to avoid fast hydrolysis of the boranes and to maintain a neutral pH to stabilize reduced rhenium intermediates.

After optimization of the stoichiometric ratio of reagents and labeling conditions such as temperature, total volume and reaction time, *fac*-[^{188}Re(CO)$_3$(H_2O)$_3$]$^+$ was obtained in relatively high yield (80–95%) based on ITLC-SG and RP-HPLC analysis (ret. time = 5.0 min,). The main radiochemical impurities identified in the HPLC γ-trace were [^{188}ReO_4]$^-$ (ret. time = 10.8 min) and a non-identified radiochemical impurity with a retention time around 14 min. This impurity disappears by decreasing the pH to 2–3 and seems to be related with the amount of $BH_3 \cdot NH_3$ used in the reaction. The presence of [^{188}ReO_2] or other colloidal species was evaluated by ITLC-SG using methanol/12 M HCl (99/1, v/v) as mobile phase. Under these analytical conditions [^{188}ReO_2] remains at the origin where less than 10% of the total radioactivity was typically found, and *fac*-[^{188}Re(CO)$_3$(H_2O)$_3$]$^+$ appears as a single peak with R_f = 0.5. It is also worth mentioning that after purification we only observed a well-defined peak related to the precursor *fac*-[^{188}Re(CO)$_3$(OH_2)$_3$]$^+$, in opposition to the literature where this precursor appears as two broad peaks using similar analytical conditions [37,38].

The stability of non-purified precursor *fac*-[^{188}Re(CO)$_3$(H_2O)$_3$]$^+$ was evaluated at room temperature, and an impressive reoxidation to [^{188}ReO_4]$^-$ was observed after 4 h (Fig. 3**A**). Furthermore, addition of a radical scavenger such as ascorbic acid did not improve the *in vitro* stability of the precursor. Purification of *fac*-[^{188}Re(CO)$_3$(OH_2)$_3$]$^+$ by anionic exchange cartridges or RP-HPLC led to a significant improvement of its radiochemical purity (>95%) and, more interestingly, enhanced extensively the *in vitro* stability, even after 24 h at room temperature (Fig. 3**B**).

The complexes **^{188}Re2**-**^{188}Re4**, analogs of the γ-gamma emitting complexes **Tc2-Tc4** and cold surrogates **Re2-Re4** (Fig. 1), were synthesized by reacting the corresponding bifunctional ligands **L2-L4**, prepared according to previously described procedures [23,24], with purified *fac*-[^{188}Re(CO)$_3$(H_2O)$_3$]$^+$. Complexes **^{188}Re3** and **^{188}Re4** were prepared in high yield (>85%) after 30 min or 45 min, respectively, at 100 °C. On the contrary, despite our efforts to optimize the radiolabeling conditions, complex **^{188}Re2** was always obtained in low yield (<20%). Moreover, after purification by Sep-Pak C18 cartridge, **^{188}Re3** and **^{188}Re4** were obtained with high radiochemical yield, high radiochemical purity (>95%) and high specific activity, whereas **^{188}Re2** could not be obtained as a pure radiochemical species. Indeed, the latter complex was always contaminated with Na[^{188}ReO_4] as well as with further unidentified radiochemical species,

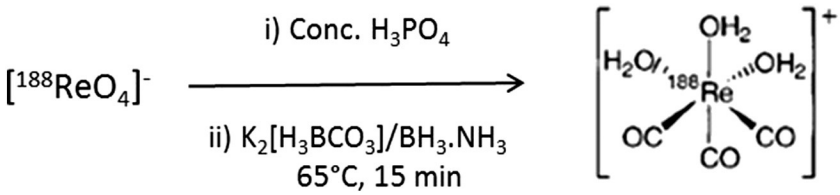

Fig. 2. Radiosynthesis of the organometallic precursor *fac*-[^{188}Re(CO)$_3$(H_2O)$_3$]$^+$.

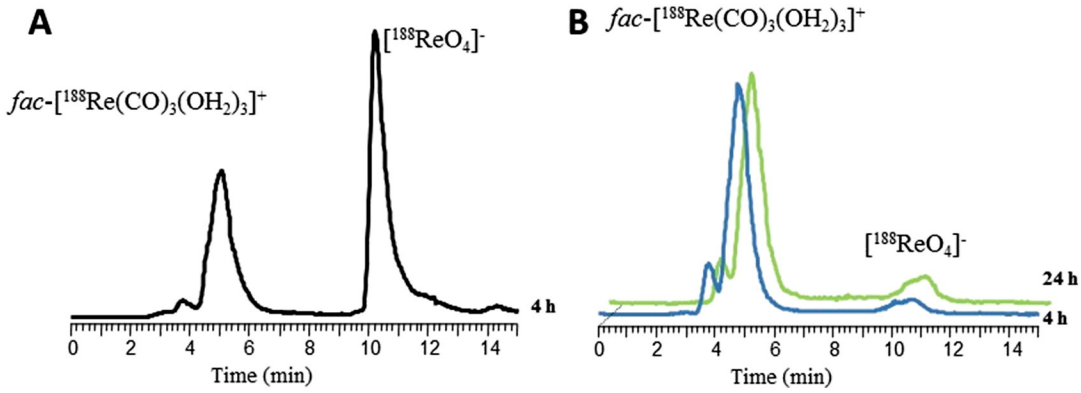

Fig. 3. Stability of *fac*-[^{188}Re(CO)$_3$(OH$_2$)$_3$]$^+$ (ret. time = 5.0 min) at room temperature; **A** – Non-purified precursor; **B** – purified precursor.

indicating instability. The chemical identity of the radioactive complexes has been ascertained by comparing their analytical RP-HPLC γ-traces with RP-HPLC UV-vis traces of the surrogate, non-radioactive, rhenium compounds **Re2**-**Re4**, prepared and characterized as previously described [23,24]. The results showed that **^{188}Re3** and **^{188}Re4** were obtained as single molecular species in contrast to ^{188}Re-HEDP.

3.2. In vitro stability of ^{188}Re3 and ^{188}Re4

In order to accomplish significant therapeutic efficacy, the **^{188}Re** complexes must be stable *in vivo* and accumulate in the target organ as long as possible. However, one of the major drawbacks associated to the use of radioactive rhenium compounds for systemic therapy is related with their higher tendency to re-oxidize to [^{188}ReO$_4$]$^-$ than the ^{99m}Tc analogs. Actually, one of the main disadvantages of ^{188}Re-HEDP is related with its low stability both *in vitro* and *in vivo*[8]. Therefore, we evaluated the *in vitro* stability of complexes **^{188}Re3** and **^{188}Re4** upon incubation with phosphate-buffered saline (PBS) pH 7.4 and in cell culture media at 37 °C up to 24 h. Analysis of samples collected at various time points by RP-HPLC and ITLC-SG demonstrated that **^{188}Re3** presented higher stability than **^{188}Re4**. In fact, some reoxidation of **^{188}Re4** to [^{188}ReO$_4$]$^-$ was observed (10% and 30% at 2 h and 24 h, respectively) whereas no relevant degradation of **^{188}Re3** was found over this incubation time both in PBS pH 7.4 and culture media. This is an important feature when comparing with the approved bone agent ^{188}Re-HEDP that according to the literature undergoes fast reoxidation to [^{188}ReO$_4$]$^-$ in PBS pH 7.4 at 37 °C (after 24 h more than 50% was already oxidized) [37].

3.3. Hydroxyapatite absorption studies

Aiming to ascertain the *in vivo* bone-targeting properties of the **^{188}Re** complexes and to assess their potential as bone therapeutic agents, we have performed hydroxyapatite (HA) adsorption studies (15 mg HA, 37 °C) with complexes **^{188}Re3** and **^{188}Re4**. The results were compared with those obtained previously for the ^{99m}Tc analogs **Tc3** and **Tc4** [23,24]. A high and fast hydroxyapatite binding was observed for complex **^{188}Re3** (Fig. 4), in agreement with the results observed for the congener **Tc3**[23]. However, a slightly different behavior was observed for **^{188}Re4**. Despite presenting relatively high binding affinity to HA the value decreased over time, which can be explained by the lower *in-vitro* stability of **^{188}Re4** (Fig. 4).

Brought together, these results prompted us to assess the *in vivo* bone seeking properties of both radioactive complexes **^{188}Re3** and **^{188}Re4**.

3.4. Biodistribution studies

As stated above, one of the major concerns related with the radioactive rhenium compounds is their tendency to re-oxidize *in vivo* to [^{188}ReO$_4$]$^-$, which might impair their use as therapeutic agents. Taking this into account, a preliminary comparative biodistribution study was performed in mice at 1 h post intravenous injection (p.i.). The results have shown that unlike **^{188}Re3**, complex **^{188}Re4** exhibited much lower bone uptake (1.0 ± 0.2% I.A./g) than that observed previously for the corresponding **Tc4** congener (17.7 ± 3.0% I.A./g), which is most likely related with the *in vivo* instability of **^{188}Re4**. This assumption was corroborated by the relatively high level of radioactivity found in the stomach (26.4 ± 10.9% I.A./g stomach), which has been attributed to accumulation of [^{188}ReO$_4$]$^-$ resulting from re-oxidation of the original metal complex.

Complete biodistribution studies with **^{188}Re3** were carried out in normal Balb/c mice at 1 h and 4 h p.i. to assess the pharmacokinetic profile, bone affinity and *in vivo* stability, and the results for the most relevant organs are summarized in Table 1. For the sake of comparison, we also present the tissue distribution profile of the analog **Tc3** [23] in the same animal model. The tissue uptake was calculated and expressed as a percentage of the injected activity per gram of tissue (% I.A./g ± SD). The whole-body radioactivity excretion was determined as a percentage of the total injected activity.

Analysis of data indicated that **^{188}Re3** has an overall biodistribution profile similar to that of **Tc3** with a fast blood clearance via both the hepatic and the renal pathways and a moderate total radioactivity excretion, superimposing the results found for the ^{99m}Tc congener. The most remarkable feature of **^{188}Re3** is the rapid accumulation and long retention time in the bone (16.1 ± 3.3% I.A./g at 1 h p.i.) even using a carrier-free formulation, in agreement with the results found for **Tc3** (17.1 ± 3.6%, I.A./g at 1 h p.i.). In contrast to **^{188}Re3**, addition of carrier perrhenate to the formulation of ^{188}Re-HEDP is needed to achieve

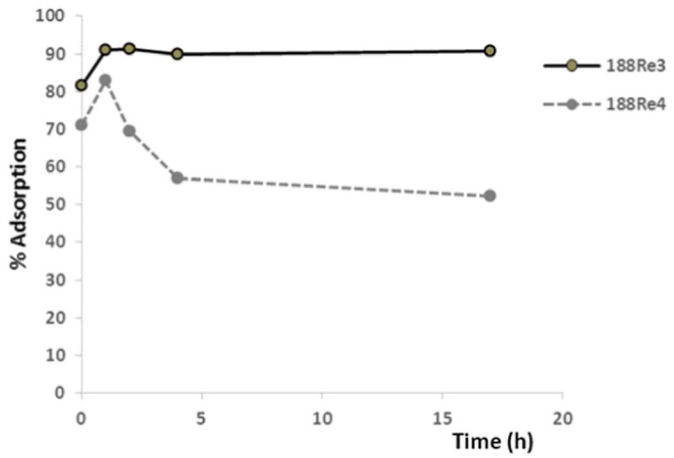

Fig. 4. Adsorption of complexes **^{188}Re3** and **^{188}Re4** onto HA (15 mg) at 37 °C as a function of incubation time.

Table 1
Biodistribution of **^{188}Re3** and **Tc3** in normal adult Balb/c mice (n = 4–5) at 1 h and 4 h p.i. (% I.A./g).

Tissue	% I.A./g ± SD			
	Tc3[a]		^{188}Re3	
	1 h	4 h	1 h	4 h
Blood	0.21 ± 0.05	0.07 ± 0.01	0.46 ± 0.07	0.17 ± 0.02
Liver	4.9 ± 0.7	4.0 ± 0.5	6.9 ± 0.8	6.5 ± 0.7
Intestine	0.49 ± 0.07	0.3 ± 0.2	2.1 ± 0.2	0.6 ± 0.1
Kidney	1.3 ± 0.2	1.1 ± 0.3	1.5 ± 0.2	1.3 ± 0.2
Muscle	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.30 ± 0.02
Bone	17.1 ± 3.6	14.3 ± 2.3	16.1 ± 3.3	13.9 ± 0.9
Stomach	0.5 ± 0.3	0.19 ± 0.09	2.8 ± 0.6	0.5 ± 0.2
Total excretion (% I.A.)	59.5 ± 4.0	67.3 ± 4.1	50.5 ± 4.5	63.6 ± 3.0

[a] From Ref. [23].

high bone accumulation [9,39]. In the case of ^{186}Re-HEDP, the formulation is carrier-added by virtue of the production mode of ^{186}Re. Indeed, the presence of macroscopic amounts of stable perrhenate in $^{186/188}$Re-HEDP preparations has a tremendous impact in their biodistribution and intravenous stability. The carrier-free **^{188}Re3** does not undergo significant re-oxidation *in vivo* to $[^{188}ReO_4]^-$ as demonstrated by the low radioactivity accumulation in stomach (2.8 ± 0.6 I.A./g and 0.5 ± 0.2% I.A./g stomach at 1 h and 4 h p.i., respectively).

The high bone uptake of **^{188}Re3** associated to the fast clearance from blood stream and soft tissues like muscle resulted in a significantly highly bone-to-blood (34.8 ± 3.4 and 84.2 ± 4.5 at 1 h and 4 h, respectively) and bone-to-muscle (39.8 ± 7.2 and 46.4 ± 2.5 at 1 h and 4 h, respectively) radioactivity ratios that increase with time (Fig. 5), confirming the ability of the complex to accumulate in areas of metabolically active bone.

Brought together, the biodistribution studies demonstrated that **^{188}Re3** is able to deliver radiation to bone in a very selective way, with low accumulation in sensitive, non-target, organs such as the kidneys.

3.5. Cell based studies

MDAMB231 human breast cancer cell line is a model for triple negative breast cancer (ER −, PR − and HER2 −) with a poor outcome compared to the other subtypes of breast cancer cells [40]. Considering that MDAMB231 cell line is known to induce bone metastases, we have selected this cell line for the cellular uptake studies and the radiotoxic effects.

3.5.1. Cellular uptake

The results of the cellular uptake as a function of incubation time are presented in Fig. 6. **^{188}Re3** presents a maximal uptake (ca. 2.2%) at 6 h incubation, which is slightly lower than the value found for its congener **Tc3** at the same incubation time (ca. 4%) under the same experimental conditions [23]. However, unlike **Tc3** that is rapidly externalized (ca. 1.5%, 24 h) the uptake value for **^{188}Re3** is kept constant at least up to 24 h. This is an interesting finding if we consider that the radiocomplex should be retained in the bone microenvironment as well as enter into the tumor cells to elicit its radiocytotoxic effect with a higher efficacy.

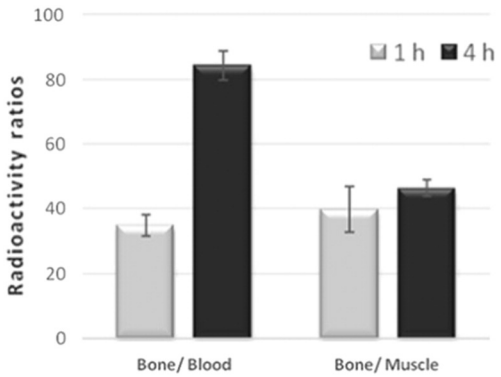

Fig. 5. Bone-to-blood and bone-to-muscle radioactivity ratios of **^{188}Re3** in Balb/c mice at different time points p.i.

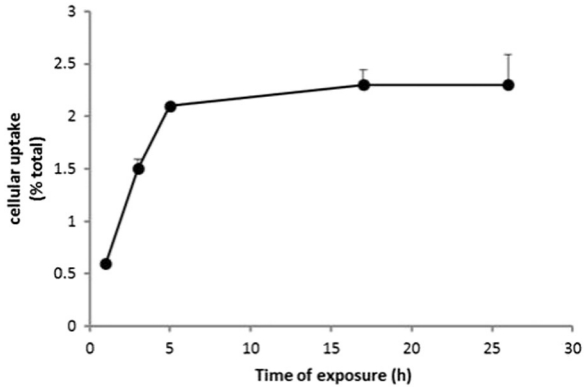

Fig. 6. The cellular uptake of **^{188}Re3** at 37 °C in the MDAMB231 breast cancer cells over the time.

3.5.2. Radiocytotoxicity

We performed radiotoxicity studies aiming to determine the radiosensitivity of MDAMB231 cells to **^{188}Re3** and to the control compound $[^{188}ReO_4]^-$ following a reported experimental procedure [41]. Briefly, the cells were incubated in media containing different radioactive concentrations of the radioactive compounds (0.05–14 MBq/200 μL) and, after 36 h incubation (equivalent to 2 half-lives), the cellular viability was determined by the MTT colorimetric assay (Fig. 7). At radioactive concentrations above 1.8 MBq/200 μL considerable loss of cellular viability was observed for **^{188}Re3**. At the same concentrations, and even at higher doses, **$[^{188}ReO_4]^-$** did not display any radiocytotoxic effect, suggesting the inability of this compound to cross cell membranes and enter the cells [41].

Moreover, cytotoxicity studies performed with the inactive complex **Re3** and respective non-coordinated ligand (**L3**) revealed that none of the molecules presented cytotoxic effect even at very high doses (IC_{50} > 200 μM). These results suggested that the radiotoxic effect of **^{188}Re** was due solely to the β^- emission of the radionuclide.

3.5.3. Genotoxicity

Ionizing radiation produces many types of DNA lesions of different complexity, with the cellular damage depending on the dose, radiation quality (*e.g.*, high/low LET) and radionuclide uptake.

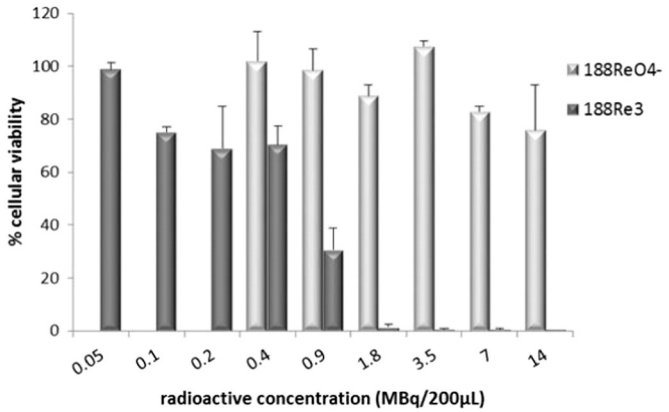

Fig. 7. The radiocytotoxic effect of **^{188}Re3** and **$[^{188}ReO_4]^-$** in MDAMB231 breast cancer cells as a function of radioactive concentration (0.05–14 MBq/200 μL). The cellular viability was determined by the MTT assay after 34 h incubation.

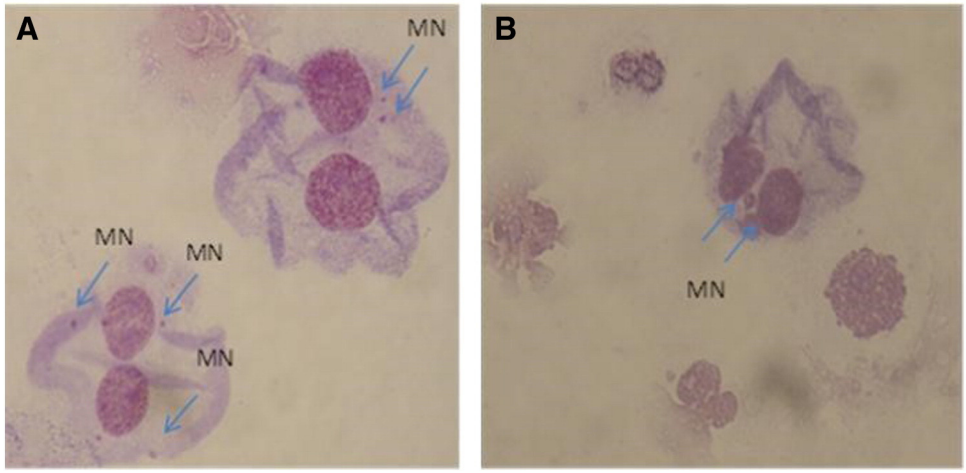

Fig. 8. The genotoxic effect of **^{188}Re3** (0.9 MBq/0.2 mL) evaluated by the cytokinesis-blocked micronucleus (CBMN) assay in the MDAMB231 cells. **A**) two binucleated (BN) cells with induced micronuclei (MN); **B**) non-viable cells, for MN scoring, with loss of membrane integrity and changes in nuclear morphology.

The genotoxicity effect of **^{188}Re3** was evaluated by the cytokinesis-blocked micronucleus (CBMN) assay in MDAMB231 breast cancer cells. MN are fragments of genetic material that contain acentric fragments (resulting from DNA breaks), whole chromosomes, or complex rearrangements that are unable to properly attach and be pulled to the poles by the mitotic spindle. As a result the induced DNA damage can be quantified by scoring the number of MN in the cytoplasm formed during anaphase [42,43]. In brief, cells were incubated with the compound at two different radioactive concentrations (0.4 and 0.9 MBq/0.2 mL) during 36 h at 37 °C. Subsequently, cytochalasin-B an inhibitor of actins was added to the medium 20 h after irradiation to arrest cytokinesis. Cytochalasin-B arrests cells in binucleated (BN) state by permanently blocking them at the G2/M cell cycle. Cells are generally incubated with cytochalasin-B for approximately 1–2 cell cycle times in order to gather the majority of the cells at the binucleated state.

In agreement with the results from the cellular viability assays, our results suggest an increased number of MN, per BN cell for both 0.4 and 0.9 MBq, when compared to the control, giving evidence for complex DNA damage in cells not undergoing either apoptosis or necrosis (Fig. 8).

Moreover, in cells exposed to 0.9 MBq it was observed an increase of the cellular damage expressed by the occurrence of 2 and 3 MN per BN cell (Fig. 9).

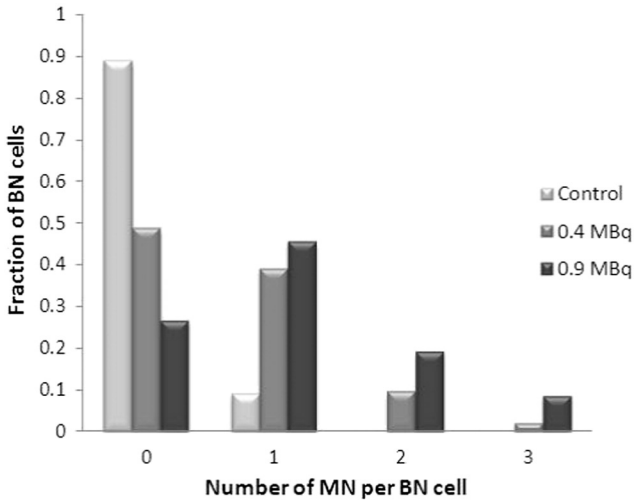

Fig. 9. Distribution of MN per 500 BN MDAMB231 cells exposed to different radioactive concentrations of **^{188}Re3**, 0.4 MBq and 0.9 MBq/0.2 mL. The control refers to non-irradiated cells.

4. Conclusions

We have successfully synthesized and characterized a set of new ^{188}Re tricarbonyl complexes (**^{188}Re2**, **^{188}Re3** and **^{188}Re4**) by reaction of the corresponding bisphosphonate-containing ligand (**L2**, **L3** and **L4**, respectively) with the precursor *fac*-$[^{188}Re(CO)_3(H_2O)_3]^+$. The most promising complex **^{188}Re3** can be easily obtained with high specific activity, high radiochemical yield and purity. Unlike the clinically tested bone-seeking agents $^{186/188}$Re-HEDP, **^{188}Re3** is obtained as a single molecular species that has been fully characterized and showed high *in vitro* and *in vivo* stability. In contrast to $^{186/188}$Re-HEDP that only the carrier-added composition accumulates in bone, the carrier-free formulation of **^{188}Re3** presents a high bone uptake, and high bone-to-blood and bone-to-muscle radioactivity ratios indicating that **^{188}Re3** is able to deliver radiation to bone microenvironment in a very selective way being an attractive candidate for further preclinical evaluation for systemic radionuclide therapy of bone metastases.

The genotoxic effect of **^{188}Re3** evaluated by the CBMN assay in the MDAMB231 breast cancer cells showed morphological changes in the nucleus and in the cytoplasmic membrane and most of the viable cells showed an increase number of MN per BN cell indicating DNA damage in cells not undergoing either apoptosis or necrosis. Furthermore, **^{188}Re3** presents a radiotoxic effect, which is much higher than that elicited by **$[^{188}ReO_4]^-$** at similar radioactive concentrations.

Acknowledgments

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