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RESEARCH ARTICLE

In situ Generated ^{212}Pb-PSMA Ligand in a ^{224}Ra-Solution for Dual Targeting of Prostate Cancer Sclerotic Stroma and PSMA-Positive Cells

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Abstract: ***Background:*** New treatments combating bone and extraskeletal metastases are needed for patients with metastatic castration-resistant prostate cancer. The majority of metastases overexpress prostate-specific membrane antigen (PSMA), making it an ideal candidate for targeted radionuclide therapy.

Objective: The aim of this study was to test a novel liquid ^{224}Ra/^{212}Pb-generator for the rapid preparation of a dual-alpha targeting solution. Here, PSMA-targeting ligands are labelled with ^{212}Pb in the ^{224}Ra-solution in transient equilibrium with daughter nuclides. Thus, natural bone-seeking ^{224}Ra targeting sclerotic bone metastases and ^{212}Pb-chelated PSMA ligands targeting PSMA-expressing tumour cells are obtained.

Methods: Two PSMA-targeting ligands, the p-SCN-Bn-TCMC-PSMA ligand (NG001), specifically developed for chelating ^{212}Pb, and the most clinically used DOTA-based PSMA-617 were labelled with ^{212}Pb. Radiolabelling and targeting potential were investigated *in situ*, *in vitro* (PSMA-positive C4-2 human prostate cancer cells) and *in vivo* (athymic mice bearing C4-2 xenografts).

Results: NG001 was rapidly labelled with ^{212}Pb (radiochemical purity >94% at concentrations of ≥15 µg/ml) using the liquid ^{224}Ra/^{212}Pb-generator. The high radiochemical purity and stability of [^{212}Pb]Pb-NG001 were demonstrated over 48 hours in the presence of ascorbic acid and albumin. Similar binding abilities of the ^{212}Pb-labelled ligands were observed in C4-2 cells. The PSMA ligands displayed comparable tumour uptake after 2 hours, but NG001 showed a 3.5-fold lower kidney uptake than PSMA-617. Radium-224 was not chelated and, hence, showed high uptake in bones.

Conclusion: A fast method for the labelling of PSMA ligands with ^{212}Pb in the ^{224}Ra/^{212}Pb-solution was developed. Thus, further *in vivo* studies with dual tumour targeting by alpha-particles are warranted.

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1. INTRODUCTION

Radiopharmaceuticals have recently gained significant attention in the treatment of metastatic cancer [1]. One alpha-emitting and several beta-emitting radiopharmaceuticals have been approved for cancer therapy in the last decade [1]. Alpha-emitting radionuclides are particularly attractive as effector arms due to their short range (< 0.1 mm) in tissue and high-linear energy transfer (LET) properties, allowing the generation of strong and irreparable cell damage [2, 3]. However, there are only a few alpha emitters suitable for routine clinical use [3-6]. Two of the most promising nuclides in terms of half-life, decay properties and large-scale manufacturing potential, ^{223}Ra ($T_{1/2}$≈11.4 days) and ^{224}Ra ($T_{1/2}$≈3.7 days), are not suitable for conjugation to targeting molecules [7, 8]. Their use as natural bone-seekers has been limited to the targeting of osteosclerotic disease [9-11]. Bismuth-212 ($t_{1/2}$≈60.6 min) can be produced in large quantities [4]. Unfortunately for clinical applications, the short half-life of ^{212}Bi and the presence of high-energy gamma emission from ^{208}Tl in the decay chain (Figs. **1** and **2A**, Supplementary Table **S1**) make this nuclide impractical to use. Therefore, several research groups have been looking into the use of its longer-lived mother nuclide, ^{212}Pb ($t_{1/2}$≈10.6 h, Figs. **1** and **2B**) as a targetable *in vivo* generator of alpha particles [12-16]. Lead-212 is a beta emitter that produces alpha parti-

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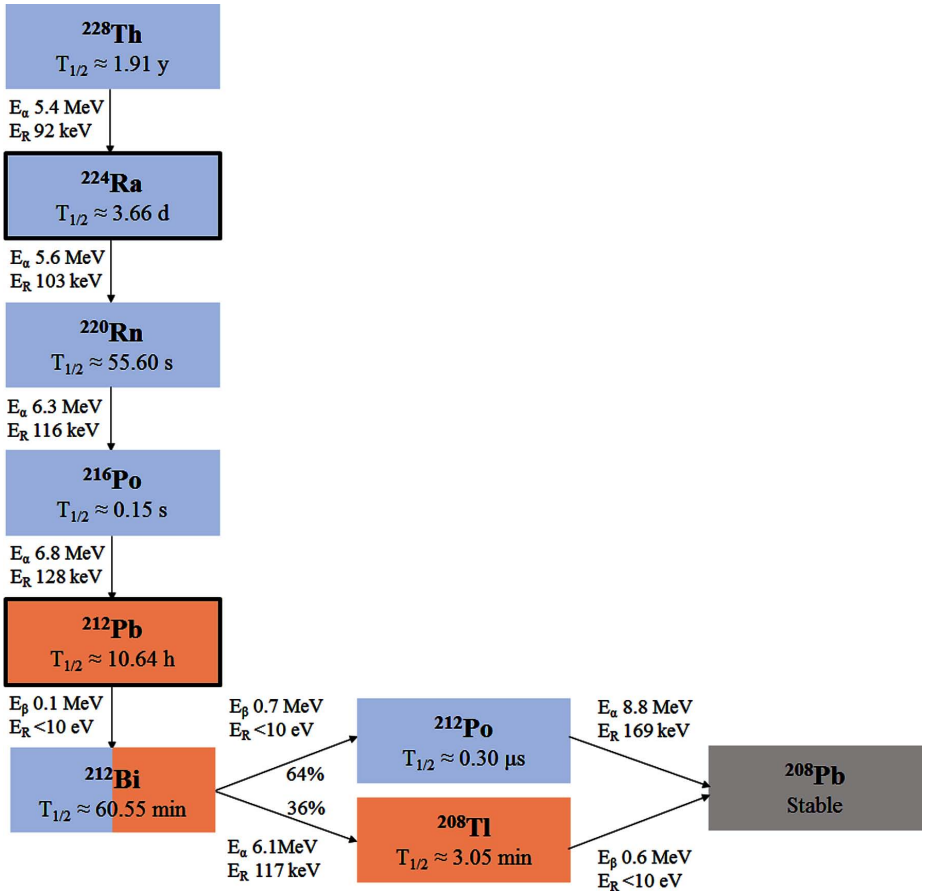

Fig. (1). The decay chain of ^{228}Th to stable ^{208}Pb. Alpha-emission energies (E_α), beta-emission energies (E_β), recoil energies (E_R) and the half-lives for ^{228}Th and daughter nuclides are specified [21]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

cle radiation via its short-lived progenies ^{212}Bi and ^{212}Po ($t_{1/2}$≈0.3 µs). It should be noted that the needed activity levels of ^{212}Pb would be much less than for a ^{212}Bi-based product [17]. This is because 1 Bq of ^{212}Pb would produce about 10 times the amount of alpha rays if integrated over its lifetime as compared to 1 Bq of ^{212}Bi. Lead-212 has suitable properties in terms of chelation chemistry, and a highly effective bifunctional chelator, S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza-1,4,7,10-tetra(2-carbamoylmethyl)cyclododecane (TCMC), was developed specifically for this radionuclide [13, 18, 19]. Lead-212 can also be chelated by the versatile S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) bifunctional chelator [13, 18, 19]. Furthermore, ^{212}Pb can be obtained at an industrial scale from the ^{224}Ra generator using ^{228}Th ($t_{1/2}$≈1.9 years) as a long-term generator [5-6, 17]. It is isolated in high yield and with high purity using, for example, actinide selective resin for immobilising or to catch ^{228}Th while ^{224}Ra is mobilised by HCl or HNO_3 solutions [17]. Disadvantages include a non-optimal half-life of ^{212}Pb, making centralised production and long-distance shipment difficult and impractical, and the considerable gamma rays associated with progenies. Another potential problem with ^{212}Pb is the retention factor of daughter nuclides in the chelator after decay. The majority of ^{212}Pb atoms are bound to the TCMC or DOTA chelator, but about one-third of ^{212}Bi atoms could dissociate from the chelator after ^{212}Pb decay [17, 20]. It is indicated that the use of ^{212}Pb for developing radiotherapeutics would require a reliable method for supplying the radionuclide from a centralised production facility to the end user and a relatively rapid and simple procedure for preparing the radiopharmaceutical injectate for administration to patients.

In recent preclinical and clinical studies, ^{212}Pb was eluted with concentrated HCl from a ^{224}Ra/^{212}Pb-generator (Fig. **3A**), as previously described by Baidoo *et al*. [19], where ^{224}Ra is absorbed on a resin obtained from Orano Med (formerly Areva Med, Bessines-sur-Gartempe, France) or Oak Ridge National Laboratory (Oak Ridge, Tennessee, USA) [12, 15, 23, 24]. After the evaporation of the generator eluate, the residue is digested with concentrated HNO_3 followed by the extraction of ^{212}Pb with dilute HNO_3 (Fig. **3A**). The ^{224}Ra/^{212}Pb-generator facilitates the on-site production of ^{212}Bi and ^{212}Pb suitable for the radiolabelling of targeting ligands or monoclonal antibodies (mAb). This is in contrast to a ^{212}Pb-chelated ligand or mAb prepared outside the hospital that must be delivered to the hospital for final use within a few hours after its preparation due to the short half-life [23].

A simple and time-efficient method of producing ^{212}Pb-labelled radioimmunoconjugates for the end user was suggested by Westrøm *et al*. [17]. The novel method presented here involves the *in situ* labelling of a tumour-targeted ligand

using a ^{224}Ra/^{212}Pb-liquid generator, in which ^{224}Ra is in transient equilibrium with daughter nuclides (Fig. **2C**). The proposed procedure (Fig. **3B**) may be advantageous compared to the ^{224}Ra-based generator described by Baidoo *et al*. [19] (Fig. **3A**) since the steps involving the handling and evaporation of concentrated acid solutions with high radioactivity levels can be avoided in the hospital, thereby reducing the on-site preparation time that only involves radiolabelling (~30 min). After ^{212}Pb-labelling, the removal of ^{224}Ra may be performed by simple desalting gel exclusion separation (~15 min) [17].

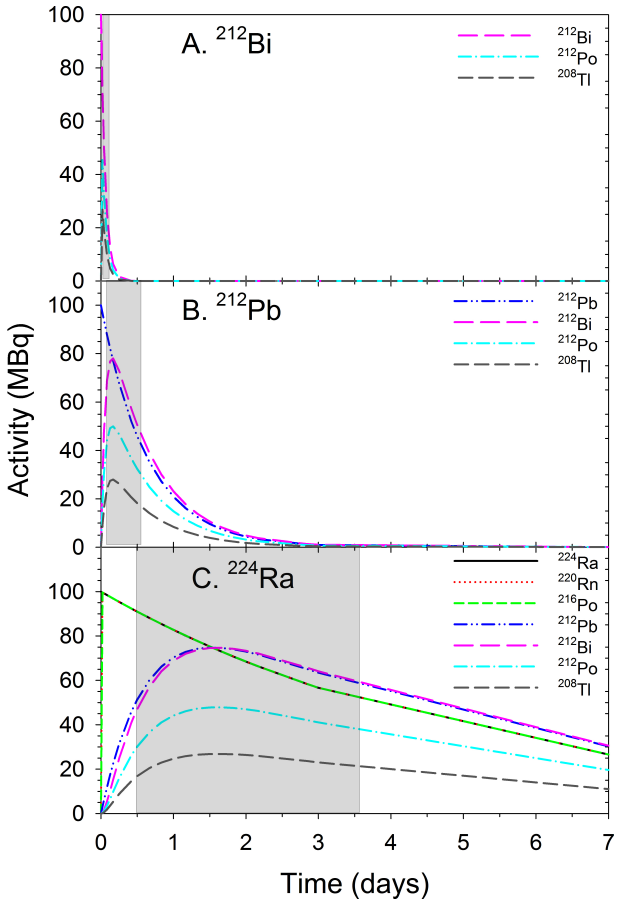

Fig. (2). The decay of pure ^{212}Bi (**A**) and ^{212}Pb (**B**) and in-growth/decay of their progenies. (**C**) Ingrowths and decays of ^{212}Pb and other progenies from ^{224}Ra in a liquid generator system starting with a pure ^{224}Ra source [22]. Grey areas present time frames for the suitable clinical use of the radiopharmaceuticals. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Prostate cancer is the second most common cancer diagnosed in men worldwide [25]. The prostate-specific membrane antigen (PSMA) is highly expressed in androgen-independent prostate cancer and exhibits only limited expression in normal tissues [26]. Thus, PSMA presents both a therapeutic and diagnostic target in patients with metastasised castration-resistant prostate cancer (mCRPC) [27-29]. A series of small molecular PSMA ligands have been synthesised and radiolabelled with ^{177}Lu, ^{225}Ac and ^{213}Bi [27, 28]. Radioligand therapy (RLT) with [^{177}Lu]Lu-PSMA-617 or [^{177}Lu]Lu-PSMA-I&T has shown high response rates, pain reduction and a favourable safety profile in patients with mCRPC [29-32]. However, about 30% of patients do not respond to beta-emitting [^{177}Lu]Lu-PSMA therapy [33-35]. Recent clinical studies have reported that targeted alpha therapy (TAT) using ^{225}Ac- and ^{213}Bi-labelled PSMA ligands has a strong anticancer activity in mCRPC patients and, importantly, also in some cases in patients failing to respond to ^{177}Lu-based PSMA RLT [29, 33, 34, 36, 37]. The high cytotoxicity including a high degree of non-re-joining DNA double-strand breaks and low oxygen enhancement ratio of high-LET radiation from alpha particles, may account for the increased anti-tumour response observed [2, 3, 38]. Unfortunately, the current supply situation for ^{225}Ac and ^{213}Bi may limit the use of these radionuclides for TAT [39]. Hence, there is a need for alternative clinical grade alpha emitters that can be produced in large scale.

Currently, bone metastases occur in up to 90% and extraskeletal metastases (regional lymph node and visceral metastases) in up to 50% of patients with mCRPC [40-42]. Hence, an ideal targeted RLT for mCRPC must combat the entire spectrum of metastases. The use of a liquid ^{224}Ra/^{212}Pb-generator would allow the preparation of a dual-alpha targeting solution, where a PSMA-targeting ligand is labelled with ^{212}Pb in the ^{224}Ra-solution without the subsequent removal of ^{224}Ra (Fig. **3B**). This solution will contain radionuclides with dual targeting properties, whereby the natural bone-seeking radionuclide ^{224}Ra will target stromal elements in osteoblastic metastatic lesions [11, 43], and the tumour cell surface-seeking ^{212}Pb-labelled PSMA ligand will target the extraskeletal metastases by selective binding to the PSMA-expressing cells.

The clinically most studied therapeutic PSMA-seeking carrier PSMA-617 gives relevant tumour to normal tissue ratios for longer-lived radionuclides, such as ^{177}Lu ($t_{1/2}$≈6.7 days) and ^{225}Ac ($t_{1/2}$≈9.9 days) but at early time points (typically a few hours after injection) shows relatively high uptake in kidneys [44, 45]. With shorter-lived radionuclides such as ^{212}Pb and ^{211}At ($t_{1/2}$≈7.2 hours), the initial kidney uptake could represent a potential toxicity problem [46]. It therefore seems advantageous to have a PSMA ligand with more rapid pharmacokinetics, resulting in moderate kidney uptake and retention and high tumour-targeting capability.

The goal of this study was to label the p-SCN-Bn-TCMC PSMA-targeting ligand NG001 [47] and PSMA-617 with ^{212}Pb by use of the ^{224}Ra/^{212}Pb-liquid generator to produce dual-targeting radiopharmaceutical solutions. We have characterised labelling efficiency with ^{212}Pb in the ^{224}Ra-solution *in situ* and investigated the targeting properties of the two radioligands *in vitro* and *in vivo*. Cell binding assays were performed to evaluate the radiolytic stability of the radioligands in dual targeting solutions. The biodistribution of the radioligands and ^{224}Ra were studied in C4-2 tumour-bearing mice.

2. MATERIALS AND METHOD

2.1. Preparation of Radionuclides and Radioactivity Measurements

Radium-224 was extracted from a generator column containing actinide resin (Eichrom Technologies, Lisle, Illinois, USA) with immobilised ^{228}Th (Eckert & Ziegler,

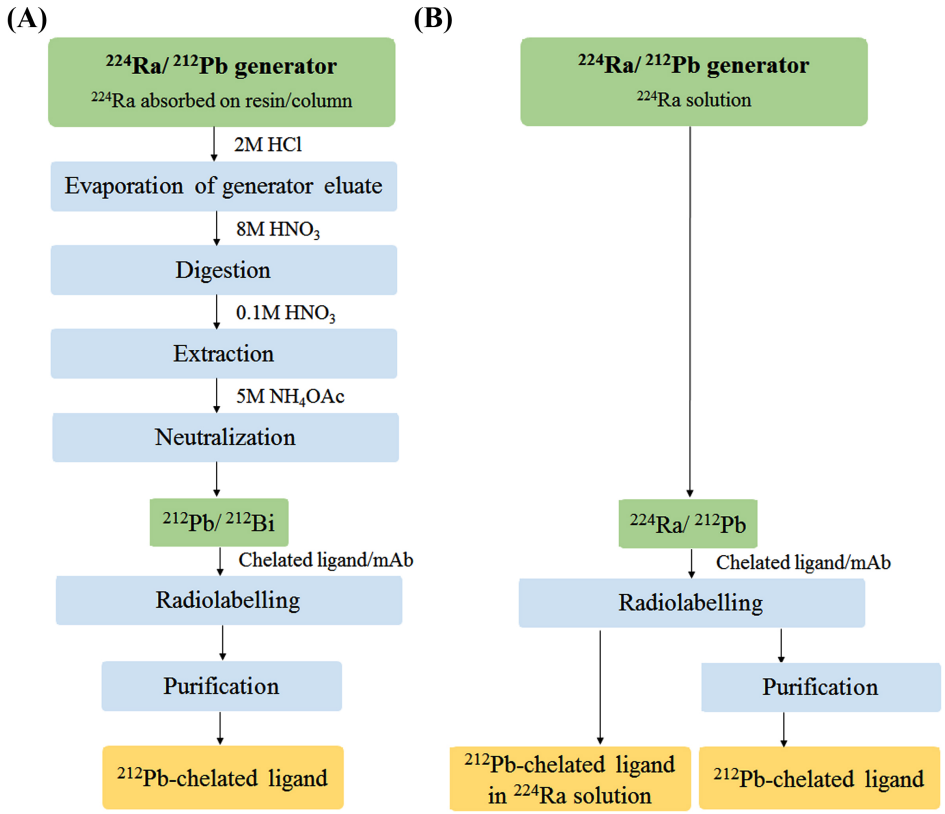

Fig. (3). A simplified scheme comparing the ^{212}Pb production and labelling of chelated ligands or monoclonal antibodies (mAb) for the end user using (**A**) a standard ^{224}Ra/^{212}Pb-generator [19] and (**B**) a ^{224}Ra/^{212}Pb-liquid generator solution [17]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Braunschweig, Germany) by eluting with 1 M HCl [17, 48]. Eichrom's actinide resin is based on the DIPEX® extractant. A column-based generator was prepared by removing 80% of the actidine resin from a column and mixing this as a slurry with ^{228}Th in 1 M HCl for approximately two hours and then the slurry was loaded onto the same column with the 20% acting as a catcher layer. Thereafter the column was washed thoroughly with 1 M HCl to ensure that only immobilised ^{228}Th was present. After one or two weeks of ingrowth, the ^{224}Ra generated was mobilised by eluting with 1 M HCl. The eluate was purified on a second actinide resin column, and the second eluate was evaporated to dryness using an evaporation vial with a cap with a gas inlet and outlet placed in a heater block at approximately 110 °C and a gentle stream of nitrogen gas. When the evaporation vial was empty of solvent, around 200-400 µl of 0.1 M HCl was added to dissolve the residue. The ^{224}Ra-solution was left for 2-3 days to reach equilibrium with daughter radionuclides. The solution was then used for producing ^{212}Pb-labelled ligands by labelling *in situ*, that is, ^{212}Pb was complexed by the ligands in the presence of ^{224}Ra.

Radioactive samples were measured on a Cobra II Auto-gamma Counter (Packard Instrument Company, Downer Grove, Illinois, USA) and a Hidex Automatic Gamma Counter (Hidex Oy, Turku, Finland) using the 50-120 keV and 60-110 keV counting windows, respectively. This setting mainly measures the ^{212}Pb activity (34.9% relative to the mother nuclide ^{224}Ra) with very little contribution from other radionuclides (1.2% relative to ^{224}Ra) in the ^{224}Ra series (Supplementary Table **S1**) [17, 48]. Radium-224 activity was indirectly determined by measuring the ^{212}Pb activity after 4-5 days when the initial ^{212}Pb had decayed and the equilibrium between ^{224}Ra and the newly formed ^{212}Pb had been reached. A radioisotope dose calibrator Capintec CRC-25R (Capintec Inc., Ramsey, New Jersey, USA) was used during the radiolabelling procedure. To determine the distribution of ^{224}Ra, ^{212}Pb and ^{212}Bi in samples, a liquid nitrogen cooled High-purity Germanium (HPGe) radiation detector (Canberra Industries Inc., Meriden, Connecticut, USA) combined with the Genie 2000 Spectroscopy Software (Canberra, Australia) was used.

2.2. Radiolabelling of NG001 and PSMA-617

The PSMA ligands NG001 and PSMA-617 were supplied by MedKoo Biosciences (Morrisville, North Carolina, USA) as a purchased synthesis service. The products were supplied as HPLC purified and dried trifluoroacetic acid salts with a purity of at least 98%. Before they were used in radiolabelling, the ligands were dissolved in 0.5 M ammonium acetate (NH_4OAc) in 0.1 M HCl at a concentration of 1 mg/ml. For radiolabelling, NG001 and PSMA-617 were added to the ^{224}Ra-solution (2-5 MBq/ml) with progenies in 0.5 M NH_4OAc in 0.1 M HCl, adjusted to pH 5-6. Different concentrations of the ligands were radiolabelled, ranging from 2.5-500 µg/ml. The reaction mixture was incubated for 30 minutes on a thermomixer (Eppendorf, Hamburg, Germany) at 37 °C and 450 rpm. In an experiment, the effect of different incubation conditions (various temperatures and

pH) on the radiolabelling of NG001 was tested. The labelling reactions were evaluated by thin layer chromatography (TLC).

2.3. Instant TLC Analysis

The radiochemical purity (RCP) of the radiolabelled ligands was measured by TLC using instant TLC (ITLC) strips (Tec-control, Biodex, New York, USA). A sample of the radiolabelled solution was diluted in a formulation buffer consisting of 7.5% human serum albumin and 5 mM EDTA in DPBS adjusted to pH 7. The mixture was left for 5-10 minutes to allow the chelation of unbound radioisotopes with EDTA. Around 1-5 µl of the reaction mixture was applied to the origin line of the ITLC strip and placed in a small beaker with 0.5 ml of 0.9% NaCl. When the solvent front had migrated to the front line, the strips were bisected and each half put in a counting tube for activity measurement using a gamma counter. In this system, unbound radioisotopes complex with EDTA and follow the solvent front (top half), while the radiolabelled ligand is retained at the baseline (bottom half). The RCP was calculated using the following equation:

RCP (%) = Activity in bottom half × 100 / (Activity in bottom half + activity in top half)

Bismuth-212 will also be present in the ^{224}Ra-solution in equilibrium with progeny. Therefore, in a separate experiment, the chelation of ^{212}Bi to NG001 was examined. The ITLC strips were measured in an HPGe detector to determine the radioactivity of ^{212}Bi (727 keV, 4.3% relative abundance), and RCP was calculated as described above.

2.4. Cell Line

The PSMA-expressing prostate cancer cell line C4-2 [49], obtained from ATCC (ATCC® CRL3314™, Virginia, USA), was grown in RPMI 1640 medium (Sigma-Aldrich Norway AS, Oslo, Norway) supplemented with 10% heat-inactivated foetal bovine serum (FBS, GE Healthcare Life Sciences, Chicago, Illinois, USA) and 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) at 37 °C in a humid atmosphere with 95% air and 5% CO_2.

2.5. Radiolytic Stability of ^{212}Pb-labelled NG001 and PSMA-617 in the Presence of ^{224}Ra

The stability of ^{212}Pb-labelled NG001 and PSMA-617 in the presence of ^{224}Ra was tested by cell binding and TLC analysis after different time points up to 48 hours. Cell binding ability was measured by incubating 0.2 ml of C4-2 cells (5-6 × 10^7 cells/ml) with 0.5-2 ng of radioligand for 1 hour at 37 °C and 450 rpm. Non-specific binding was estimated by blocking cells with an excess of unlabelled ligand before the addition of radioligand. Added activities were measured in a gamma counter before the cells were washed 3 times with 0.5% BSA in PBS. Cell bound activities were then measured, and the cell-binding fraction (%) was estimated by subtracting the non-specific cell bound activity from the total cell bound activity.

The effect of the radioprotectants ascorbic acid and/or human serum albumin [16, 50, 51] on the cell binding ability of the radiolabelled NG001 was tested. Human serum albumin (HSA, Sigma-Aldrich) and/or L-ascorbic acid (Sigma-Aldrich) were added to the ^{224}Ra-solution immediately after the radiolabelling of NG001. Stability and cell binding ability were tested at different time points up to 48 hours.

2.6. Separation of ^{212}Pb-labelled NG001 from ^{224}Ra

For one of the biodistribution studies, ^{212}Pb-labelled NG001 and PSMA-617 were purified using PD Minitrap G-10 columns prepacked with Sephadex G-10 resin (exclusion limit, M_r cut-off of 0.7 kDa) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to remove ^{224}Ra and other unconjugated daughter nuclides.

2.7. Animals

Twenty six Hsd: Athymic Nude-Foxn1^{nu} mice bred at the Department of Comparative Medicine at the Norwegian Radium Hospital (Oslo University Hospital, Oslo, Norway) were used in this study and maintained under specific pathogen-free conditions with *ad libitum* access to food and water. Cages were housed in a scantainer, which was maintained at a constant temperature (24 °C) and humidity (60%). The mice were around 10-12 weeks in age, weighing between 25-35 g at the start of the study. The mice were inoculated subcutaneously in both flanks with 10^7 C4-2 cells in RPMI 1640 medium without supplements mixed 1:1 with Matrigel Matrix (Corning, New York, USA) in a total volume of 200 µl. Prior to cell inoculation, the absence of murine contaminations in the human cell line was verified by a PCR-based pathogen test (IMPACT I, IDEXX Bioanalytics, Ludwigsburg, Germany). The tumours were grown to reach a volume of 250-1000 mm^3 before the animals were used in the studies. The mice were monitored two times per week for changes in weight and tumour size and for any sign of illness or discomfort. Humane end points were >15% weight loss from the initial weight, tumours that exceed 15 mm in any direction, ulcerate or interference with normal behaviour or any signs of severe sickness or discomfort. No mice became ill or died prior to the experimental end point.

2.8. Biodistribution of [^{212}Pb]$PbCl_2$, [^{212}Pb]Pb-NG001, [^{212}Pb]Pb-PSMA-617 and ^{224}Ra in Mice with C4-2 Xenografts

The radiolabelled ligands were diluted in 0.9% NaCl and sterile-filtered immediately before injections. The mice bearing C4-2 tumours were injected intravenously via tail vein with 27 kBq (0.17 nmol) of [^{212}Pb]Pb-NG001 in the presence of ^{224}Ra (33 kBq), 18 kBq (0.20 nmol) of [^{212}Pb]Pb-PSMA-617 in the presence of ^{224}Ra (15 kBq), 44 kBq of [^{212}Pb]$PbCl_2$ in the presence of ^{224}Ra (40 kBq), 29 kBq (0.24 nmol) of purified [^{212}Pb]Pb-NG001 or 79 kBq (0.49 nmol) of purified [^{212}Pb]Pb-PSMA-617 in a volume of 100 µl per mouse.

Two hours after injection, the mice were given gas anaesthesia (~3.5% Sevofluran [Baxter, Illinois, USA] in oxygen at a flow rate of 0.5 l/min) for blood collection by cardiac puncture. The mice were then euthanised by cervical dislocation and different organs/tissues were harvested. The weight and activity of each tissue sample were measured in a gamma counter. The obtained values were corrected for the decay of ^{212}Pb and the ingrowth of ^{212}Pb from ^{224}Ra. Percent-

age injected dose per gram tissue (%ID/g) was calculated. Samples of the injectates were used as references in the measurement procedures.

2.9. Statistics

The datasets were analysed for significance using one-way ANOVA with Tukey's multiple comparison post-test using SigmaPlot 14.0 software (Systat Software, Inc. San Jose, California, USA). A p-value of <0.05 was considered statistically significant.

3. RESULTS

3.1. Radiolabelling of Ligands

The ^{212}Pb-labelling of NG001 and PSMA-617 in the ^{224}Ra-solution in transient equilibrium with progeny was tested at different ligand concentrations (2.5-500 µg/ml), temperatures and pH values. Radiolabelling was efficient with RCP above 90% at a ligand concentration of 10 µg/ml and above 94% at concentrations of 15 µg/ml and higher (Fig. **4**, Supplementary Table **S2**), indicating that a variety of specific activities can be achieved. For NG001, the RCP of ^{212}Bi was around 45% at a ligand concentration of 10 µg/ml and around 65-75% at concentrations above 50 µg/ml, demonstrating that ^{212}Bi is also conjugated by the TCMC chelator though not to the same extent as ^{212}Pb (Fig. **4**, Supplementary Table **S2**). The radiolabelling of NG001 was successful with RCP >93% at a range of various temperatures and pH values (Table **1**). There was a slight decline in RCP at pH 8, but RCP was above 88%.

3.2. Radiolytic Stability

The radiolytic stability of ^{212}Pb-labelled NG001 and PSMA-617 in the ^{224}Ra-solution was tested by performing cell binding and TLC analysis at different time points up to 48 hours. The initial activity of ^{224}Ra in the solutions was between 2.8-5 MBq/ml, causing absorbed radiation doses to the solutions of 1-1.8 kGy after 24 hours and 1.8-3.2 kGy after 48 hours. The radioligands maintained a high RCP even after 48 hours (Table **2**), indicating that the PSMA ligands are capable of complexing ^{212}Pb continuously as it is generated from ^{224}Ra during storage. The chelation of ^{212}Bi to NG001 was also stable up to 48 hours (Supplementary Table **S3**).

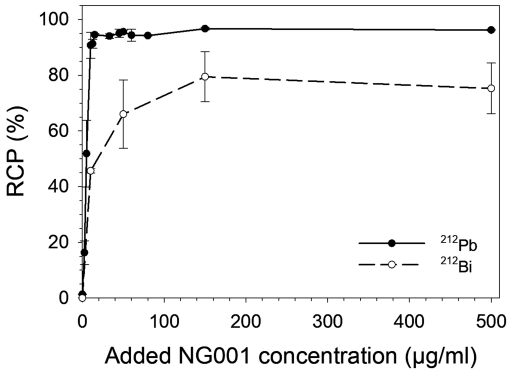

Fig. (4). Radiochemical purity (RCP) values as mean ± SD of ^{212}Pb and ^{212}Bi after the radiolabelling of different concentrations of NG001 in the ^{224}Ra-solution in transient equilibrium with progeny. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Both radioligands were stable over 4 hours at room temperature but showed decreased cell binding ability when incubated for 24 and 48 hours (Table **2**). Therefore, the effect of the radioprotectants L-ascorbic acid and HSA, alone or in combination, on the stability of ^{212}Pb-labelled NG001 was evaluated.

The addition of scavengers did not interfere with the continuous complexing of ^{212}Pb by the ligand, indicated by the high RCP values at all time points (Fig. **5**, Supplementary Table **S4**). However, only the combination of L-ascorbic acid and HSA prevented radiolysis and resulted in the radioligand maintaining a higher cell binding ability after 24 and 48 hours (cell binding fraction of 61.14±1.77% and 56.00±10.24, respectively).

3.3. Biodistributions of ^{212}Pb and ^{224}Ra in Mice Bearing Human Prostate C4-2 Xenografts

Before injection into the mice, the binding of the radiolabelled ligands was verified by measuring cell binding ability in C4-2 cells. The cell binding fraction was between 45-65%

Table 1. Radiochemical purity (RCP) values as mean ± SD of ^{212}Pb-labelled NG001 (33 µg/ml) after radiolabelling in the ^{224}Ra-solutions of different pH values at 37 ºC or at different incubation temperatures (pH=5-6). N, the number of independent experiments.

Incubation Conditions	RCP (%)	N
pH		
4	94.59±0.89	2
5-6	94.04±0.87	7
8	88.25±0.82	2
Temperature (°C)		
4	93.81±0.40	2
20	94.86±0.43	2
37	94.04±0.87	7

for both radioligands. The mice were euthanised 2 hours after the injection of radioligands.

The measured activity values were corrected for the decay of ^{212}Pb and ingrowth of ^{212}Pb from ^{224}Ra, and %ID/g values of ^{212}Pb without ^{212}Pb ingrowth from ^{224}Ra (Fig. **6A**, Supplementary Table **S5**), ^{212}Pb ingrowth from ^{224}Ra (Supplementary Table **S5**) and ^{224}Ra (Fig. **6B**, Supplementary Table **S6**) were calculated. The highest activities of the carrier-free ^{212}Pb (^{224}Ra&^{212}Pb group) were observed in the kidneys, urine, liver, bones and blood, while the activities of [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 were highest in the urine, kidneys and tumour (Fig. **6A**). The uptake values (%ID/g) for the tumour and kidneys at 2 hours post-injection were 1.71±1.29 and 48.47±7.57 for [^{212}Pb]$PbCl_2$, 15.01±4.73 and 21.39±9.55 for [^{212}Pb]Pb-NG001 and 18.71±4.78 and 74.17±25.59 for [^{212}Pb]Pb-PSMA-617 after intravenous injection of the ^{224}Ra-solutions (Fig. **6A**, Supplementary Table **S5**). The tumour-to-kidney ratios for [^{212}Pb]$PbCl_2$, [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 were 0.04, 0.70 and 0.25, respectively. Compared with [^{212}Pb]Pb-PSMA-617, [^{212}Pb]Pb-NG001 showed comparable tumour uptake ($P>0.05$) and 3.5-fold lower kidney uptake ($P<0.01$). Radium-224 had high skeletal uptake with similar uptake in the femur and skull in all groups ($P>0.05$): 19.40±1.97 and 19.62±1.28 %ID/g for [^{212}Pb]$PbCl_2$, 18.90±3.74 and 17.85±5.45 %ID/g for [^{212}Pb]Pb-NG001, and 25.57±0.81 and 21.98±2.73 %ID/g for [^{212}Pb]Pb-PSMA-617 (Fig. **6B**, Supplementary Table **S6**).

Purified ^{212}Pb-labelled ligands were prepared from the ^{224}Ra/^{212}Pb-solution using a standard gel filtration column. The removal of ^{224}Ra and other unconjugated daughter nuclides from the solutions with NG001 or PSMA-617 did not influence their biodistribution (Table **3**). The uptake values (%ID/g) of purified ^{212}Pb-labelled ligands for tumour and kidneys at 2 hours post-injection were similar to the corresponding values of ^{212}Pb-labelled ligands in ^{224}Ra-solution (Table **3**).

4. DISCUSSION

The current study has evaluated the feasibility of using ^{224}Ra as a liquid generator solution for ^{212}Pb in the preparation of ^{212}Pb-labelled PSMA-binding ligands. It indicates that the ^{224}Ra-based solution is suitable for such use. A relatively fast method (~30 min) for the labelling of NG001 or PSMA-617 with ^{212}Pb was developed. Here, ^{224}Ra is in transient equilibrium with progeny at pH 4-6. It was shown that the labelling reaction would occur *in situ* when the reaction solution was stored up to at least 48 hours (Table **2**) at room temperature. The use of an ^{224}Ra-solution generator system provides an opportunity for centralised production and long-distance shipment within a time window of three to four days (Fig. **3B**).

The use of ^{212}Pb to label PSMA-targeting ligands may be a promising option for TAT. PSMA has an internalisation function, a feature which is very attractive for ^{212}Pb-labelled products as this would minimise the translocalisation of ^{212}Pb progenies due to the decay-induced release of ^{212}Bi from the chelator [17, 20]. The decay-induced release of daughters from the chelator may be a problem for alpha-emitting radionuclides. In the case of ^{225}Ac, the four consecutive decay daughters (^{221}Fr, ^{217}At, ^{212}Bi and ^{209}Pb) receive high recoil energies (typically about 100 keV) when the alpha particle is emitted [52, 53]. This energy is about 1000 times higher than the chemical bond energies of any chelating agent, which means that the recoiling daughter will

Table 2. Radiochemical purity (RCP) values and cell binding fraction expressed as mean ± SD of ^{212}Pb-labelled NG001 and PSMA-617 in the ^{224}Ra-solution when incubated at activity concentrations of 2.8-5 MBq/ml up to 48 h (at room temperature, pH 5-6, n=2-6).

Incubation Time	RCP (%)		P-value	Cell Binding Fraction (%)		P-Value
	[^{212}Pb]Pb-NG001	[^{212}Pb]Pb-PSMA-617		[^{212}Pb]Pb-NG001	[^{212}Pb]Pb-PSMA-617	
1 h	94.04±1.23	95.49±1.65	0.140	57.62±4.59	51.62±4.84	0.122
4 h	94.79±0.59	94.91±1.31	0.893	52.27±0.05	48.35±5.45	0.495
24 h	93.46±0.67	94.67±1.75	0.269	45.92±5.46	46.07±5.30	0.969
48 h	92.52±1.39	94.55±1.32	0.095	40.12±5.31	44.86±3.76	0.194

Table 3. Percentage of injected activity per gram of tissue (%ID/g) ± SD of purified [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 at 2 hours after intravenous injection of [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 in athymic mice bearing human prostate C4-2 cancer xenografts (n=3-11 mice).

Organ	%ID/g		P-value
	[^{212}Pb]Pb-NG001	[^{212}Pb]Pb-PSMA-617	
Tumour	17.61±6.76	17.93±2.90	0.931
Kidney	21.07±10.33	52.82±26.62	0.013

always break free from chelating agents [52, 53]. Thus, free daughter radionuclides may accumulate in healthy organs and cause high toxicity [53, 54]. The recoil energies from beta emitters are low (< 10 eV) [55]. However, conversion electron cascades associated with the ^{212}Pb decay are probably responsible for some release of ^{212}Bi from the chelator [20].

The rapid elimination of unbound radioligands from normal tissues is mandatory when using shorter-lived radionuclides such as ^{212}Pb. A potential problem is the high initial kidney uptake of these small radioligands, but it appears that the use of NG001 instead of PSMA-617 as a carrier for ^{212}Pb significantly reduced the kidney exposure (Fig. **6A**).

The ^{224}Ra/^{212}Pb-based generator used here was designed to overcome challenges with the original ^{228}Th/^{212}Pb-based generators, including the radiolytic damage of the parent nuclide materials that decreased the yield as well as serious radiation safety issues [17, 56, 57]. To avoid the potential radiolysis of the radioligand, the mixing of the ligand and the ^{224}Ra/^{212}Pb-liquid generator solution could take place just before injection (Fig. **3B**). The ^{212}Pb-labelled ligand could be used as a purified single-agent product and not only as a dual-targeting product in tandem with ^{224}Ra.

An interesting feature with the ^{212}Pb-labelling of the PSMA-targeting molecule is the great variation in labelling conditions that can be used for preparing the radioligand (Fig. **4** and Table **1**). With the ^{177}Lu-labelling of PSMA-617, the product has to be heated (80-98 °C) to obtain efficient radiolabelling of the ligand [58, 59]. Regarding ^{212}Pb-labelling, the current data show that NG001 and PSMA-617 can be radiolabelled in an efficient way using a ^{224}Ra-solution in transient equilibrium with daughters at pH 4-6 and without the need for heating the reaction solution (Fig. **4**, Table **1**, Supplementary Table **S2**). It was found that the majority of ^{212}Bi present in the labelling solutions was also complexed by the chelators (Fig. **4**). This observation is compatible with that reported by Westrøm *et al.* for TCMC-conjugated mAb [17].

A noteworthy aspect with the ^{224}Ra-based generator solution is that the activity level curve for ^{212}Pb is quite flat from about 20 to about 96 hours when starting with a pure ^{224}Ra source (Fig. **2C**). For example, an ^{224}Ra source of 100 MBq at day 0 can be mixed with the radioligand for *in situ* ^{212}Pb-labelling, which would yield a product of 60 MBq of ^{212}Pb-labelled radioligand three days later (Fig. **1C**). Thereby, the liquid generator system significantly improves the suitability of the centralised production and shipment of the ^{212}Pb product kit.

When using RLT, radiolysis could be a potential problem for product integrity. The oxidation of PSMA ligands has been shown to occur when the PSMA ligands are labelled with the ^{177}Lu isotope and can be minimised by the addition of ascorbic acid [60]. The presence of the oxidised forms of NG001 and PSMA-617 was also observed in our studies after 24 and 48 hours. This oxidation somewhat influenced the binding of ^{212}Pb-labelled NG001 and PSMA-617 to cells (Table **2**). The addition of ascorbic acid alone did not improve cell binding at 24 and 48 hours (Fig. **5**), nor did the addition of another potential radioprotectant, HSA [51], alone proved efficient to prevent the radiolysis of NG001 (Fig. **5**). However, when both of them were added to the ^{224}Ra-solution, they prevented the radiolysis of [^{212}Pb]Pb-NG001 even at higher activity concentrations (2.8-5 MBq/ml) without interfering with the continuous complexing of ^{212}Pb by the ligand (Fig. **5**).

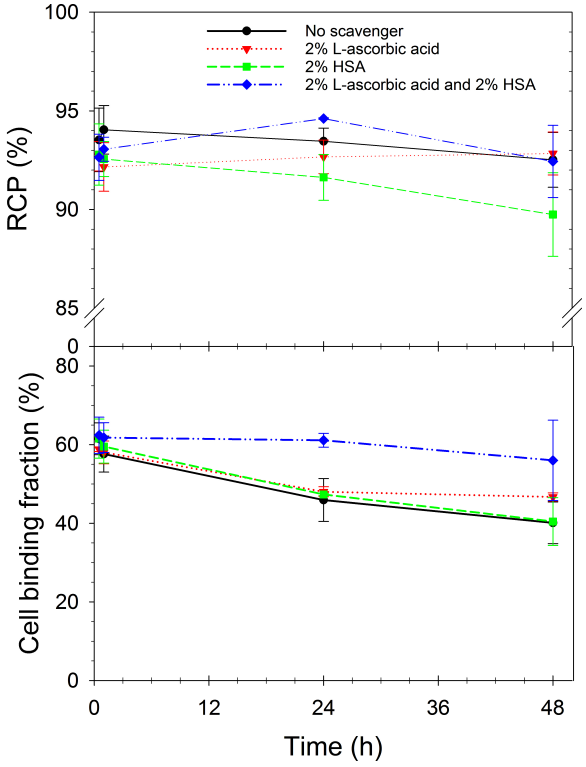

Fig. (5). Radiochemical purity (RCP) values (top) and cell binding fraction (bottom) expressed as mean ± SD of ^{212}Pb-labelled NG001 in ^{224}Ra-solution containing no scavengers, 2% L-ascorbic acid, 2% HSA or both scavengers when incubated at activity concentrations of 2.8-5 MBq/ml up to 48 hours (at room temperature, n=2-3). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The current study is the first presentation in the scientific literature regarding the labelling of NG001 and PSMA-617 with ^{212}Pb in the ^{224}Ra-solution in transient equilibrium with daughter nuclides and where [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 in the ^{224}Ra-solution efficiently bind to PSMA-positive C4-2 cells *in vitro* and *in vivo* (Table **2**, Figs. **5** and **6A**). Recently, several novel PSMA-seeking ligands (CA008, CA009/NG001, CA011, CA012, L1, L2, L3, L4 and L5) were synthesised and labelled with ^{203}Pb, a surrogate for ^{212}Pb [14, 15]. Two PSMA ligands, ^{203}Pb-labelled CA009 (identical to the structure of NG001, which is patented by Larsen [61]) and CA012, were presented by Dos Santos *et al.* [14]. In C4-2 tumour-bearing BALB/c nu/nu mice, [^{203}Pb]Pb-CA012 showed a tumour uptake of 8.4 %ID/g at 1 hour after injection [14], which was considerably lower than the tumour uptake of 22.5 %ID/g reported for [^{203}Pb]Pb-L2 in NOD-SCID mice bearing PC3-PIP tumours [15]. In the present work, [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 showed similar tumour uptakes of 15.01 %ID/g and 18.71 %ID/g in C4-2 tumour-bearing Hsd: Athymic Nude-Foxn1^{nu} mice after 2 hours, respectively (Fig. **6A**). The obtained val-

ues are higher than those reported for ^{203}Pb-CA012 [14] for the same tumours and lower than those for [^{203}Pb]Pb-L2 where PC3-PIP tumours were studied [15]. PC3-PIP cells have higher PSMA expression than C4-2 cells [49, 62, 63]. The direct comparison of new PSMA ligands is not easy since different mouse strains and cell lines were used in the various studies. However, our results indicate that the efficient targeting of PSMA-tumours can be achieved with radioligands labelled in the ^{224}Ra-solution.

Herein, ^{224}Ra showed high uptake in the femur and skull in all groups (Fig. **6B**), demonstrating that it is possible to effectively chelate ^{212}Pb without reducing the bone-seeking properties of ^{224}Ra in radiopharmaceutical solutions containing the two radionuclides. The novel ^{224}Ra/^{212}Pb liquid generator opens opportunities to use ^{212}Pb-labelled NG001 either as a purified compound by itself for targeting prostate cancer cells or together with ^{224}Ra in a dual bone and soft-tissue targeting solution. Such solutions could be used to extend TAT to prostate cancer patient groups which are not eligible for Xofigo ([^{223}Ra]$RaCl_2$) as bone metastases therapy since Xofigo is not indicated for patients with documented extraskeletal disease [9].

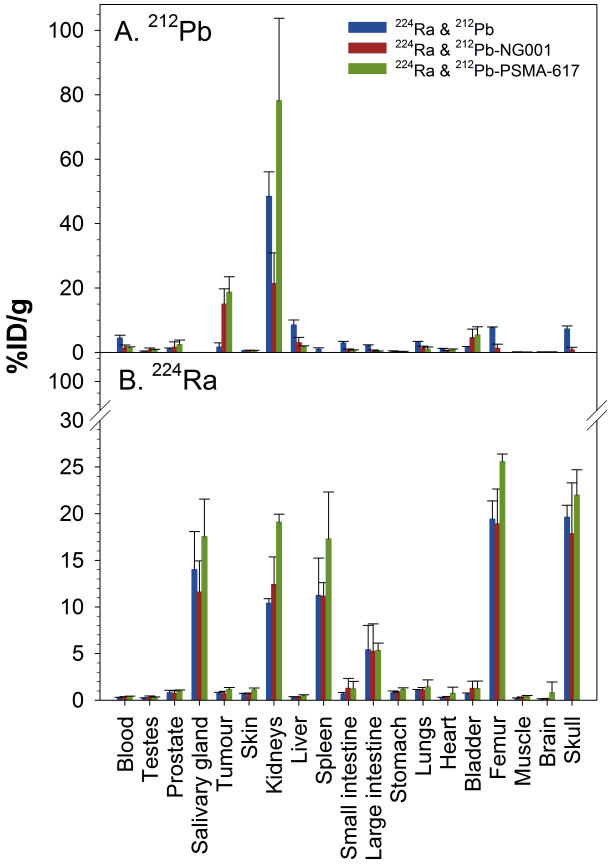

Fig. (6). Percentage of injected activity per gram of tissue (%ID/g) ± SD of [^{212}Pb]$PbCl_2$, [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 (top) and radium-224 (bottom) at 2 hours after intravenous injection of ^{224}Ra-solution containing [^{212}Pb]$PbCl_2$, [^{212}Pb]Pb-NG001 or [^{212}Pb]Pb-PSMA-617 in athymic mice bearing human prostate C4-2 cancer xenografts (n=3-5 mice per group). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The main route of excretion of radiolabelled PSMA ligands is via renal excretion/elimination [64]. Kidneys receive the highest radiation doses, and they are considered the dose-limiting organ [14, 15, 65]. Importantly, kidney uptake was 3.5-fold lower for [^{212}Pb]Pb-NG001 compared to [^{212}Pb]Pb-PSMA-617 at the 2 hour time point (Fig. **6A**) without compromising tumour-targeting, indicating that NG001 may be better suited for ^{212}Pb-targeting than PSMA-617.

Salivary gland toxicity is a common side effect of [^{225}Ac]Ac-PSMA-617 therapy [29, 35, 44]. Preventative approaches to mitigate this side effect have included intraglandular injection of botulinum toxin, performing sialendoscopy or deescalating the dose schedule of [^{225}Ac]Ac-PSMA-617 [29, 37, 66, 67]. Only traces of [^{212}Pb]Pb-NG001 and [^{212}Pb]Pb-PSMA-617 were found in the salivary glands (Fig. **6A**). It is unclear the extent to which mouse data can be indicative of salivary gland uptake of the PSMA-targeting ligands in humans. A significant uptake of ^{224}Ra was observed in the kidneys and salivary glands (Fig. **6B**). Radium-224 was used earlier for many years for the treatment of different non-cancerous diseases [43]. To our knowledge, salivary gland toxicities have not been linked to the use of ^{224}Ra. Kidney uptake is not a problem in humans as more than 90% of radium is excreted by intestinal clearance in man compared with about 70% renal clearance of radium in mice [68, 69].

Radium-224 has a high affinity for sclerotic bone tumours while PSMA binding ligands have shown a great affinity for PSMA-positive tumour lesions. The current work demonstrates the possibility to combine these two modes of targeting in one radiopharmaceutical solution using the ^{224}Ra and ^{212}Pb radionuclide combination.

CONCLUSION

A fast and efficient method for the labelling of NG001 or PSMA-617 with ^{212}Pb in the ^{224}Ra-solution in transient equilibrium with progeny at pH 4-6 and without the need for heating the reaction solution was developed. High RCP and stability of [^{212}Pb]Pb-NG001 were demonstrated over 48 hours in the presence of ascorbic acid and albumin. Thus, the use of shippable ^{224}Ra sources for the preparation of ^{212}Pb would allow shipment to the end user of a ready-to-use product or a simple kit that can be used to prepare the injectable radiopharmaceutical just prior to administration. Biodistribution data indicated similar tumour-targeting and less kidney uptake of radiolabelled NG001 compared with PSMA-617. [^{212}Pb]Pb-NG001 could be prepared as a purified product by simple desalting gel exclusion separation. Alternatively, a dual-alpha solution of [^{212}Pb]Pb-PSMA ligand for cell surface targeting and cationic ^{224}Ra for bone-targeting could be prepared by using the reaction solution without purification. Thus, further *in vivo* studies with [^{212}Pb]Pb-NG001 in the absence or presence of ^{224}Ra are warranted.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Committee on Research Animal Care, Department of Comparative

Medicine, Oslo University Hospital, Norway and the Norwegian Food Safety Authority, Brumunddal, Norway (Approval No: FOTS ID 8724, 2016/111707).

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. All procedures and experiments involving animals in this study were performed in accordance with the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education (New York Academy of Sciences, New York, NY) and the EU Directive 2010/63/EU for animal experiments.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analysed during this study are included in this published article and its supplementary information files.

FUNDING

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CONFLICT OF INTEREST

R. H. L., Ø. S. B. and V. Y. S. hold an ownership interest in Nucligen AS.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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