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Optimizing labelling conditions of ^{213}Bi -DOTATATE for preclinical applications of peptide receptor targeted alpha therapy

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Abstract

Background: ^{213}Bi Bismuth (^{213}Bi , $T_{1/2} = 45.6$ min) is one of the most frequently used α -emitters in cancer research. High specific activity radioligands are required for peptide receptor radionuclide therapy. The use of generators containing less than 222 MBq ^{225}Ac (actinium), due to limited availability and the high cost to produce large-scale $^{225}\text{Ac}/^{213}\text{Bi}$ generators, might complicate in vitro and in vivo applications though.

Here we present optimized labelling conditions of a DOTA-peptide with an $^{225}\text{Ac}/^{213}\text{Bi}$ generator (< 222 MBq) for preclinical applications using DOTA-Tyr³-octreotate (DOTATATE), a somatostatin analogue. The following labelling conditions of DOTATATE with ^{213}Bi were investigated; peptide mass was varied from 1.7 to 7.0 nmol, concentration of TRIS buffer from 0.15 mol.L⁻¹ to 0.34 mol.L⁻¹, and ascorbic acid from 0 to 71 mmol.L⁻¹ in 800 μL . All reactions were performed at 95 °C for 5 min. After incubation, DTPA (50 nmol) was added to stop the labelling reaction. Besides optimizing the labelling conditions, incorporation yield was determined by ITLC-SG and radiochemical purity (RCP) was monitored by RP-HPLC up to 120 min after labelling. Dosimetry studies in the reaction vial were performed using Monte Carlo and in vitro clonogenic assay was performed with a rat pancreatic tumour cell line, CA20948.

Results: At least 3.5 nmol DOTATATE was required to obtain incorporation $\geq 99\%$ with 100 MBq ^{213}Bi (at optimized pH conditions, pH 8.3 with 0.15 mol.L⁻¹ TRIS) in a reaction volume of 800 μL . The cumulative absorbed dose in the reaction vial was 230 Gy/100 MBq in 30 min. A minimal final concentration of 0.9 mmol.L⁻¹ ascorbic acid was required for ~ 100 MBq ($t = 0$) to minimize radiation damage of DOTATATE. The osmolarity was decreased to 0.45 Osmol/L.

Under optimized labelling conditions, ^{213}Bi -DOTATATE remained stable up to 2 h after labelling, RCP was $\geq 85\%$. In vitro showed a negative correlation between ascorbic acid concentration and cell survival.

Conclusion: ^{213}Bi Bismuth-DOTA-peptide labelling conditions including peptide amount, quencher and pH were optimized to meet the requirements needed for preclinical applications in peptide receptor radionuclide therapy.

Keywords: Somatostatin, ^{213}Bi Bismuth, Targeted alpha therapy, Radiochemical purity, Ascorbic acid, Absorbed dose

Background

Peptide receptor radionuclide therapy (PRRT) is an effective treatment for metastatic and inoperable neuroendocrine tumours (van der Zwan et al. 2015; Bergsma et al. 2012; Bodei et al. 2012). The most common radionuclides used for PRRT include ^{177}Lu (β^- -emitter, $T_{1/2} = 6.71$ days, 149 and 497 keV) and ^{90}Y (β^- -emitter, $T_{1/2} = 64.1$ h, 935 and 2284 keV).

α -Emitters have been demonstrated to offer an additional treatment option for patients refractory to standard PRRT with β^- -emitters or chemotherapy (Kratochwil et al. 2011; Jurcic et al. 1995; Cordier et al. 2010). Moreover, α -particles have short path length in tissue (~ 80 μm), sparing non-target tissues from radiation. ^{213}Bi ($T_{1/2} = 45.6$ min) is an α -emitter and has been applied in several (pre) clinical research studies of targeted alpha therapy (TAT) (Kratochwil et al. 2011; Jurcic et al. 1995; Norenberg et al. 2006; Song et al. 2008; Wild et al. 2011).

PRRT is based on receptor-mediated processes. In order to achieve treatment success, a sufficient cytotoxic dose of radio-peptide must be delivered to the targeted cells (Konijnenberg 2014). Moreover, the number of receptors available on the cell membrane is limited, so high specific activity (SA, expressed in MBq per nmol of peptide) of labelled peptides is advantageous for administration in small animals e.g. mice (~ 25 g) (Breeman et al. 2001; Breeman et al. 1995; de Jong et al. 1999). Besides high SA, other requirements for preclinical applications include high stability of the radio-peptide at physiological conditions in vitro (~ 0.3 Osmol.L $^{-1}$, pH ~ 7.4) and in vivo. In addition, a high osmolarity of the drug-containing solution is inconsistent with maintenance of physiological conditions for in vitro and in vivo applications.

Due to the limited availability and the high cost to produce large-scale $^{225}\text{Ac}/^{213}\text{Bi}$ generators, preclinical studies with ^{213}Bi are often being performed using relatively low activity generators (e.g. 222 MBq ^{225}Ac). Under these conditions, the standard clinical labelling procedure for ^{213}Bi , which was designed for high activity generators (up to 4 GBq) with a reaction volume of 2 mL at > 0.7 Osmol.L $^{-1}$ (Kratochwil et al. 2014), probably needs to be adjusted. Therefore, the labelling conditions for preclinical applications required modifications.

In this study, we systematically studied the consequences using the somatostatin analogue [DOTA 0 , Tyr 3]-octreotate (DOTATATE) as a model for optimizing labelling conditions of ^{213}Bi for preclinical applications, starting from the standard labelling protocol.

Herein we varied the labelling conditions such as peptide amount, quencher and pH. The stability of the labelled peptide and the radiochemical purity (RCP) was monitored up to 2 h after labelling. The absorbed dose rates of ^{213}Bi , ^{213}Po , ^{209}Tl and ^{209}Pb (mGy.s $^{-1}$) as function of reaction volume were calculated. The use of the absorbed dose rate is investigated as a possible surrogate indicator for the ionization probability and consequential radiolysis to the peptide in the reaction vial. And lastly, in vitro clonogenic assay was performed to investigate the influence caused by labelling conditions used.

The objective of this study was to establish a practical, ready to use, and reproducible labelling procedure of ^{213}Bi -DOTATATE to meet the constraint for the use of peptide as radiopharmaceutical for preclinical applications.

Methods

²¹³Bi-elution

The ²²⁵Ac/²¹³Bi generator (222 MBq) was supplied by the Institute for Transuranium Elements (ITU), Germany. Prior to elution, the column was rinsed with diluted HCl (0.01 mol.L⁻¹). ²¹³Bi was eluted from the ²²⁵Ac/²¹³Bi generator by NaI/HCl (0.1 mol.L⁻¹/0.1 mol.L⁻¹) in a volume of 600 µL. After elution the column was rinsed and stored with diluted HCl (0.01 mol.L⁻¹) (Morgenstern et al. 2012).

Standard protocol radiolabelling of ²¹³Bi-DOTATATE

DOTATATE (DOTA-DPhe-Cys-Tyr-DTrp-Lys-Thr-Cys-Thr, *M_w* 1436 g/mol) was purchased from BioSynthema (St. Louis, MO, USA). All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

The following standard labelling protocol was applied: ²¹³Bi (600 µL) was added to DOTATATE (7.0 nmol), TRIS buffer (0.34 mol.L⁻¹), ascorbic acid (71 mmol.L⁻¹) and MilliQ water in a final volume 800 µL at pH 8.7. The reaction was incubated for 5 min at 95 °C. The radionuclide incorporation reaction was halted by cooling the mixture for 2 min in ice and through the addition of 50 nmol diethylenetriaminepentaacetic acid (DTPA, Erasmus MC Pharmacy) to chelate any unbound or “free” ²¹³Bi.

Labelling optimization for preclinical studies

Standard labelling protocol was used as described above, while varying the following conditions:

1. Peptide mass; labelling was performed with 1.7, 3.5 and 7.0 nmol DOTATATE .
2. pH dependence study; 7.0 nmol DOTATATE was labelled in a reaction containing 71 mmol.L⁻¹ ascorbic acid and 0.15 mol.L⁻¹ and 0.34 mol.L⁻¹ TRIS buffer.
3. Quencher dependence study; in absence of ascorbic acid and 0.1, 0.3, 0.9, 2.6, 7.9, 24 and 71 mmol.L⁻¹ ascorbic acid.

Quality control of radiolabelled peptide

The quality control of labelled peptides was determined by ITLC-SG (instant thin layer chromatography silica gel, Varian) and HPLC (high performance liquid chromatography).

Incorporation yield (expressed as mean ± SD) was determined by ITLC-SG using sodium citrate (0.1 mol.L⁻¹, pH 5) as mobile phase. The ²¹³Bi activity was determined by HPGe detector with a pulse height multichannel analysis (MetorX, Goedereede, The Netherlands and Software Genie 2000 Canberra) at fixed geometry, all measurements were performed for the 440 keV γ-emission by ²¹³Bi with a yield of 0.261 per decay. The counting efficiency of the HPGe detector at 440 keV γ-emission was determined using a known amount of ²²⁵Ac activity provided and calibrated by ITU, Germany (Morgenstern et al. 2012; Ma et al. 2001; McDevitt et al. 1999).

The RCP (expressed as percentage ± SD intact radio-peptide of interest, compared to other detectable radioactive compounds in the same analysis) and stability of the labelled peptide as function of time were determined by HPLC. HPLC-grade methanol and trifluoroacetic acid (TFA) were purchased from Mallinckrodt Baker (Deventer, the Netherlands). The HPLC system (Waters 2695 separation module, Alliance, Waters,

Etten-Leur, The Netherlands) consisted of a quaternary pump and an autosampler, Waters 2996 photodiode array detector (Waters, Etten-Leur, The Netherlands), radiometric sodium iodide detector (Canberra, Canberra, Genie 2000) and symmetry C₁₈ 5 μm column, 4.6 mm × 250 mm (Waters, Etten-Leur, The Netherlands). The mobile phase consisted of buffer A (0.1 % TFA in water) and buffer B (Methanol). The gradient used for the analysis was as described earlier (de Blois et al. 2011). The retention times of the unlabelled DOTATATE and ²¹³Bi-DOTATATE were 12.0 ± 0.3 min and 13.0 ± 0.3 min, respectively.

Dosimetry model of ²¹³Bi exposure in reaction vials

The degradation of radiopharmaceuticals by radiolysis is dependent on the amount of energy absorbed within the ligand. Most of the α-particle energy emitted by ²¹³Bi and its daughter ²¹³Po will be absorbed within the reaction vial with the ²¹³Bi-labelled compound, as their particle ranges in water are smaller than the vial dimensions. The energy emitted by the α- and β⁻-particles from ²¹³Bi itself and its daughters ²⁰⁹Tl and ²⁰⁹Pb, however, will not be completely absorbed within the small vials. The activity as a function of time for ²¹³Bi, ²¹³Po, ²⁰⁹Tl and ²⁰⁹Pb was calculated by the Bateman equations, see Additional file 1: Equation 1. The β⁻-emission spectra are summarized in Table 1 together with their ranges in water, from the NIST Star database (<http://www.nist.gov/pml/data/star/>; assessed 27-11-'15). The α-particle energies from ²¹³Bi and ²¹³Po are also indicated with their projected ranges as calculated with the Stopping and Range of Ions in Matter (SRIM version 2013.00 software; www.SRIM.org). The recoil energies from the α-particle emissions are 112 keV (²¹³Bi) and 160 keV (²¹³Po) (Eckerman KF MIRD2008 2999).

Radiation transport was calculated for ²¹³Bi containing liquid inside reaction vials to determine the absorbed energy and absorbed dose to the hot liquid. The Monte Carlo codes MCNP5 (MCNP Team The Monte Carlo codes MCNP52005 2999) and MCNPX (Hendricks JS MCNPX Extensions Version 2 5 02005 2999) were used for the calculations. Calculations for the α-particles from ²¹³Bi and ²¹³Po were performed with MCNPX using the α-particle energies listed in Table 1. The β⁻-spectra and low-energy internal conversion and Auger electron spectra for ²¹³Bi, ²⁰⁹Tl and ²⁰⁹Pb from MIRD Radionuclide data and decay schemes book (Eckerman KF MIRD2008 2999) were used

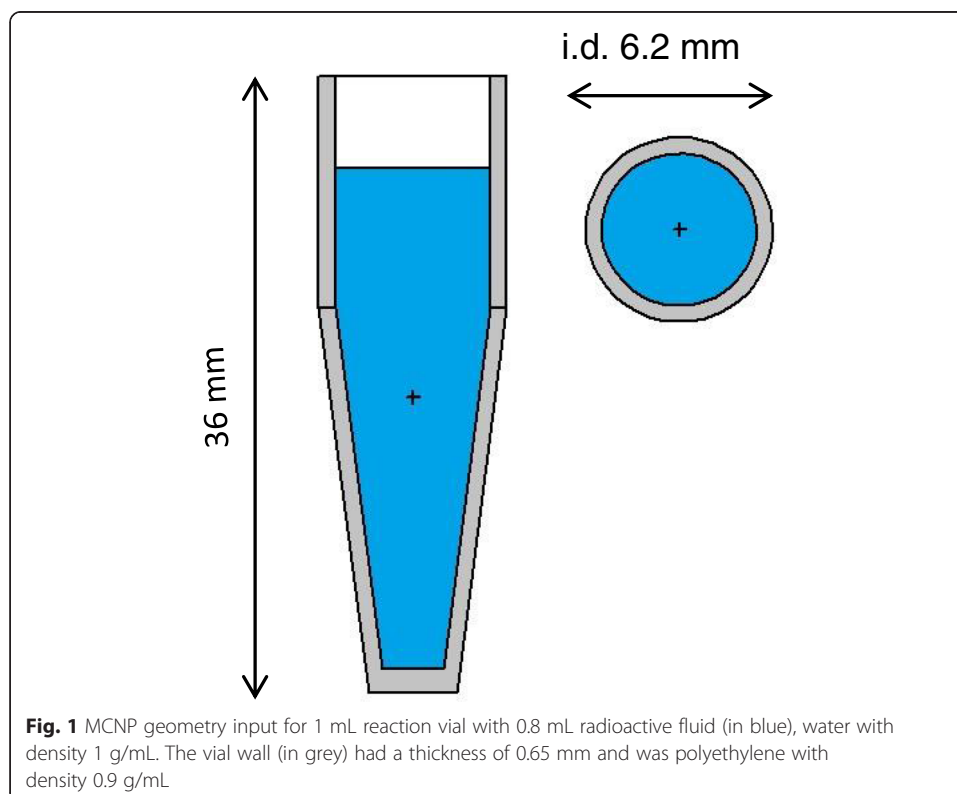
Table 1 α- and β-particle emissions by ²¹³Bi and its daughters, emission abundances per decay of ²¹³Bi or daughter nuclide and energies are from the MIRD Radionuclide data and decay schemes book (Eckerman, 2008). Particle ranges (mm) in water were determined from the NIST Star database (electrons) and with the SRIM code (α-particles). Abundance (Ab.) is expressed in % decay and energy (E) in MeV

α-particles	Ab. (%/decay)	E (MeV)	range (μm)		
²¹³ Bi	1.94	5.87	46.47		
²¹³ Po	100	8.38	81.75		
β-particles	Ab. (%/decay)	E _{mean} (MeV)	range (mm)	E _{max} (MeV)	range (mm)
²¹³ Bi	97.91	0.434	1.45	1.422	6.65
²⁰⁹ Tl	100	0.655	2.55	1.944	9.48
²⁰⁹ Pb	100	0.197	0.44	0.644	2.49

in MCNP5. Particulate radiation emissions with the given energy spectra were simulated so as to be uniformly distributed within a conical reaction vial with isotropic direction emission. All physics processes were taken into account by choosing either the α (MODE A) or the photon–electron mode (MODE P E) and the default PHYS cards with the default cut-off energy at 1 keV for electrons and photons and 4 MeV for α -particles. The *F8 tally determined energy absorption within the hot reaction fluid. Sufficient particle histories (NPS) were used to reduce the variation in the data to be less than 5 % for most cases; NPS was set to 1×10^7 particles. A conically shaped 1 mL reaction vial was modelled with various volumes of radioactive fluid inside the vial. The MCNP-model geometry for a vial containing 0.8 mL liquid is shown in Fig. 1. The labelling volumes were modelled: 10, 50, 100, 200, 400 and 800 μL by adjusting the reaction fluid level accordingly. Average dose rates were determined in the vial volume.

In vitro clonogenic assay

For in vitro clonogenic assays the CA20948 cell line (derived from a rat pancreas tumour at our institute Erasmus MC) was used. CA208948 is a rat pancreatic tumour cell line with relative high expression of somatostatine receptor type 2 (SSTR₂), and was cultured in DMEM supplemented with 10 % fetal calf serum (FBS, Gibco, Life technologies). Cells (500 cells/well) were seeded in 6-wellplates pre-coated with poly-l-lysine 24 h before exposure to the compounds used for labelling, except for peptide and ²¹³Bi. For clonogenic assay, 400 μL of standard labelling procedure was used, and diluted in 8 mL incubation medium containing 30 $\text{mmol}\cdot\text{L}^{-1}$ HEPES and 0.25 % BSA. The concentration of TRIS, ascorbic acid, NaI, HCl and DTPA corresponded to 16, 3.4,



3.6, 3.6, and 6.1×10^{-3} mmol.L⁻¹, respectively. Cells were incubated with compounds for 1 h at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air. Untreated CA20948 cells were used as control. After 1 h, incubation medium containing labelling compounds was removed from cells, cells were washed twice with PBS and incubated with culture medium containing 10 % FBS for 12 days. Every 2 or 3 days, culture medium was replaced by fresh medium. At day 12, colonies were fixed with 100 % ethanol and stained with hematoxyline. Survival colonies were counted and the survival CA20948 was determined. Clonogenic assay with CA20948 as function of ascorbic acid was also performed with the method described above.

Results

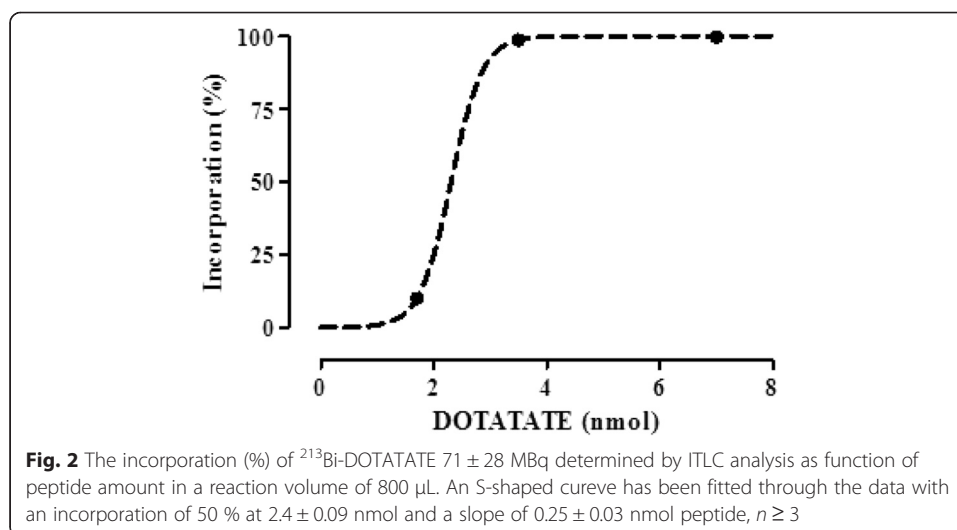
Labelling

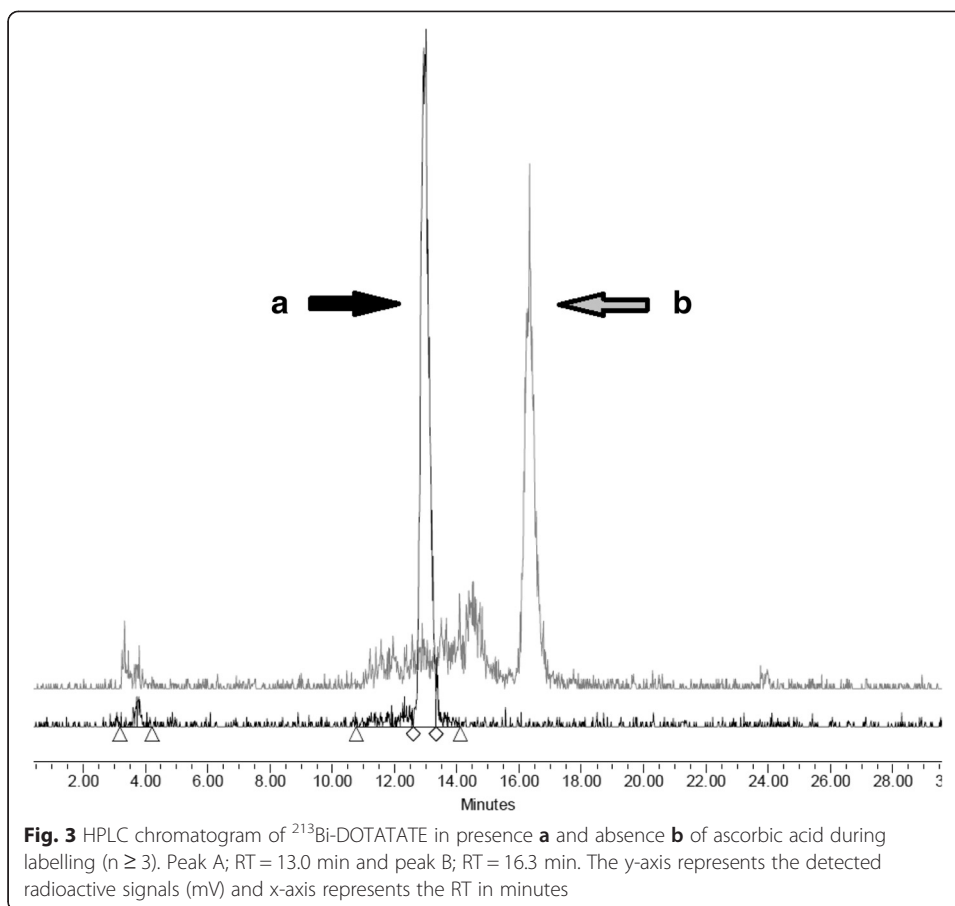
The standard labelling protocol ²¹³Bi-DOTATATE resulted in an incorporation > 99 % and a RCP > 85 % at pH 8.7 for up to 2 h after labelling. The calculated osmolarity of the labelling using the standard procedure was 0.7 Osmol.L⁻¹.

Labelling with 1.7 nmol DOTATATE in 800 μL resulted in low incorporation of 6 ± 4 % and poor RCP of 5 ± 5 % (median: 6.9 % (range: 0–8.6 %)), see Fig. 2. A high incorporation and high RCP were found when labelled with 3.5 nmol (incorporation was 99 ± 1 %, RCP was 89 ± 4 %) and 7.0 nmol peptide (incorporation was 99 ± 1 %, RCP was 88 ± 6 %).

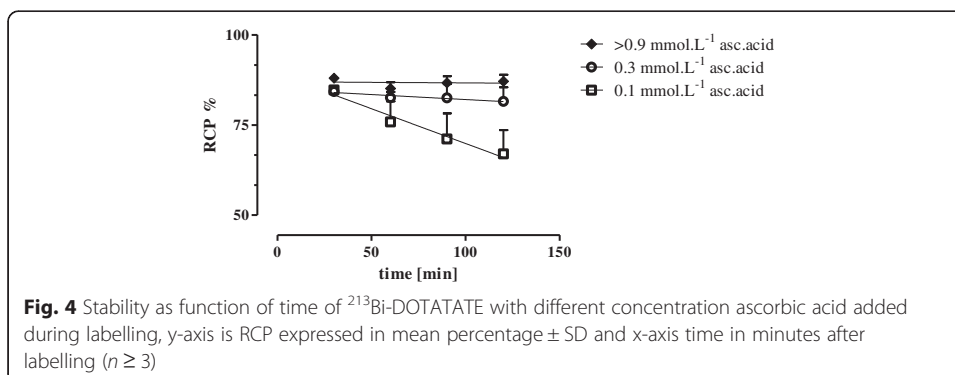
Reducing the amount of TRIS buffer in the labelling did not show an effect on the incorporation of the labelled compound, incorporation of > 99 % and a RCP of > 85 % were found for labelling with 0.15 mol.L⁻¹ and 0.34 mol.L⁻¹ TRIS, corresponding to pH 8.3 and 8.7, respectively.

After labelling in the presence of ascorbic acid under the standard procedure, an incorporation of > 99 % and a RCP of > 85 % were found, Fig. 3a. Labelling in absence of ascorbic acid however showed a high incorporation of 99 ± 1 % but at poor RCP, < 5 % ²¹³Bi-DOTATATE remained intact after labelling, as shown in the HPLC chromatogram, Fig. 3b.





To decrease the osmolarity ^{213}Bi -DOTATATE labelling, labelling as function of ascorbic acid was performed, it showed that at least 0.9 mmol.L^{-1} ascorbic acid was required to maintain high incorporation and high RCP ($>85\%$), The labelled peptide showed a RCP $>85\%$ up to 2 h after labelling. A RCP of $75 \pm 8\%$, $83 \pm 1\%$, $87 \pm 1\%$, $86 \pm 1\%$, $86 \pm 1\%$, $87 \pm 1\%$, $88 \pm 0.4\%$ were found by addition of 0.1 mmol.L^{-1} , 0.3 mmol.L^{-1} , 0.9 mmol.L^{-1} , 2.6 mmol.L^{-1} , 7.9 mmol.L^{-1} , 24 mmol.L^{-1} and 71 mmol.L^{-1} ascorbic acid, respectively. Addition of less than 0.9 mmol.L^{-1} ascorbic acid resulted in high incorporation directly after radiolabeling, however the RCP decreased over time, see Fig. 4.



The adjusted labelling conditions in our experiment were 3.5–7.0 nmol peptide, in 0.15 mol.L⁻¹ TRIS buffer containing 2.6 mmol.L⁻¹ ascorbic acid in a reaction volume of 800 μL with 71 ± 28 MBq ²¹³Bi, with a calculated osmolarity of 0.45 Osmol.L⁻¹.

Dosimetry

The absorbed fraction of energy from the α-particles from ²¹³Bi was almost complete within the reaction fluids, 98.9 % for the 10 μL reaction volume and further rose to 99.7 % for 800 μL. The higher energy α-particles from ²¹³Po showed a little more energy transfer to the vial wall; 97.2 % was absorbed in 10 μL and 99.2 % in 800 μL. The absorbed dose rate caused by ²¹³Bi, ²¹³Po, ²⁰⁹Tl and ²⁰⁹Pb were predominantly contributed by the α-particles from ²¹³Bi and ²¹³Po, see Fig. 5a. The β⁻-particles and low energy electrons were less absorbed within the reaction fluid, e.g. the absorbed fraction of energy by β⁻-particles from ²¹³Bi ranges from 44 % (10 μL) to 77 % (800 μL). The relative contribution of the β⁻-particles to the total ²¹³Bi S-value thus raised from 58 to 70 %. The absorbed dose rate caused by α-particles (initially 165 mGy.s⁻¹) continued to be high compared to the absorbed dose rate of β⁻-particles (initially 7 mGy.s⁻¹), see Fig. 5b.

Based on the absorbed dose rate, see Table 2, the estimated absorbed dose for 100 MBq was > 230 Gy at 30 min in a reaction vial containing 800 μL hot reaction fluid. The absorbed dose was predominantly caused by the decay of α-particles, see Fig. 5b.

Clonogenic assay

In all cells treated in present of ascorbic acid (corresponding to 3.4 mmol.L⁻¹ in incubation medium), a significant decrease of survival was found compared to control cells, $P < 0.05$, see Fig. 6. Control cell survival was 100 % with a standard deviation of 12 %. The mean cell survival ± SD of CA20948 after exposure to ascorbic acid or in combination with TRIS, NaI, HCl and DTPA was listed in Table 3. The concentration of TRIS, NaI, HCl, and DTPA in incubation medium was 16 mmol.L⁻¹, 3.6 mmol.L⁻¹, 3.6 mmol.L⁻¹ and 6.1×10^{-3} mmol.L⁻¹, respectively.

Reducing the concentration of ascorbic acid during the labelling, resulted in an increase of cell survival, see Fig. 7.

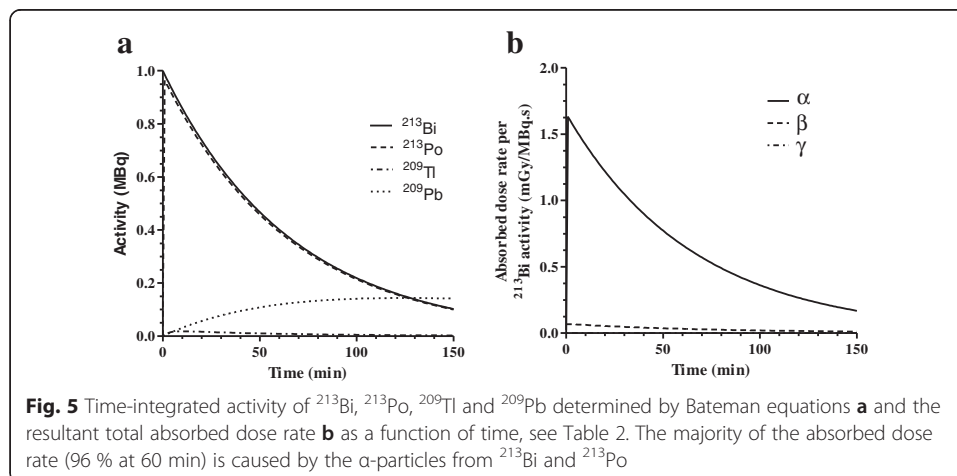


Table 2 Absorbed dose rates per unit activity (S-values, in mGy/MBq.s) in the reaction fluid for different volumes of ^{213}Bi , ^{213}Po , ^{209}Tl and ^{209}Pb . The different contributes to the S-value (α , β , low energy electrons and γ -rays) are indicated separately

^{213}Bi	10 μL	50 μL	100 μL	200 μL	400 μL	800 μL
5.97 MeV α	1.94	0.39	0.19	0.097	0.049	0.024
β -particles	2.98	0.80	0.44	0.24	0.13	0.066
Auger + IC e^-	0.20	0.048	0.026	0.013	0.0067	0.0034
γ -rays	0.0054	0.0019	0.0012	0.0007	0.0005	0.0003
<i>Total</i>	<i>5.12</i>	<i>1.24</i>	<i>0.67</i>	<i>0.35</i>	<i>0.18</i>	<i>0.094</i>
^{213}Po						
8.38 MeV α	130	26.4	13.3	6.6	3.3	1.7
^{209}Tl						
β -particles	3.17	0.94	0.55	0.30	0.17	0.08
Auger + IC e^-	0.36	0.08	0.04	0.02	0.01	0.01
γ -rays	0.04	0.02	0.01	0.01	0.00	0.00
<i>Total</i>	<i>3.57</i>	<i>1.03</i>	<i>0.60</i>	<i>0.33</i>	<i>0.18</i>	<i>0.09</i>
^{209}Pb						
β -particles	2.33	0.53	0.28	0.14	0.07	0.04

Discussion

A cytotoxic dose within the tumour cells is required to cause a therapeutic effect of TAT, when using a receptor-mediated process as dose delivery method. In the clinical situation using β^- -emitters, this cytotoxic dose is in the order of 200 Gy for neuroendocrine tumours (Ilan et al. 2015; Pauwels et al. 2005) and in the preclinical situation this dose is in the order of 70 Gy for cure of CA20948 (rat pancreatic) tumour (Verwijnen et al. 2004). The limited number of receptors available on the cell membrane and relative short half-life of ^{213}Bi however, require radioligands with high SA to obtain a

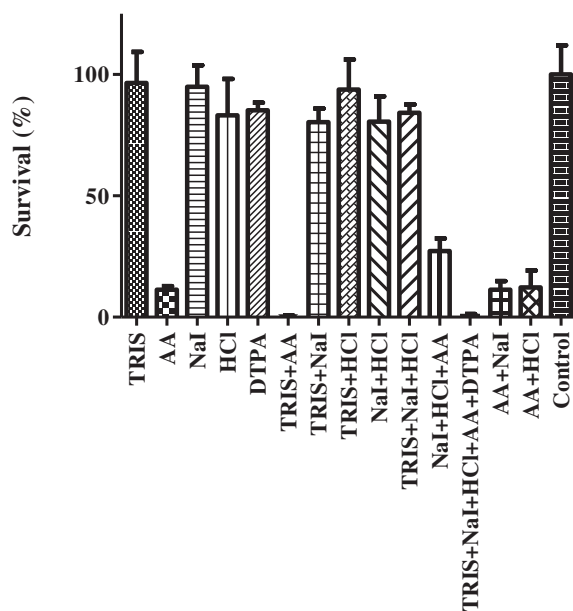


Fig. 6 Survival (%) \pm SD of CA20948 after exposure to the compounds used in standard labelling procedure, $n = 3$. Concentration in incubation medium was; [TRIS] = 16 mmol.L $^{-1}$, [AA] = ascorbic acid, 3.4 mmol.L $^{-1}$, [NaI] = 3.6 mmol.L $^{-1}$, [HCl] = 3.6 mmol.L $^{-1}$ and [DTPA] = 6.1×10^{-3} mmol.L $^{-1}$

Table 3 CA20948 clonogenic survival in mean percentage \pm SD after exposure to ascorbic acid or in combination with TRIS, NaI, HCl and DTPA, $n = 3$. Concentration in incubation medium was; [TRIS] = 16 mmol.L⁻¹, [AA] = ascorbic acid, 3.4 mmol.L⁻¹, [NaI] = 3.6 mmol.L⁻¹, [HCl] = 3.6 mmol.L⁻¹ and [DTPA] = 6.1×10^{-3} mmol.L⁻¹

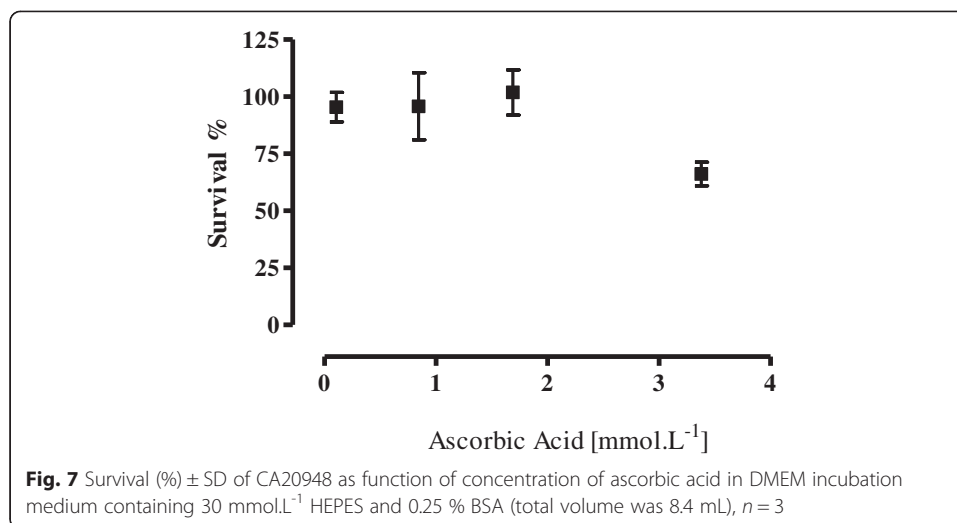
Compounds	Survival %
AA	11 \pm 1
TRIS + AA	1 \pm 1
NaI + HCl + AA	27 \pm 5
TRIS + NaI + HCl + AA + DTPA	1 \pm 1
AA + NaI	11 \pm 4
AA + HCl	12 \pm 7

curative tumour dose. The optimal characteristics of radioligands for TAT applications in a preclinical setting include a low injection volume, physiologic osmolarity, high SA, and suitable stability. Labelling with a radionuclide generator yielding relatively low radioactivity is a barrier for high SA radiolabelling.

The most commonly techniques used to increase the SA are purification of the elution by ion-exchange chromatography or by radiolabelling using fractionated elution to reduce the reaction volume which may improve the reaction kinetics. This may allow high incorporation yield with lower peptide amounts and thus increase the SA of the radio-peptide. To mimic this concept, ²¹³Bi-DOTATATE labelling was performed with half of the elution volume to reach lower reaction volume (total 400 μ L), high incorporation yield was achieved even at reduced amount of peptide, e.g. 1.7 nmol (data not shown).

The most practical method to achieve high SA radiolabelling for preclinical applications is the use of a higher activity ²²⁵Ac/²¹³Bi generator to accomplish the requirements needed for preclinical applications. Purification, e.g. by HPLC or solid phase extraction, might increase the SA. However, introduction of an extra purification step also increases the time interval prior to injection and thus reduces the ²¹³Bi radioactivity. Moreover, HPLC purification often introduces organic compounds e.g. methanol or acetonitrile into the radiolabelling, which might be harmful to animals and limit further translation to clinical studies.

We found a correlation of ascorbic acid on cell survival; at high concentration of ascorbic acid used for labelling (71 mmol.L⁻¹, corresponding to 3.4 mmol.L⁻¹ in incubation medium), a significant reduction of cell survival was observed. A lower reduction of cell survival was found with only or in combinations with NaI, HCl, TRIS and DTPA compared to control cells, probably caused by the high osmolarity of NaI, HCl, TRIS or Zn-depletion to cells by DTPA (Cho et al. 2007), however this reduction was not significant. Ascorbic acid, functioning as pro-oxidant, is known to be selectively toxic, especially in vitro to certain type of tumour cells at high concentration (Park 2013). C₁₈ solid phase extraction can be an option to prevent this by removing ascorbic acid from the labelling reaction. However, radiolysis continues to occur after labelling, and removing ascorbic acid might increase peptide damage caused by radiolysis. After optimizing the labelling conditions, the concentration of ascorbic acid used for labelling (2.6 mmol.L⁻¹, corresponding to 0.13 mmol.L⁻¹ in incubation medium) did not



interfere with the outcomes of *in vitro* studies, therefore direct application can be performed and no purifications steps were required for further *in vitro* and *in vivo* preclinical applications.

In theory, one DOTA-peptide molecule can incorporate one atom ²¹³Bi, thus at a molar ratio of 1 metal *vs* DOTA, under ideal condition the maximum theoretical SA will be 155 GBq ²¹³Bi /nmol DOTA-peptide. In a study investigating the highest achievable SA, 7.0 nmol DOTATATE was labelled with different mole ratios ²⁰⁹/²¹³Bi using the standard labelling procedure. At mole ratio of 0.6 ²⁰⁹/²¹³Bi *vs* DOTATATE, the incorporation was ≥ 99 %. At mole ratio >0.6, the incorporation started to decline, possibly caused by the presence of other cations (derived from decay product of ²¹³Bi, quencher, buffer, elution solution) in the labelling reaction which interfered with the incorporation of ²¹³Bi into DOTA-moiety (Breeman et al. 2005; Zhernosekov et al. 2007). In theory, a SA of 93 GBq/nmol DOTATATE (at mole ratio ²¹³Bi 0.6) can be achieved under these labelling conditions.

Radiolabelling of ²¹³Bi is less pH-dependent (in the range tested) as is the case of ¹¹¹In and ¹⁷⁷Lu with formation of insoluble ¹¹¹InOCl or ¹⁷⁷LuOCl (Breeman et al. 2003). Radiolabelling with ²¹³Bi can be performed at pH values ranging from 4.0 to 10 (Norenberg et al. 2006; Hassfjell et al. 2003; Le Gac et al. 2011), due to the formation BiL₄⁻/BiL₅²⁻ after elution with 0.1 M/0.1 M HCl/NaI (Morgenstern et al. 2012). However, deprotonation of the carboxyl group of the DOTA appears faster at higher pH and resulting in more rapid radiolabelling kinetics (Wu et al. 1997; Szilagyí et al. 2000).

In this study, we highlighted that ITLC-SG alone as quality control is not sufficient, see labelling in absence and presence of ascorbic acid. HPLC provides beside the incorporation yield also information of peptide damage caused by α -track or radiolysis. The formation of free radicals is dependent on the type of radiation, the amount radioactivity present in the reaction mixture and on the duration of exposure, all of which have been shown to induce radiation damage of the peptide. Quenchers such as gentisic acid in combination with ascorbic acid and ethanol are often used to prevent radiolysis in radiolabelling with ¹¹¹In or ¹⁷⁷Lu (de Blois et al. 2012). Radiolysis leads to damage of peptide and will decrease the RCP.

With α -radiation more water radicals, such as peroxide, will be formed besides the direct radiation damage from the high LET α -particle tracks (Deutsch 1998). Stanton et al. observed more biological efficacy on SV40 viral DNA in vitro caused by low LET compared to high LET and Sugo et al. demonstrated less degradation of TODGA, a tridentate complexing agent, after irradiation with helium ion beam than that by that of γ -ray irradiation (Sugo 2009; Stanton et al. 1993). Since the activated radicals are formed along the α -particle tracks and therefore decreasing the probability for damage by both the direct and the radical induced effect in the reaction solution, thus a lower amount of quenchers is required to prevent radical damage to the peptide. In our case, ascorbic acid alone showed sufficient protection capacity to prevent damage to the peptide, so no other quenchers were introduced to the labelling, also to prevent further increase of the osmolarity. Higher osmolarity would require further diluting of the injection matrix for small-animals applications, leading to lower radioactivity injections for effective TAT in small animals.

Here we present detailed results obtained with MCNP calculations of the absorbed dose of ^{213}Bi in the reaction vial. The calculated absorbed dose is an average absorbed dose estimated in a vial of 800 μL , mostly caused by α -particles, but also including doses from β^- -particles and γ -rays emitted by the decay daughters of ^{213}Bi . As shown in Fig. 4, 0.9 $\text{mmol}\cdot\text{L}^{-1}$ ascorbic acid was required to protect the peptide in a vial containing 71 ± 28 MBq in 800 μL , with an absorbed dose of ~ 200 Gy. At higher radioactivities of ^{213}Bi or smaller reaction volumes, more ascorbic acid was required to protect the peptide, since the absorbed dose in the reaction vial increased and formation of radicals also increased. These doses are much lower than the absorbed doses considered to cause radiation damage in DTPA and TODGA, which are in the order of 100 kGy (Sugo 2009; Stanton et al. 1993). The main sensitive region for radiation damage will therefore be in the DOTATATE's disulfide bond and its other ionized parts, as the DOTA cage may be considered not to be damaged at the "low" absorbed doses considered.

The activation energy for the DOTA ring inversion process is in the order of 65 kJ/mol (Lima et al. 2015). The initial dose rate by the α -radiation in the vial with 100 MBq ^{213}Bi amounts 165 mGy/s, which corresponds to an absorbed energy rate of 0.13 mJ/s in 800 μL with 7 nmol compound, or 19 kJ/mol.s. This indicative calculation shows that the momentary dose rate is on average not energetic enough to create configuration changes within the DOTA ring. The energy of 65 kJ/mol is reached within 3.5 s.

Stability, incorporation yield and SA of ^{213}Bi -labelled peptides are influenced by the affinity/stability of the metal-chelator complex, radiolysis or by the recoil effect both during and after labelling. High LET α -particles cause reactive hydrogen radicals in an aqueous environment (Bibler 1972). These radicals damage the peptide, which can lead to losses of affinity of the peptide to its receptor. The relative high recoil energy of ^{213}Po , 160 keV, is a critical challenge for the choice of chelator, since complexation of metal and chelator is dependent on the binding energy. This complex should remain stable until reaching the targeted receptor. DOTA (with a Log K of 30.3) is not considered to be the most suitable chelator for ^{213}Bi , due to relative poor labelling kinetics compared to derivatives of DTPA, e.g. CHX-A-DTPA (Brechtel 2001; Stavila et al. 2006). Nevertheless, we demonstrated that

^{213}Bi formed a highly stable complex with DOTA in a relative short radiolabelling time of 5 min. Furthermore, under optimized radiolabelling conditions, the labelling remained stable up to 2 h after labelling.

Conclusion

The optimized ^{213}Bi labelling conditions demonstrated to be suitable for labelling of DOTATATE and proofed ready-to-use for preclinical applications. The labelling procedure presented herein resulted in high incorporation yield, high RCP and high stability up to 2 h after labelling. The addition of quenchers such as ascorbic acid during labelling appeared essential for the protection of the peptide, since the absorbed dose rate within the reaction was high; $> 165 \text{ mGy}\cdot\text{s}^{-1}$ with 100 MBq of ^{213}Bi .

Additional files

Additional file 1: Bateman equations used to determine the activity of ^{213}Bi , ^{213}Po , ^{209}Tl and ^{209}Pb . (DOCX 17 kb)

Competing interests

Author Ho Sze Chan declares that she has no conflict of interest.
Author Erik de Blois declares that he has no conflict of interest.
Author Mark W. Konijnenberg declares that he has no conflict of interest.
Author Alfred Morgenstern declares that he has no conflict of interest.
Author Frank Bruchertseifer declares that he has no conflict of interest.
Author Jeffrey P. Norenberg declares that he has no conflict of interest.
Author Fred J. Verzijlbergen declares that he has no conflict of interest.
Author Marion de Jong is the chair of the Translational Molecular Imaging and Therapy Committee of the EANM.
Author Wouter A. Breeman declares that he has no conflict of interest.

Authors' contributions

HSC, EB, MWK, AM and FB are involved in the starting up of the study. HSC, EB, MWK and WAP are involved in the study design. HSC, EB and MWK performed the experiments and data analysis. All authors prepared the manuscript, read and approved the final manuscript.

Funding

This study was funded by the DINSE Stiftung, Germany.

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Received: 2 March 2016 Accepted: 5 May 2016

Published online: 14 May 2016

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