RESEARCH ARTICLE

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2018, 9, 1761Development of a [⁶⁸Ga]-ghrelin analogue for PET
imaging of the ghrelin receptor (GHS-R1a)[†]C. L. Charron,^a M. S. McFarland,^a S. Dhanvantari ^{bc} and L. G. Luyt ^{*ade}

The ghrelin receptor is a member of the growth hormone secretagogue receptor (GHS-R) family and is present at low concentrations in tissues such as the brain, kidney, cardiovascular system, and prostate. The ghrelin receptor plays an important role in cellular proliferation, apoptosis, invasion, and migration associated with the progression of many cancers, including prostate, breast, ovarian, testicular, and intestinal carcinomas. Ghrelin, the endogenous ligand, is a 28 amino acid peptide (IC₅₀ = 3.1 nM) known to have poor *in vivo* stability. Herein, we report the synthesis and evaluation of [Dpr³(octanoyl),Lys¹⁹(Ga-DOTA)]ghrelin(1-19). This new ghrelin analogue has a binding affinity (IC₅₀ = 5.9 nM) comparable to that of natural ghrelin. Preliminary *in vivo* evaluation shows higher uptake of [Dpr³(octanoyl),Lys¹⁹(⁶⁸Ga-DOTA)]ghrelin(1-19) in HT1080/GHSR-1a xenografts than the non-transfected HT1080 xenografts in NOD-SCID mice, although considerable uptake is observed in the kidneys. This is the first example of ghrelin receptor PET imaging in a xenograft model using a peptide derived directly from the endogenous ligand and serves as motivation for developing more effective ghrelin-based radiopeptides.

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Introduction

The endogenous ligand for the ghrelin receptor was discovered in 1999 by Kojima *et al.* and subsequently named ghrelin.¹ This 28 amino acid peptide hormone was found to possess a unique post-translational modification that is not commonly seen in other peptide hormones. Through the action of ghrelin-*O*-acyltransferase (GOAT), natural human ghrelin contains an octanoylated serine (Ser) at residue 3 that has been found to be essential for the affinity to the receptor. In fact, without this modification, the IC₅₀ decreases from 3.1 nM (Fig. 1, 1) to >10 μM (Fig. 1, 2).² The highest concentration of ghrelin can be found in the stomach and is responsible for diverse functions, most importantly growth hormone (GH) release, gastric acid secretion, adiposity and gut motility.³

The ghrelin receptor is a member of the growth hormone secretagogue (GHS-R) family that contains two isoforms, types 1a and 1b. GHS-R1a is a 366 amino acid polypeptide and G-protein coupled receptor (GPCR) containing seven transmembrane domains. The third transmembrane domain has been recognized as the ligand-receptor binding pocket for human ghrelin and therefore we refer to it as the ghrelin receptor.⁴ In contrast, GHS-R1b is a 5-transmembrane receptor that has no binding affinity to human ghrelin and is not known to exhibit any biological functions.⁵ The ghrelin receptor is present at low concentrations in tissues such as the brain, kidney, cardiovascular system and prostate.^{3,6}

In addition to regulating the growth and differentiation of normal tissues, the ghrelin receptor has been shown to play important roles in proliferation, apoptosis, cell invasion and migration associated with cancer progression.^{7–12} More specifically, it is expressed at higher levels in prostate, breast, ovarian, testicular and intestinal carcinomas when compared to healthy tissue.^{13–16}

Structure-activity studies on native ghrelin have shown that in addition to octanoylation, the N-terminal portion of the peptide is required for receptor interaction.^{2,16–21} For example, the binding affinities of C-terminally truncated ghrelin(1-14) and ghrelin(1-5) were 9.6 nM and 55 nM (IC₅₀), respectively.² The utility of lower molecular weight ghrelin analogues in detecting the ghrelin receptor has been demonstrated in both prostate cancer and cardiomyopathy using peptides with an optical-dye tag. Fluorescein-ghrelin(1-19) showed 4.7-times higher localization in cancerous prostate

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Fig. 1 The structure of natural ghrelin(1-28) (1), des-acyl ghrelin(1-28) (2), optical-dye (3) and gallium-labelled ghrelin analogues (4) as well as the corresponding IC_{50} for the ghrelin receptor. *Reported as EC_{50} .

tissue than normal and benign hyperplasia tissue.¹⁷ In another study, cyanine5-ghrelin(1-19) was able to track the expression of the ghrelin receptor in P19-derived cardiomyocytes.²² Both dye-labelled analogues retained receptor affinity, with IC_{50} of 9.5 nM for the fluorescein analogue and 25.8 nM for the Cy5 analogue (Fig. 1, 3).²² There is also a growing body of literature describing ghrelin analogues as PET and SPECT radiolabelled peptides. [^{68}Ga -NODAGA]ghrelin(1-16) (Fig. 1, 4) exhibited good receptor affinity and the biodistribution profile was evaluated in male Wistar rats along with metabolic stability studies.²³ Ghrelin was also developed as a SPECT radiolabelled peptide by incorporation of technetium-99m into the ghrelin sequence using various chelating moieties. These analogues also demonstrated good receptor affinity as well as good biological properties for further development of ghrelin into diagnostic radiopharmaceuticals.²⁴

To date, gallium-68 has been incorporated into many peptides, such as bombesin and somatostatin, to visualize various tumour models using PET.²⁵ Gallium-68 is a preferred isotope for clinical imaging because it has a short half-life (68 minutes), decays 89% by positron emission, and is available on-site from a generator.

Based upon our prior work on ghrelin(1-19) analogues as fluorescent probes, we hypothesized that a similar approach using a ^{68}Ga -DOTA complex would provide a high affinity radiolabelled peptide capable of detecting the ghrelin receptor *in vivo* using PET imaging. Herein, we describe the synthesis and evaluation of [Dpr³(octanoyl), Lys¹⁹(Ga-DOTA)]ghrelin(1-19) as well as *in vitro* and preliminary *in vivo* results.

Results and discussion

Design and synthesis

The published structure-activity studies on the ghrelin amino acid sequence indicate that five or more N-terminal amino acids are required for affinity to GHS-R1a. In 2009, we

reported an investigation of the PET and SPECT potential of ghrelin(1-14) analogues by functionalizing the peptide with fluorine-19 and rhenium-185/187 in various locations.¹⁶ Installing a fluorine-19 at the end of a Dpr³(*n*-dodecyl) chain resulted in the highest affinity analogue (IC_{50} = 27.9 nM) but when a larger rhenium chelating group was incorporated in a similar location, the binding affinity decreased (IC_{50} = 174 nM). This has led to investigating other areas of truncated ghrelin analogues capable of accommodating a chelating moiety without negatively affecting binding affinity. Using a lysine to incorporate a chelator is ideal for two reasons; the amine can be easily functionalized using facile chemistry and the 4-carbon side chain provides space between the peptide backbone and the chelator. As described in earlier publications, ghrelin(1-19) can tolerate modifications to the side-chain of lysine-19 while maintaining binding affinity to the ghrelin receptor.^{17,22} Therefore, we proposed the installation of a tris-*tert*-butyl 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (DOTA) chelator on the side chain of lysine-19 to permit gallium-68 radiolabelling of ghrelin(1-19).

The DOTA-ghrelin(1-19) analogue (7) was synthesized as the precursor for subsequent coordination with gallium-69/71 and gallium-68. The first 19 N-terminal amino acids were synthesized using automated Fmoc solid-phase peptide synthesis. To reduce the occurrence of *in vivo* enzymatic hydrolysis of the Ser octanoyl ester, Ser-3 was replaced by diamino-propionic acid (Dpr). Orthogonal protecting groups were installed at Dpr-3 and Lys-19 for selective modification of the side chain amines on-resin (Scheme 1). Octanoic acid was coupled to Dpr-3 to mimic the natural octanoyl modification (5) and DOTA was coupled to Lys-19 to incorporate a radionuclide *via* a pendant labelling approach.²⁵ The fully modified analogue was cleaved from the resin and purified by preparative HPLC resulting in peptide 7.

The non-radioactive [$^{69/71}Ga$]-7 standard was synthesized using gallium(III) nitrate. This analogue is used as a non-

Scheme 1 Synthesis of [Dpr³(octanoyl), Lys¹⁹(DOTA)]ghrelin(1-19) (7).

radioactive alternative for *in vitro* studies and as a chromatography standard for radiochemistry.

Radiochemistry

Radiolabelling conditions were optimized (see ESI[†], Table S1) to maximize specific activity. In all cases, the reaction mixture was heated at 90 °C for 15 minutes before Sep Pak reverse-phase purification. Radiolabelling of 50 µg of 7 in 1 M HEPES buffer resulted in the best yield of 96% (d.c) but did not result in a desirable specific activity. Reduction of the amount of 7 to 20 µg resulted in yields of 54-83% (d.c) and resulted in the highest specific activity of 10.2–22.8 GBq µmol⁻¹. The identity of the radiolabelled [⁶⁸Ga]-7 was confirmed by co-injection with [^{69/71}Ga]-7 on RP-HPLC resulting in consistent retention times for the radiolabelled and product standard (Fig. 2).

In vitro analysis

Competitive binding assays were carried out on 7, and [^{69/71}Ga]-7 against [¹²⁵I]-ghrelin(1-28) to determine ghrelin re-

Fig. 2 Analytical HPLC traces. [^{69/71}Ga]-7 standard (dashed) was co-injected with [⁶⁸Ga]-7 (dotted) to confirm the identity of the isolated radiolabelled product.

ceptor affinity (Fig. 3). The natural ligand 1 has the best binding affinity to the ghrelin receptor of 3.1 nM. When comparing 7 and [^{69/71}Ga]-7, [^{69/71}Ga] chelation improves the binding affinity from 14.9 nM to 5.9 nM. Matsumoto reports that elimination of the C-terminal carboxylic acid by amidation results in an eight-fold increase in potency to GHS-R1a.¹⁸ This same trend is observed upon [^{69/71}Ga] chelation to 7. The synthesized standard, [^{69/71}Ga]-7, possesses comparable binding affinity to the endogenous ligand 1.

Serum stability

Natural human ghrelin(1-28) is known to degrade in human serum rather quickly, with a human serum half-life of 240 min.²⁶ Previously mentioned modifications, such as amidation of the C-terminus and substitution of an ester linkage to an amide linkage, are known to increase the serum stability of peptides. Serum stability of [^{69/71}Ga]-7 was performed in human serum over a 24 hour period. After 360 minutes, no loss of [^{69/71}Ga]-7 was observed, showing increased stability when compared to ghrelin(1-28). After 24 hours, 71 ± 8% remained intact while the only other metabolite observed was the demetalated analogue. Therefore, the aforementioned modifications to natural ghrelin resulted in improved serum stability for [^{69/71}Ga]-7.

Fig. 3 Competitive binding curves. HEK-293 cells expressing the ghrelin receptor were incubated with 1, 7 and [^{69/71}Ga]-7 in the presence of [¹²⁵I]-ghrelin(1-28). IC₅₀ values were 3.1 nM, 14.9 nM and 5.9 nM, respectively.

In vivo evaluation

Preliminary *in vivo* studies were performed in NOD-SCID male mice with two cell lines used for xenograft studies: HT1080 stably transfected with GHS-R1a (HT1080/GHS-R1a, $n = 1$) and parental HT1080 ($n = 1$). Each mouse was injected with 7–10 MBq of [^{68}Ga]-7 in a saline solution and immediately underwent a 60 minute PET dynamic scan. The dynamic scans were processed into 10 minute intervals to monitor the uptake and accumulation of [^{68}Ga]-7 in the tumour and other organs and tissues. A coronal PET image (Fig. 4, A) and a three-dimensional projection (Fig. 4, C) from the small animal PET scanner was taken at the 30 to 40 minute time interval in the negative control HT1080 mice. Uptake was highest in the kidneys (k) and bladder (b). The tumour (t) is present on the right flank and accumulated a small amount of residual radioactivity. The residual radioactivity can be attributed to circulation of the [^{68}Ga]-7 and its metabolites through the highly vascularized tumour rather than association to receptors in the HT1080 cell line. Images were also acquired in the same way in mice bearing positive HT1080/GHS-R1a tumours (Fig. 4, B and D) Similar to the control, there was high uptake in the kidneys (k) and the bladder (b). More radioactivity was localized and retained in the tumour (t) on the left flank. The localization of radioactivity to the kidneys is a common trait seen in radiolabelled peptides, as this is known to occur through tubular reabsorption.²⁷

To more accurately compare the uptake of [^{68}Ga]-7 in both tumour models, standard uptake values (SUV) were calculated by designating volumes of interest (VOI) for each xenograft over 10 min intervals using AsiPro software. The SUV

Fig. 5 Graphical representation of standard uptake values (SUV) for [^{68}Ga]-7 in HT1080/GHS-R1a and HT1080 xenografts over 60 minutes. Each value represents $n = 1$.

for HT1080/GHS-R1a xenografts was approximately 5 times greater than the HT1080 xenografts (Fig. 5). These results show [^{68}Ga]-7 is selective to xenografts with higher GHS-R1a expression compared to those with limited receptor expression, such as the control HT1080 cell line.

Although these results show promise for imaging GHSR-1a using a ghrelin-based radiopeptide, this particular analogue has a poor pharmacokinetic profile that was not supportive of further *in vivo* evaluation. Therefore, a complete *in vivo* study was not pursued but rather, efforts were focused on creating shorter ghrelin analogues to optimize the pharmacokinetic profile of these analogues.²¹

Conclusions

The objective of this work was to develop a ghrelin-based PET imaging probe capable of targeting GHS-R1a with comparable affinities to the natural ligand. Truncating ghrelin (1) to the first 19 N-terminal amino acids and modifying positions 3 and 19 increased the stability and introduced a bi-functional chelator, and maintained a high affinity to GHS-R1a. Competitive binding studies determined that the $^{69/71}\text{Ga}$ -ghrelin complex had an improved IC_{50} value compared to the non-coordinated 7. When evaluated *in vivo*, [^{68}Ga]-7 showed localization in HT1080/GHS-R1a xenografts within the first 30 minutes of circulation, but not in the HT1080 negative control. The SUVs over 60 minutes revealed approximately five times greater uptake [^{68}Ga]-7 in the presence of GHS-R1a. The high localization of [^{68}Ga]-7 in the kidneys suggests the need for further optimization of this ghrelin analogue. This is the first example of ghrelin receptor PET imaging of a xenograft model using a peptide derived directly from the endogenous ligand. Our work demonstrates that natural ghrelin can be used as a targeting peptide for detecting the ghrelin receptor, and addresses the need to continue developing new ghrelin analogues for diagnostic imaging.

Experimental

Materials and methods

All common solvents were purchased from Fisher Scientific. All protected amino acids, coupling reagents and resins were

Fig. 4 A 60 minute dynamic uPET scan showing uptake of [^{68}Ga]-7 from 30–40 minutes in HT1080 and HT1080/GHS-R1a xenografts. (A) HT1080 – coronal. (B) HT1080/GHS-R1a – coronal, (C) HT1080 – 3D projection and (D) HT1080/GHS-R1a – 3D projection. t: tumour, k: kidney, b: bladder.

purchased from Novabiochem, Peptides International and Chem-Impex and were used without further purification unless otherwise stated. All reagents were obtained from Sigma-Aldrich with the exception of gallium(III) nitrate hydrate that was obtained from Strem Chemicals, HEPES free acid that was obtained from AMRESCO and tris-*t*-butyl DOTA that was obtained from CheMatech. RP-tC18 Sep-Pak SPE cartridges were purchased from Waters. [¹²⁵I]-Ghrelin was purchased from Perkin Elmer. The germanium/gallium generator and automated synthesis unit were purchased from Eckert and Ziegler Strahlen- und Medizintechnik AG. For analytical HPLC-MS, a Sunfire RP-C18 4.6 × 250 mm, 5 μm column was used. For preparative HPLC-MS work, a Sunfire RP-C18 19 × 150 mm, 5 μm column was used. A gradient solvent system was used containing 0.1% TFA in acetonitrile (solvent A) and 0.1% TFA in water (solvent B). For analytical UHPLC-MS, studies were performed on a Waters, Inc. Acquity UHPLC H-Class system, combined with a Xevo QT of mass spectrometer (ESI+, cone voltage = 30 V). For analytical UHPLC-MS studies, a Waters Acquity UHPLC BEH C18 2.1 × 50 mm, 1.7 μm column was used with a gradient solvent system consisting of 0.1% formic acid in acetonitrile (solvent C) and 0.1% formic acid in water (solvent D). Analytical radio-RP-HPLC (Sunfire™ RP-C18 column 4.6 × 150 mm, 5 μm) was performed on a Waters 1525 Binary HPLC pump containing a Waters 2487 dual λ absorbance detector, Waters in-line degasser, a gamma detector and Breeze software (version 3.30).

Synthesis of [Dpr³(octanoyl), Lys¹⁹(DOTA)]ghrelin (1-19) amide (7)

Peptide synthesis was carried out manually using Fmoc-based solid-phase peptide chemistry. Peptides were synthesized at a 0.1 mmol scale on Rink Amide MBHA resin (0.51 mmol g⁻¹). The resin was initially swelled with *N,N*-dimethylformamide (DMF), followed by Fmoc deprotection using 2 mL of 20% piperidine in *N,N*-dimethylformamide (DMF) for two cycles (10 minutes, 5 minutes). Amino acids were preactivated by combining 3 eq. of Fmoc-protected amino acid, 3 eq. of HCTU and 6 eq. of *N,N*-diisopropylethylamine (DIPEA) in 2 mL of DMF. The mixture was added to the resin and coupled for 30 minutes and repeated again for 60 minutes. These cycles were repeated until all 19 N-terminal amino acids were coupled to the resin (5).

Methyl trityl deprotection was carried out by mixing the resin with 2 mL of 2% trifluoroacetic acid (TFA) + 5% triisopropylsilane (TIS) in dichloromethane (DCM) solution for 2 minutes and repeating for 8 cycles, or until the yellow colour no longer persisted. To neutralize residual TFA, the resin was treated with 200 μL of DIPEA in DMF for 5 minutes. Octanoic acid was coupled to the resulting free amine using 3 eq. octanoic acid, 3 eq. HCTU and 3 eq. of DIPEA in DMF. The mixture was left to couple overnight (6).

Allyloxycarbonyl deprotection was performed under inert atmospheric N₂ conditions. DCM was dried over sieves for 24

hours before adding 1 mL to the resin. 2 eq. of phenylsilane in 1 mL dry DCM was then added to the peptide resin followed by 0.045 eq. of tetrakis(triphenylphosphine) palladium (0) in 1 mL dry DCM. The peptide vessel was removed from inert conditions and allowed to react for 30 minutes. The resulting free amine was coupled to the chelator using 2 eq. of tris-*t*-butyl DOTA, 2 eq. of HCTU and 6 eq. of DIPEA in DMF. The reaction mixture was left to couple overnight.

Full deprotection of 7 was performed by adding a 2 mL mixture of 95% TFA, 2.5% TIS and 2.5% water to the resin and allowed it to mix for 5 hours. The cleaved peptide was precipitated from solution using ice-cold *tert*-butyl methyl ether (TBME) and centrifuged (3000 rpm, 10 minutes) resulting in a crude peptide pellet. The supernatant was decanted and the resulting peptide pellet was dissolved in 20% acetonitrile in water, frozen at -78 °C and lyophilized to a white crude powder. Purification was performed using preparative HPLC-MS on a gradient of 20% to 60% solvent A in B over 10 minutes (see ESI,† Fig. S1). Purity of the resulting peptide was analysed using analytical UHPLC on a gradient of 10% to 60% solvent C in D over 4 minutes. Pure peptide was isolate in a 21% yield. HRMS (ESI+): *m/z* calculated for C₁₁₈H₁₉₅N₃₈O₃₇, [M + 2H]²⁺ = 1370.2927, observed [M + 2H]²⁺ = 1370.1941.

Synthesis of [[Dpr³(octanoyl), Lys¹⁹([^{69/71}Ga]-DOTA)]ghrelin(1-19) amide ([^{69/71}Ga]-7)

Purified 7 (5 mg) was dissolved in 1 mL of distilled water in a 5 mL pear-shaped round bottom flask. Ga(NO₃)₃ (20 eq., 8 mg) was added to the flask and stirred at 60 °C for one hour. The reaction mixture was diluted with 3 mL of distilled water and loaded onto a tC18 Sep Pak SPE cartridge. The cartridge was rinsed with 2 mL of distilled water to remove excess gallium, and [^{69/71}Ga]-7 was eluted from the cartridge with 3 mL of 50% ACN/H₂O (3 × 1 mL aliquots). Each aliquot was analysed by RP-UHPLC to confirm the identity and purity of [^{69/71}Ga]-7 (See ESI,† Fig. S2). Pure [^{69/71}Ga]-7 was isolated in 86% yield. HRMS (ESI+): *m/z* calculated for C₁₁₈H₁₉₃N₃₈O₃₇⁶⁹Ga, [M + 2H]²⁺ = 1403.6979, observed [M + 2H]²⁺ = 1403.8301.

Radiochemistry

A sterile 10 mL reaction vial was loaded with 7 (10–100 μg) in HEPES buffer (0.5–1 M, pH 4); all conditions can be found in Table S1.† Fresh radioactive ⁶⁸Ga³⁺ was eluted from a ⁶⁸Ge/⁶⁸Ga generator using 0.1 M hydrochloric acid (HCl) and trapped on a Strata X C cation exchange column. The column was eluted with 0.1 M HCl in acetone to transfer ⁶⁸Ga³⁺ to the reaction vial. In all cases, the solution was heated to 90 °C for 15 minutes followed by dilution with 3 mL of milliQ water. The diluted mixture was loaded onto a precondition tC18 Sep Pak SPE cartridge to remove unchelated ⁶⁸Ga³⁺. [⁶⁸Ga]-7 was eluted with 1 mL of ethanol into a sterile product vial. Specific activities were calculated by assuming all unlabelled 7 remains present in the product vial. The optimal

manual labelling conditions were obtained with 20 μg of 7 in 1 M HEPES buffer. [^{68}Ga]-7 was obtained in a 54–83% decay corrected yield and specific activity of 10.2–22.8 $\text{GBq } \mu\text{mol}^{-1}$. Radiochemical purity was assessed using RP-HPLC coupled to a gamma detector, prior to use in *in vitro* and *in vivo* assays.

Competitive binding assay (IC_{50})

Ghrelin receptor binding affinities of [$^{69/71}\text{Ga}$]-7 and 7 were determined using a radioligand binding assay, as we have done previously.^{21,28} Assays were performed using HEK293 cells transfected with GHS-R1a and human [^{125}I]-ghrelin(1-28) (PerkinElmer Inc.) as radioligand. Human ghrelin(1-28) was used as reference to ensure the validity of the results. IC_{50} values were determined by nonlinear regression analysis to fit a 4 parameter dose response curve using GraphPad Prism (version 6.0c). All binding assays were performed in triplicate.

Serum stability

[$^{69/71}\text{Ga}$]-7 (1.4 mg) was dissolved in 10 μL of DMSO and vortexed until completely dissolved and warmed in a 37 $^{\circ}\text{C}$ water bath for 10 min. PBS (315 μL) and human serum (125 μL) were also warmed in a 37 $^{\circ}\text{C}$ water bath for 10 min. The PBS and human serum were added to the dissolved [$^{69/71}\text{Ga}$]-7 and vortexed to ensure complete confluence. The mixture was warmed at 37 $^{\circ}\text{C}$ for 10 min to reach equilibrium before removing at 15 μL aliquot as $T = 0$. Removed aliquots were added to 40 μL of 4% NH_4OH dissociation solution before being loaded onto an Oasis HLB extraction plate to remove serum additives. The extraction plate was rinsed with 200 μL of dissociation solution before eluting the peptide with 60 μL of 20% methanol in water. The elution solution was collected for evaluation using LCMS. Samples were injected onto a UHPLC and the area under the curve was calculated to determine the total intact peptide. Aliquots were removed at 0, 60, 120, 240, 360, and 1440 minutes.

In vivo evaluation

The Animal Use Subcommittee of the Canadian Council on Animal Care (CCAC) at Western University approved the protocols for all mouse handling and treatment procedures described in this study (protocol 2012-033). The CCAC is the national organization responsible for setting and maintaining standards for the ethical use and care of animals in science in Canada.

In vivo imaging studies were carried out in two cell lines, HT1080/GHS-R1a (receptor positive, stably transfected) and HT1080 (endogenous expression). HT1080 xenografts were grown on the right upper flank of male NOD/SCID mice by injecting two million HT1080 cells ($n = 1$) while HT1080/GHS-R1a xenografts were grown on the left flank by injecting two million HT1080/GHS-R1a cells ($n = 1$). Tumours of approximately 1 cm in diameter were present two weeks post injection. Prior to the imaging session, animals were warmed using a heat lamp and anaesthetized with <1.5% isoflurane

by inhalation. The mouse head and body were positioned on the scanning bed using medical tape and a catheter was placed in the lateral tail vein for administration of [^{68}Ga]-7. Each mouse was injected with 7–10 MBq of [^{68}Ga]-7. A 60-minute dynamic scan was performed on both cell lines using a Siemens Inveon preclinical PET scanner (Fig. 4A–D).

Small animal PET scans were reconstructed using CarimasTM software developed at the Turku PET Centre (Turku, Finland).²⁹ To reduce variability due to body weight and injected dose, all radiotracer accumulation was quantified using standardized uptake values (SUV) and normalized to the kidney uptake; therefore PET scans are represented as SUV ratio (SUVr) values. SUVs were determined using AsiPro software (Concorde Microsystems, Knoxville, Tenn, USA). Volumes of interest (VOI) were contoured by hand resulting in 3D reconstruction of the tumour. The image-derived radioactivity concentration (Bq cc^{-1}) was determined for each VOI based on the injected dose. SUVs were calculated using the equation, $\text{SUV} = C(t)/[\text{ID}(t)/W]$, where $\text{SUV}(t)$ is the standard uptake value at time t , $C(t)$ is the imaged-derived radioactivity concentration in Bq cc^{-1} , $\text{ID}(t)$ is the injected dose at time t , and W is the weight of the mouse in grams.

Conflicts of interest

There are no conflicts to declare.

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