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Synthesis and evaluation of Lys^1(α,γ-Folate)Lys^3(^{177}Lu-DOTA)-Bombesin(1-14) as a potential theranostic radiopharmaceutical for breast cancer

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HIGHLIGHTS

- ^{177}Lu-Folate-BN improves recognition of breast cancer cells positive to FR and GRPR.
- ^{177}Lu-Folate-BN shows theranostic (imaging and radiation therapy) properties.
- The heterobivalent ^{177}Lu-Folate-BN tracer interacts with different targets on tumors.

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ABSTRACT

The aim of this work was to synthesize Lys^1(α,γ-Folate)-Lys^3(^{177}Lu-DOTA)-Bombesin (1-14) (^{177}Lu-Folate-BN), as well as to assess its potential for molecular imaging and targeted radiotherapy of breast tumors expressing folate receptors (FR) and gastrin-releasing peptide receptors (GRPR). Radiation absorbed doses of ^{177}Lu-Folate-BN (74 MBq, i.v.) estimated in athymic mice with T47D-induced breast tumors (positive to FR and GRPR), showed tumor doses of 23.9 ± 2.1 Gy. T47D-tumors were clearly visible (Micro-SPECT/CT images). ^{177}Lu-Folate-BN demonstrated properties suitable as a theranostic radiopharmaceutical.

1. Introduction

^{177}Lu-labeled molecules with target-specific recognition, have been proposed as a new class of theranostic radiopharmaceuticals because of ^{177}Lu therapeutic (β^-_{max} emission of 497 keV) and diagnostic (γ-emission of 113 and 208 keV) properties (Banerjee et al., 2015; Ferro-Flores et al., 2015). ^{177}Lu-radiopharmaceuticals offer an ideal opportunity for theranostics since the diagnostic dose can be increased to obtain therapeutic effect (Banerjee et al., 2015).

Over the last two decades, several experimental evidences have suggested that the gastrin-releasing peptide (GRP) and other bombesin-like peptides act as growth factors in many types of cancer (Sancho et al., 2011). Overexpression of gastrin-releasing peptide receptors (GRPR) is present in 96% of breast cancer tissues (Dalm et al., 2015). Different bombesin analogs with high affinity for GRPR have been evaluated in preclinical studies for imaging and therapy (Sancho et al., 2011). Clinical studies in women using radio-bombesin derivatives have shown successful radionuclide imaging of breast tumors (Santos-Cuevas et al., 2008; Scopinaro et al., 2002; Shariati et al., 2014; Van de Wiele et al., 2008).

In particular, the GRPR is highly expressed in T47D human breast cancer cells, and that is the reason for which these cells have been used as tumoral models to evaluate new bombesin probes (Dalm et al., 2015; Parry et al., 2007; Prasanphanich et al., 2009).

Folate receptor-α (FRα) is a membrane-bound protein with high affinity for binding and transporting folate into cells. Folate is a basic component of cell metabolism and DNA synthesis and

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repair. Cancer cells, which rapidly divide, have an increased requirement for folate to maintain DNA synthesis, an observation supported by the widespread use of antifolates in cancer chemotherapy (Kelemen, 2006; Teng et al., 2012).

The overexpression of the FRα protein has been confirmed in all clinical breast cancer subtypes comprised of estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), human epidermal growth factor receptor-positive (HER2+), and triple negative (ER^-, $HER2^-$, PR^-) tumors (Necela et al., 2015; O'Shannessy et al., 2012; Zhang et al., 2014). Recent polymerase chain reaction studies confirmed that FRα is highly expressed in T47D cells (Renukuntla et al., 2015).

The development of radiolabeled heterobivalent molecules that interact concomitantly with different targets on tumor cells is an important strategy employed for specific, sensitive, and non-invasive tumor imaging and therapy (Fischer et al., 2013). Therefore, a heterobivalent conjugate of bombesin and folate is expected to improve both the recognition of breast cancer cells positive to FR and GRPR, and its theranostic (imaging and radiation therapy) properties when labeling with ^{177}Lu.

The aim of this work was to synthesize the Lys^1(α,γ-Folate)-Lys^3(^{177}Lu-DOTA)-Bombesin (1-14) (^{177}Lu-Folate-BN) heterobivalent conjugate, as well as to assess its *in vitro* and *in vivo* potential for molecular imaging and targeted radiotherapy of breast tumors expressing folate receptors (FR) and gastrin releasing peptide receptors (GRPR).

2. Experimental methods

2.1. Synthesis of Lys^1(α,γ-Folate)Lys^3(DOTA)-Bombesin (1-14) [Folate-BN]

All reagents were purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA) and were used as received. H_2N-Lys^1Lys^3(DOTA)-Bombesin (1–14) (DOTA-BN) peptide with a purity of > 90% (MALDI+, m/z=1745), was obtained from piChem Laboratory (Graz, Austria). The synthesis and chemical characterization of DOTA-Gly-Gly-Cys-NH_2 (DOTA-GGC) was carried out according to the detailed procedure previously reported with a purity of >95% (MALDI+) m/z=621.12 [M+H]+ (calculated 621.68) (Luna-Gutiérrez et al., 2012).

2.1.1. Activation of the folic acid carboxylate group

Eighty microliters of folic acid solution [2.25 μmol; 1 mg in 80 μL of dimethylformamide (DMF)] was added to a mixture containing 50 μL of 0.2 M diisopropylethylamine (DIPEA, to provide a basic medium) (9.3 μmol; 1.2 mg in 50 μL of DMF) and 50 μL of the carboxylate activating agent [HATU=(*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluoro phosphate); 2.63 μmol, 1 mg in 50 μL of DMF]. The reaction mixture was incubated for 15 min at room temperature (20 °C).

2.1.2. Conjugation

To 80 μL of the above solution (1 μmol of activated folate), 0.5 mg (1.16 μmol) of DOTA-BN, previously dissolved in 20 μL of DMF, was added. The reaction mixture was incubated for 90 min at room temperature (20 °C). DMF was removed under vacuum. The reaction mixture was reconstituted in 0.5 mL of PBS and purified using a size exclusion high-performance liquid chromatography (HPLC, 1 mL loop) system (YMC-Pack-Diol-60, 8 mm × 500 mm I. D., 1 mL/min, PBS/acetonitrile 70/30) with a photodiode array UV–vis detector. The retention time (t_R) for the DOTA-Folate-BN was 12.40 min. For the DOTA-BN and Folate, it was 13.03 min and 16.2 min, respectively. The collected fraction, with a t_R of 13.6 min, was dried under high vacuum, obtaining a slightly yellowish solid. The solid was dissolved with 0.5 mL of PBS to obtain a conjugate concentration of approximately 1 mg/mL. For comparative studies, DOTA-GGC was conjugated to folate under the same procedure.

2.2. Labeling of Lys^1(α,γ-Folate)Lys^3(DOTA)-Bombesin (1-14) with ^{177}Lu [^{177}Lu-Folate-BN]

Radiolabeling was carried out by adding $^{177}LuCl_3$ (50 μl, 1850 MBq, n.c.a., ITG, Germany) to the DOTA-BN-Folate conjugate (100 μg in 150 μL of 1 M sodium acetate buffer, pH 5) and incubated at 92 °C during 20 min. The final solution was diluted with 2 mL of 0.9% NaCl solution containing 20 mg of ascorbic acid. For comparative studies, ^{177}Lu-DOTA-GGC-Folate (^{177}Lu-Folate) and ^{177}Lu-Lys^1Lys^3(DOTA)-Bombesin (^{177}Lu-BN) were also prepared under the same procedure.

2.3. Radiochemical purity

The RP was determined by reversed-phase HPLC on a C-18 column (μBondapak C18 column, Waters) using a Waters Empower system with an in-line radioactivity detector and a gradient of water/acetonitrile containing 0.1% TFA from 95/5 (v/v) to 20/80 (v/v) over 35 min at a flow rate of 1 mL/min. Using this system, free $^{177}LuCl_3$ shows a t_R=3–3.5 min and the radioconjugate, 13–14 min.

2.4. Stability in human serum

To determine the stability of ^{177}Lu-Folate-BN in serum, 200 μL of radiotracer was diluted at a ratio of 1:10 with fresh human serum and incubated at 37 °C. Radiochemical stability was determined by 100 μL samples taken at different time points from 10 min to 24 h for radio HPLC size exclusion analysis (ProteinPak 300SW, Waters, 1 mL/min, PBS). A shift in the radioactivity profile to larger molecular weight indicates protein binding, while lower molecular weight indicates labeled catabolites or serum cysteine binding.

2.5. In vitro affinity studies

A 96-well microplate (Corning, NY, USA) was coated with a 100 μL/well of poly-L-lysine (0.1 mg/mL) and incubated at room temperature (20 °C) for 20 min. The solution was removed and the plate dried at 37 °C. Each well was coated with 100 μL (150 ng) of purified human folate receptor protein (Sigma-Aldrich, SL, USA) or 100 μL (150 ng) of gastrin-releasing peptide receptor/GRPR recombinant protein (Novus Biologicals, LLC, USA) solution in coating buffer (25 mM Tris–HCl, pH=7.4, 150 mM NaCl, 1 mM $CaCl_2$) and was incubated at 4 °C for 20 h. The plates were washed twice with binding buffer (0.1% bovine serum albumin (BSA) in coating buffer). The wells were blocked for 2 h with 200 μL of unspecific protein (blocking buffer, 1% BSA in coating buffer), which was then discharged. Then, 140 μL of binding buffer containing 70 kBq of Lys^1(α,γ-Folate)Lys^3(^{177}Lu-DOTA)-Bombesin (1-14), and appropriate dilutions (from 10,000 nM to 0.01 nM) of Lys^1(α,γ-Folate)Lys^3(DOTA)-Bombesin, were incubated in the wells at 25 °C for 1 h. After incubation, the plates were washed three times with binding buffer. The wells were cut out and counted in a gamma counter. IC_{50} values (the concentration of Folate-BN inhibiting 50% of radioligand binding) were calculated by non-linear regression analysis. Each data point was the average of six wells (n=6).

2.6. In vitro uptake studies.

The T47D human breast cancer cell line (obtained from the