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Comparing two TAG-72 binding peptides previously identified by phage display as potential imaging agents

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Abstract

The aim of this study was to evaluate the targeting property in vitro and in vivo of two TAG-72 binding peptides, previously identified in this laboratory by phage selection using different elution conditions.

Methods—The peptides GGVSCMQTSPVCENNL (A2-6) and NPGTCKDKWEICLLNGG (A3-10) were radiolabeled with ^{99m}Tc using NHS-MAG₃ as chelator or biotinylated. The specificity of the two peptides for the TAG-72 expressing LS-174T cancer cells was demonstrated in vitro both by flow cytometry analysis using the biotinylated peptides and by competitive binding using the ^{99m}Tc -labeled peptides. The in vivo biodistributions of the peptides were evaluated in TAG-72 expressing LS-174T tumor bearing mice by small animal SPECT/CT imaging.

Results—As evidence of specific binding, both peptides showed a significant increase in percent binding with increasing peptide concentration by flow cytometry analysis to LS-174T cells, but not to TAG-72 negative HT-29 cells. The ^{99m}Tc -labeled A2-6 peptide bound LS-174T cells with an IC₅₀ of 46.5nM compared to 420 nM for the A3-10 peptide. In mice, accumulation of both peptides was highest in kidneys and gallbladder. Tumors were clearly visible by SPECT/CT imaging for both ^{99m}Tc -labeled peptides through 60 min, although the tumor accumulation was higher for the A3-10 peptide.

Conclusion—A3-10 peptide with lower, yet reasonable binding affinity compared to the A2-6 peptide, showed both favorable specific binding and tumor accumulation to be considered further as a potential imaging agent for TAG-72 positive cancers.

Keywords

phage display; TAG-72; tumor imaging; peptide biomarker

Introduction

Human cancers over-express receptors on their cell surface, the identification and development of new ligands with specific targeting properties to these receptors is an area of interest in the field of diagnostic cancer imaging. Identifying new radiolabeled peptides that

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specifically target cancer cells of interest, with minimal accumulations in healthy tissues, is a continuing process designed to improve diagnostic imaging and radiotherapy.

The tumor-associated glycoprotein 72 (TAG-72) shows high levels of expression in a number of cancers including colonic adenocarcinoma, invasive ductal cancer of the breast, non small cell lung cancer, epithelial ovarian cancer, as well as pancreatic and gastric esophageal cancers [1] as opposed to trace levels of expression in normal tissues [2,3]. Accordingly, TAG-72 has been previously recognized as an attractive target for the development of antitumor antibodies and peptides in connection with tumor imaging [4–6].

Screening of phage display peptide libraries is an effective technology for identifying novel peptides with high affinity and specificity for cancer targets. In our previous study [7] using the f88-4/cys6 phage peptide library, we identified a phage clone expressing the sequence NPGTCKDKWEICLLNGG (both the phage clone and the expressed peptide are here identified as A3-10), showed evidence of specific binding when presented as the radiolabeled phage to TAG-72 expressing tumor cells in culture and in excised tumor xenografts. In a following study, the same phage library was again used along with a similar selection strategy, but with an extended elution time in the second selection cycle to identify higher affinity binding peptides [8] resulting in the identification of a second phage clone (A2-6) expressing the peptide, GGVSCMQTSPVCENNL. Unlike the A3-10 peptide, the A2-6 peptide was synthesized and in its free form showed specific binding to LS-174T cells [8].

In this investigation, both the A2-6 and A3-10 peptides were synthesized, biotinylated or radiolabeled with ^{99m}Tc and compared in vitro and in vivo as potential diagnostic imaging agents for tumors over-expressing TAG-72.

Materials and Methods

The LS-174T and HT-29 cell lines, both human colon adenocarcinomas, were obtained from the American Type Culture Collection (Rockville, MD). The LS-174T cells were cultured in Eagle's minimal essential medium (MEM) with nonessential amino acids, supplemented with 10% fetal bovine serum (FBS) while the HT-29 cells were cultured in McCoy's 5a medium modified with 1.5 mM L-glutamine, adjusted to contain 2.2 g/l sodium bicarbonate, and supplemented with 10% FBS. The sulfo-NHS-LC-biotin was from Pierce Biotechnology (Rockford, IL). The mouse monoclonal anti-biotin antibody and PE conjugated sheep anti-mouse IgG F(ab')₂ were from Sigma Aldrich (St. Louis, MO). The ^{99m}Tc -pertechnetate was eluted from a ^{99}Mo - ^{99m}Tc generator (Perkin-Elmer, Billerica, MA). All other chemicals were from standard suppliers and were used as received.

Peptide conjugations and radiolabeling

The peptides A2-6 and A3-10 were synthesized commercially in their native L configurations (New England Peptide LLC, Gardner, MA) with an amino hexyl linker on the terminal amine for conjugation and provided at 97% purity as determined by reversed phase C18 HPLC (RP-HPLC).

The peptides were biotinylated by adding 0.5 mg of the peptide, at 2.5 mg/ml in phosphate buffer pH 6.5 to sulfo-NHS-LC-biotin as a dry powder, at a biotin to peptide molar ratio around 20:1. Biotinylation of the peptide was confirmed by RP-HPLC (Vydac, The Nest Group, Southborough, MA) on a Waters system (Milford, MA) equipped with an in-line dual wavelength UV detector (Waters 2487). All analytic runs were performed with a linear gradient over 45 min from 10% to 90% acetonitrile in 0.08% TFA at a flow rate of 1 ml/min. The biotinylated peptides were used without purification since free excess biotin would

be removed in studies involving their use such as the washing steps prior to flow cytometry analysis.

The peptides were conjugated with S-acetyl NHS-MAG₃ (MAG₃) using methods standard in this laboratory [9,10]. In brief, the peptides were dissolved in 0.3 M HEPES buffer (pH 8) to a concentration of 2.5 mg/ml and then added to a tube containing the MAG₃ powder, with a peptide to MAG₃ molar ratio of 1:20. The suspension was mixed immediately on a vortex to a clear solution and left undisturbed for 1 h at room temperature. The product was purified from free MAG₃ on a 1×20 cm Biogel P2 size exclusion column (Bio-Rad, Hercules, CA) using 0.25 M ammonium acetate pH 7.0 as eluant.

The MAG₃ peptides were radiolabeled with ^{99m}Tc using methods standard in this laboratory [9,10]. In brief, 4 μl of the MAG₃ conjugated peptide was added to a combined solution of 45 μl 0.25 M ammonium acetate, 15 μl 50 mg/ml tartrate buffer followed by 2 μl of fresh stannous chloride (SnCl₂·2H₂O) prepared at 10 mg/ml in 10 mM HCl with 1.0 mg/ml ascorbic acid. After vortexing, 2–10 μl ^{99m}Tc-pertechnetate generator eluate was added, and the solution heated at 95 °C for 20 min. The sample was purified on a P2 column. Radiochemical purity was determined by both instant thin-layer chromatography (ITLC) with acetone as the solvent (ITLC-SG; Gelman Sciences, Ann Arbor, Michigan) and by paper chromatography (Whatman no. 1; VWR) with saline as the solvent. The chromatography strips were cut into 1-cm sections and the radioactivity determined in a NaI(Tl) gamma-well counter (Cobra II Auto-Gamma; Packard Instrument Co.). The radiochemical purity for both peptides was greater than 90% as determined by ITLC and paper chromatography. By calculating area under the peak, the radiochemical purity as determined by RP-HPLC was more than 90% for both peptides. The labeled peptide was used either immediately or stored at –80°C.

In vitro measurements

Cell binding was evaluated by flow cytometry analysis as previously described [8]. Briefly, the biotinylated peptides were incubated with LS-174T cells, which express TAG-72 glycoprotein, or HT-29 cells, that show no evidence of TAG-72 and served as a negative control [8], at increasing concentrations from 10^{–7} to 2×10^{–4} mol/l for 1 h with rocking at room temperature. After three washes, mouse anti-biotin antibody followed by PE-anti-mouse antibody was used to detect cell-bound peptides. Analysis was on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Background fluorescence was measured on cells in PBS only or treated with the PE-anti-mouse antibody alone.

An in vitro competitive binding study was performed to evaluate specific binding and to measure the inhibition constant at 50% (IC₅₀) of the two peptides. The LS-174T cells (10⁴) were seeded into a 96-well filter plate (Millipore, Billerica, MA) in culture medium. After an overnight incubation the culture medium was replaced with 150 μl of Dulbecco's PBS (D-PBS) containing 22 ng of ^{99m}Tc-labeled peptide (2×10⁶ cpm/well) and unlabeled native peptide at concentrations varying from 10^{–12} to 10^{–4} mol/l. The plate was incubated at room temperature for 1 h with rocking before the D-PBS along with unbound peptide was removed by vacuum washing, the wells were then washed three times with 200 μl of D-PBS. Finally, the filter membranes were removed and counted in a gamma well counter and the data was analyzed using GraphPad Prism software.

Biodistribution by imaging

The biodistribution of the ^{99m}Tc-labeled A2-6 and A3-10 peptides was evaluated in mice with LS-174T tumors in the left shoulder by imaging on a NanoSPECT/CT small animal camera (Bioscan, Washington, DC). The ^{99m}Tc-labeled peptides (20 μg, with specific

activity of 1.68 MBq/nmol) were injected in a tail vein ($n = 2-3$ per study group) with imaging 20 min post administration starting with one CT scan followed by two sequential SPECT acquisitions. The CT scanning was performed at standard resolution, using a 45 kVp voltage and 500 milliseconds exposure time. The SPECT image parameters were 1.0 mm/pixel, 256×256 frame size and 60 sec per projection with 24 projections. Acquisition time was approximately 30 min. During imaging, the animal was anesthetized with 1.8% isoflurane in 1.5 l/min O₂. The CT and SPECT reconstructions were performed by InVivoScope 1.37 software (Bioscan, Washington, DC). In the case of the A3-10 peptide, a group of mice were injected with 100 µg unlabeled peptide 30 min before administration of the ^{99m}Tc-labeled A3-10 to determine binding specificity with imaging as described above. Volume of interest (VOI) was drawn around the tumor and normal organs in all images by InVivoScope 1.37 software and the percentage injected dose (%ID) was calculated.

Statistical analysis

The student's t-test was used to test for significance where indicated.

Results

In vitro measurements

Biotinylation of A2-6 and A3-10 peptides was confirmed by RP-HPLC that showed in both cases a shift from 14.3 min for each native peptide to 16.3 min after biotinylation (data not presented). The biotinylated peptides were incubated with TAG-72 positive LS-174T cells and TAG-72 negative HT-29 cells at increasing concentrations for 1 h and binding detected by flow cytometry using a mouse anti-biotin antibody followed by PE-anti-mouse antibody. Fig 1A and 1B demonstrate that both biotinylated peptides showed an increase in percent binding with increasing peptide concentration, but only to the TAG-72 expressing LS-174T cells, indicating specific binding in both cases. At 10⁻⁴ mol/l, the binding was 3.3-fold greater to LS-174T cells compared to the HT-29 cells for the A3-10 peptide ($p < 0.05$) and 1.8-fold greater for the A2-6 peptide ($p < 0.05$).

The in vitro competitive binding study was performed to determine the IC₅₀ of the two ^{99m}Tc-labeled peptides. The LS-174T cells were incubated with 2 × 10⁶ cpm ^{99m}Tc-labeled A2-6 or A3-10 peptide along with increasing concentrations of the respective unlabeled native peptide from 10⁻¹² mol/l to 10⁻⁴ mol/l. As shown in Fig 2, at 10⁻⁴ mol/l of unlabeled peptide, binding of the labeled A2-6 was reduced by 49% and binding of the labeled A3-10 by 73%. The IC₅₀ for binding to LS-174T cells was therefore 46.5 nM (95% confidence interval: 27 nM to 80.2 nM) and 420 nM (95% confidence interval: 199 nM to 862 nM) for A2-6 and A3-10, respectively.

Biodistribution by imaging

Fig 3 shows a SPECT/CT acquisition obtained with ^{99m}Tc-labeled A2-6 and A3-10 peptides in mice bearing LS-174T tumors in the shoulder. At the same color scaling of the SPECT/CT images, tumors are clearly visible with both the ^{99m}Tc-labeled peptides at 30 min through 1 h, and not detectable at 3 h (data not shown). As is evident in the figure, at 30 min the tumor accumulation of ^{99m}Tc-A3-10 was higher at 1.4% ID compared to 0.6% ID for ^{99m}Tc-A2-6 as calculated by VOI. In both cases, the kidneys showed the highest accumulation of radioactivity with intestine and gallbladder also high. Nonspecific retention in other organs was limited. The A2-6 peptide appears to also show higher liver accumulations than the A3-10 peptide.

The ^{99m}Tc-A3-10 peptide was shown to accumulate specifically in tumor cells by competition with excess unlabeled A3-10 peptide (Fig 3). LS-174T tumor bearing mice were

injected with 100 µg unlabeled A3-10 peptide through the tail vein 30 min before the administration of the labeled peptide. The VOI results indicated that 30 min after injection of radiolabeled A3-10 peptide, the tumor accumulation of ^{99m}Tc -A3-10 peptide was blocked by 81%. The excess peptide did not lead to a decrease in peptide accumulation in normal organs including intestine and kidneys. This result provides evidence that the tumor accumulation of the A3-10 peptide was receptor mediated, in contrast to the accumulations in the kidney and intestines.

Discussion

The goal of this project is to identify a number of phages with specificity to TAG-72 and from this group identify those with the best binding properties and pharmacokinetics. The A3-10 and A2-6 phages were selected in earlier selection studies already reported [7,8] using the same f88-4/cys6 peptide library with a similar selection strategy, but in the case of the A2-6 phage with an extended elution time in the second cycle of phage selection in an attempt to select for higher affinity binding peptides. Thus, in the current study, the A3-10 and A2-6 free peptides were biotinylated and radiolabeled with ^{99m}Tc to compare the in vitro binding and the in vivo properties in tumor-bearing mice.

To be useful as a cancer biomarker, an agent must exhibit specific and sensitive binding to the cancer cell of interest such that accumulations in healthy tissues become lower than the target tissue. By comparing accumulations in TAG-72 positive LS-174T cells with accumulations in TAG-72 negative HT-29 cells, both biotinylated peptides demonstrated specific binding (Fig 1) and by measuring the IC_{50} values for the radiolabeled peptides, the binding affinity of the A2-6 peptide to LS-174T cells was found to exceed that of the A3-10 peptide (Fig 2), which is expected since the A2-6 phage was selected with an extended elution time in the second cycle.

By SPECT/CT imaging tumors are clearly visible with the ^{99m}Tc -labeled A2-6 and A3-10 peptides at 30 min through 1 h (Fig 3), with a 2.3 fold higher uptake in tumor for the ^{99m}Tc -A3-10 peptide in comparison to the ^{99m}Tc -A2-6 peptide calculated by VOI at 30 min after administration. This result is unexpected because ^{99m}Tc -A2-6 peptide showed a higher affinity to LS-174T cells compared to ^{99m}Tc -A3-10. ^{99m}Tc -A2-6 peptide showed high liver accumulation which may be due to non-specific binding of the peptide to serum protein and this also maybe one reason for less being available to tumor.

In conclusion, the A3-10 peptide, with reasonable but lower binding affinity compared to the A2-6 peptide, shows favorable specificity of binding and tumor accumulation to be considered further as a potential imaging agent for TAG-72 positive cancers.

Acknowledgments

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Abbreviations

TAG-72	tumor-associated glycoprotein 72
^{99m}Tc	technetium-99m
MAG₃	S-acetyl NHS-MAG ₃
IC_{50}	inhibition constant at 50%
D-PBS	Dulbecco's PBS

VOI volume of interest

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Fig 1.

Flow cytometry analysis of biotinylated peptides A2-6 (A) and A3-10 (B) as a function of concentration from 10^{-7} to 2×10^{-4} mol/l to both LS-174 cells (black bars) and HT-29 cells (grey bars). Mean of $n=3$ with error bars signifying one SD.

Fig 2. Binding of ^{99m}Tc -A2-6 (A) and ^{99m}Tc -A3-10 (B) with increasing concentration of unlabeled native peptide. Mean of $n=6$ with error bars signifying one SD.

Fig 3. SPECT/CT scans at 30 and 60 min post injection of ^{99m}Tc A2-6 or A3-10 peptide in LS-174T tumor bearing mice. Also shown for the A3-10 peptide are images (block) of one animal receiving 100 μg unlabeled A3-10 peptide prior to the labeled A3-10 peptide. Arrows show the location of tumor.