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Preparation and evaluation of the tumor-specific antigen-derived synthetic mucin 1 peptide: A potential candidate for the targeting of breast carcinoma

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ABSTRACT

Purpose: The goal of this study was to prepare a synthetic peptide derived from breast tumor associated antigen and to evaluate its potential as a breast cancer imaging agent.
Methods: A mucin 1 derived peptide was synthesized by solid-phase peptide synthesis and examined for its radiochemical and metabolic stability. The tumor cell binding affinity of ^{99m}Tc-MUC1 peptide was investigated on MUC1-positive T47D and MCF7 breast cancer cell lines. In vivo biodistribution was studied in normal Balb/c mice and in vivo tumor targeting and imaging in MCF7 and T47D tumor-bearing nude mice.
Results: The synthesized MUC1-derived peptide displayed high radiochemical and metabolic stability. In vitro tumor cell-binding on T47D and MCF7 cell lines demonstrated high affinity of ^{99m}Tc-MUC1 peptide towards human breast cancer cells (binding affinities in nanomolar range). Pharmacokinetic studies performed on Balb/c mice are characterized by an efficient clearance from the blood and excretion predominantly through the urinary system. In vivo tumor uptake in nude mice with MCF7 tumor xenografts was 2.77 ± 0.63% ID/g as early as 1 h p.i. whereas in nude mice with T47D human ductal breast epithelial cancer cells, the accumulation in the tumor was found to be 2.65 ± 0.54% ID/g at 1 h p.i. Also tumor lesion was detectable in γ-camera imaging. The tumor uptake values were always higher than the blood and muscle uptake, with good tumor retention and good tumor-to-blood and tumor-to-muscle ratios. A low to moderate (< 5% ID/g) accumulation and retention of ^{99m}Tc-MUC1 was found in the major organs (i.e., lungs, stomach, liver, intestines, kidneys, etc.) in both normal and tumor-bearing mice.
Conclusion: This study suggests that ^{99m}Tc-MUC1 tumor-antigen peptide may be a potential candidate for the targeted imaging of MUC1-positive human tumors and warrants further investigation.

1. Introduction

One of the major challenges of nuclear oncology is to improve non-invasive methods for early and accurate detection of tumors, which is necessary for the success of cancer imaging and therapy [1,2]. To meet this vital challenge, there is a need for a continuous search of new potential targets together with the development of new and more effective tumor targeting agents. Differentially regulated proteins on the surface of cancer cells, such as the tumor-specific antigens, are potential molecular targets for the development of alternative and effective anticancer agents. One such target that is located on the apical surface of normal epithelial cells but gets aberrantly overexpressed in various cancers is human epithelial mucin 1 (MUC1) [2,3]. MUC1 was chosen as a model tumor antigen because it is the best characterized human tumor cell-surface antigen that has been developed as a tumor marker for clinical use, in particular as a marker of human breast cancer [4]. In normal adult female breast, MUC1 is localized on the apical region of duct epithelial cells. However in malignant tissue, MUC1 expression is up-regulated, and its distribution is no longer restrained to the apical region [5]. MUC1 is highly expressed by the majority of cancers [6–8], and in particular, by primary and metastatic breast cancers [6,8]. MUC1 overexpression enhances tumor invasiveness and aggressiveness and promotes metastasis in breast cancers [3,9,10]. Because overexpression of MUC1 correlates with high metastatic potential and poor patient survival, the ability to target such tumors may be highly beneficial in clinical settings [6,8,11]. It is worth mentioning that MUC1 overexpression is not limited to breast cancer alone and so this may have applications for both imaging and treatment of a variety of human tumors.

The measurement of circulating MUC1 levels in the serum, as determined by the CA15–3 assay (approved by the US Food and Drug Administration), has been used to monitor the clinical course of patients with breast cancer during treatment and to detect early disease recurrence; and the elevated levels of serum MUC1 are always linked with poor survival [6,8]. Many studies have examined overexpression of MUC1 as a marker of poor prognosis in breast, prostate, lung and thyroid cancers [6–8,11]. The abnormal overexpression of the transmembrane mucins in various carcinomas and the identification of MUC1 as an

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oncogene have established it as a highly attractive target for the development of peptides, antibodies, vaccines and therapeutic inhibitors. However, there are currently no approved agents directed against MUC1 or the other transmembrane mucins [6]. Nonetheless, there are important leads suggesting that MUC1 is a promising target for the development of vaccines. A number of MUC1 peptide based cancer vaccines are currently in clinical trials [6–8], and it is anticipated that in the near future some of them may find applications in clinical settings. In addition, several anti-MUC1 antibodies have been generated against MUC1. However, their therapeutic potential is currently unknown [6,8]. Development of small peptide-based agents for targeting MUC1 expressing tumors is more desirable because of their low immunogenic response and favorable biokinetics, together with high affinity and selectivity for target receptors.

MUC1 is a breast cancer-associated transmembrane glycoprotein, of which the extracellular domain is formed by the repeating 20-amino acid sequence N-***PDTRP***APGSTAPPAHGVTSA-C [4,12,13]. The unique extracellular domain of MUC1 is defined by the presence of the amino acid sequence PDTRP, which is the minimal MUC1 core peptide sequence (shown in bold above) [2,6,14–17]. The same pentamer sequence is also recognized by several highly tumor-specific anti-mucin monoclonal antibodies [14,18–21], as well as some patient's sera [22]. In addition, it has been suggested that the PDTRP core peptide sequence attains a structure closer to the native conformation and is believed to be immunodominant in humans [8,17–18]. Thus it seems logical to assume that this sequence could be useful to design a MUC1-based peptide for targeting MUC1-positive breast carcinoma. Although no prior information is available about the presence of specific receptors and/or affinity of the tumor antigen derived peptide MUC1 towards the breast tumor, there was an interest to explore the potential of the synthetic peptide derived from the breast tumor-associated antigen. It is anticipated that the high expression of MUC1 on breast cancer would allow target-specific imaging and therapy using synthetic MUC1-derived peptide. It is not possible from this study to determine whether the existence of circulating MUC1 may sequester the MUC1 peptide. The in vivo integrity of the ^{99m}Tc-MUC1 peptide however is clearly demonstrated owing to its favorable biokinetics and good tumor targeting properties.

In this study, we have synthesized by solid-phase synthesis a novel MUC1-derived peptide based on PDTRP sequence and coupled it to Gly-Gly-Cys chelating sequence to facilitate radiolabeling with technetium-99m (^{99m}Tc). In addition, a negatively-charged glutamic acid residue was inserted as a spacer between the peptide and the chelating sequence to keep the chelating-site distant from the receptor-binding region and to avoid any possible steric hindrance resulting from the ^{99m}Tc-MUC1 complex. The use of a negatively charged spacer group is expected to increase the hydrophilicity of the ^{99m}Tc labeled peptide, which often resulted in faster renal excretion and improved target to background ratios. The Gly-Gly-Cys triamide-thiol (N_3S) based chelating sequence is used because of its ease of synthesis and direct coupling to the targeting peptide and its well defined chelation chemistry [23,24]. ^{99m}Tc is the radionuclide of choice in the development of peptide-based radiopharmaceuticals owing to its wide availability (from ^{99m}Tc-generator system), convenient half-life (6 h), and ideal γ-energy (140 KeV) for medical diagnostic imaging [1]. We here present the synthesis, radiolabeling with ^{99m}Tc and in vitro and in vivo evaluation of a new MUC1-derived peptide for the detection of breast cancer.

2. Materials and methods

2.1. General

All standard reagents, solvents and Fmoc-amino acids for the peptide synthesis were purchased from commercial sources and used as received. ^{99m}Tc in the form of sodium pertechnetate [Na $^{99m}TcO_4$] was eluted with sterile 0.9% NaCl from a ^{99}Mo/^{99m}Tc generator (Elumatic III, CIS bio international, Cedex, France). The structure of the synthesized peptide was confirmed by positive-ion electrospray ionization–mass spectrometry (ESI-MS) (Waters Micromass Quattro Premier XE, Manchester, UK). Reversed-phase high performance liquid chromatography (RP-HPLC) analysis was performed on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV-VIS detector (Shimadzu Corporation, Kyoto, Japan), set at 220 nm, a γ-radioactivity detection system and the Lauralite chromatogram analysis program (LabLogic Systems Ltd., Sheffield, UK). Radioactive samples from in vitro and in vivo studies were measured using a γ-counter (Mucha, raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

2.2. Preparation of MUC1-derived peptide

The MUC1-derived peptide (Ac-Gly^9-Gly^8-Cys^7-Glu^6-Pro^5-Asp^4-Thr^3-Arg^2-Pro^1-$CONH_2$) was synthesized manually in a silanized 15 mL glass reaction vessel by solid-phase peptide synthesis following standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry, using Rink amide MBHA (4-methylbenzhydrylamine) resin (100–200 mesh) on a 0.2 mmol scale according to a general method of peptide synthesis described previously [24]. Briefly, the peptide chain was elongated in cycles of Fmoc-deprotection followed by coupling of the subsequent Fmoc-amino acid to the resin. Nine repeated cycles were performed with Fmoc-protected amino acids, in the following order: proline (Fmoc-Pro-OH), arginine (Fmoc-Arg(Pbf)-OH), threonine (Fmoc-Thr(tBu)-OH), aspartic acid (Fmoc-Asp(OtBu)-OH), proline (Fmoc-Pro-OH), glutamic acid (Fmoc-Glu(OtBu)-OH), cysteine (Fmoc-Cys(Trt)-OH), glycine (Fmoc-Gly-OH), and glycine (Fmoc-Gly-OH). After incorporating all the desired amino acids to the sequence, the N-terminal Fmoc-protecting group was removed and the peptide-resin acetylated with acetic anhydride in the presence of triethylamine. Cleavage of the crude peptide and concomitant removal of the side-chain protecting groups was obtained with a mixture of 94% trifluoroacetic acid, 1% triisopropylsilane, 2.5% 1,2-ethanedithiol, and 2.5% water. The purity of the peptide was confirmed by HPLC and its structural identity by mass spectrometry.

2.3. Radiolabeling with ^{99m}Tc

A 50–100 μL of the peptide solution (1 mg/mL CH_3CN/H_2O) was mixed with 200 μL 0.2 M citrate–phosphate buffer (pH 9), 250 μL sodium potassium tartrate (40 mg/mL aqueous solution) and 20 μL 5% ascorbic acid. To this, freshly prepared 100 μL of $SnCl_2.2H_2O$ in 0.05 N HCl (20 mg of $SnCl_2.2H_2O$ in 5 mL of 0.05 N HCl) was added followed by 200 μL of $^{99m}TcO_4^-$ (5–10 mCi). The labeling mixture was then heated at 90 °C for 10 min and allowed to cool to room temperature prior to HPLC analysis. The preparation was filtered through 0.22-μm pore syringe filter to remove any precipitate.

2.4. HPLC purification and analysis

The HPLC analysis and purification of the peptide was performed on a Shimadzu HPLC system using Econosphere C_{18} reversed-phase column (10 μm, 250 × 4.6 mm). For HPLC experiments, a gradient solvent system of 0.1% (*v*/v) TFA (trifluoroacetic acid) in water (solvent A) and 0.1% (*v*/v) TFA in acetonitrile (solvent B) at a flow rate of 1.1 mL/min was used [25]. The main peak of the radiopeptide was isolated, and acetonitrile then evaporated under a stream of nitrogen gas. The HPLC-purified compound was reconstituted in sterile saline and used for in vitro and in vivo experiments.