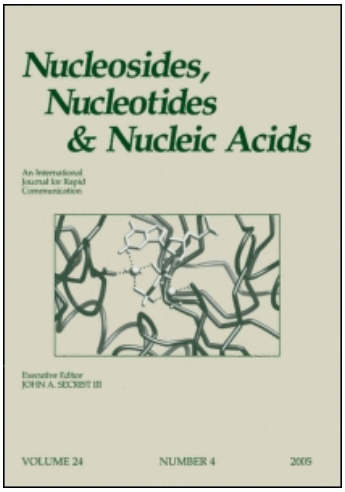

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TUMOR-TARGETING PEPTIDE-PNA-PEPTIDE CHIMERAS FOR IMAGING OVEREXPRESSED ONCOGENE mRNAS

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▫ *We have optimized a method involving continuous solid phase synthesis of chelator-peptide-PNA-peptide probes in order to noninvasively image oncogene mRNAs overexpressed in tumors. The PNA (peptide nucleic acid) probes carry cyclized peptide ligand analogs specific for receptors overexpressed on malignant breast or colorectal cancer cells, and chelators to bind radioactive metal ions, or a fluorophore. In vivo scintigraphic imaging of MCF7 xenografts in immunocompromised mice indicated that CCND1 and MYC [^{99m}Tc]chelator-PNA-D(CSKC) probes concentrated in MCF7 cells up to 7 times more than the corresponding mismatch controls.*

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INTRODUCTION

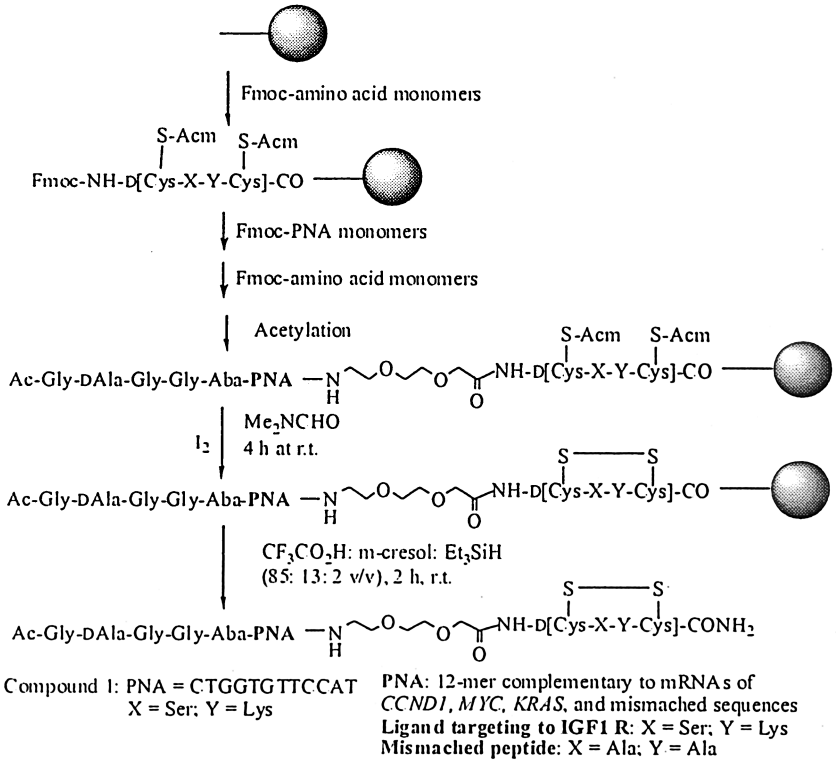
Mammography and physical examination miss up to one half of early breast cancers, the most common noncutaneous cancer in US women.[1] Moreover, if an abnormality is found, an invasive diagnostic procedure must be performed to determine if the breast contains atypia or cancer, even though two thirds of abnormalities are benign.[2] Colorectal cancer is the third most common neoplasm in the United States and the third leading cause of cancer-related mortality, responsible for ~10% of cancer-related deaths in the United States.[3]

Computerized tomography, the noninvasive imaging modality of choice for colorectal cancer, detects only metastases ≥ 5 mm.[4] This sensitivity and specificity is insufficient for early detection of metastases during staging, monitoring of established disease progression, or post-operative surveillance for disease recurrence.[5] Activation of *IGF1R*, *HER2*, *CCND1*, *myc*, and *Kras* oncogenes are early events in malignant transformation and are frequently overexpressed in breast cancer cells and colon cancer cells. *IGF1R* and *CCND1* overexpression are characteristic of estrogen receptor-positive breast cancer cells, while *HER2* and *myc* overexpression are characteristic of estrogen receptor-negative breast cancer cells. Oncogenic mutations in the *Kras* gene are present in approximately 30% of all human cancers.[6] *Kras* mutations occur frequently in the early stage of colorectal cancer. Guanylyl cyclase C (GC-C) is the receptor for *Escherichia coli* heat-stable enterotoxin (STa), an 18-amino acid peptide that is a major cause of secretory diarrhea in animals and humans. GC-C is expressed in adult humans only on the surface of intestinal epithelial cells, not by non-intestinal tissues. Of significance, expression of GC-C has been detected in all primary and metastatic colorectal tumors, but not in any non-intestinal tissues or non-intestinal tumors examined.[7] Radioimaging of receptor-targeted antisense PNA, which combines the superior sensitivity of radionuclide detection and the high specificity of both antisense-sense and ligand-receptor interaction, might detect the alterations in overexpressed oncogene mRNA that are already present in precancerous disease and cancer and identify sites of neoplastic transformation non-invasively at relatively early stages, providing opportunities for early diagnostic and therapeutic interventions.

METHODS

Design and Synthesis of the Antisense Probes for Breast Cancer Imaging

The synthesis of peptide-PNA-peptide chimeras has been reported previously, using a peptide synthesizer and a DNA synthesizer together.[8] However, the scale of the method was limited to 2 μmol due to the limited capacity of the DNA synthesizer. To enable scale-up to 100 μmol and simplify the synthesis, a new method has been developed, using a peptide synthesizer (Scheme 1). Using this



SCHEME 1 Synthesis of peptide-PNA-peptide conjugates.

approach, the coupling time for amino acid monomers is 45–60 min and 35 min for PNA monomers.

Design and Synthesis of Antisense Probes for Detection of Colon Cancer

Studies of structure-activity relations in STa revealed that the toxic domain of 13 amino acid residues from Cys5 to Cys17 has full enterotoxigenic activities, and that the three intramolecular disulfide bonds are between Cys5 and Cys10, Cys6 and Cys14, and Cys9, and Cys17 each modulate the affinity of STa in binding to GC-C.[9] Further chemical modification found that a minimum of two disulfide bridges is required for potent binding to GC-C. The disulfide bond between Cys6 and Cys14 is absolutely required.[10]

Our strategy for the synthesis of colon cancer hybridization probes is 1) to design a relatively simple analog of STa and 2) to optimize the synthetic conditions, in particular for orthogonal cyclization of the two disulfide bonds in the chelator-PNA-STa chimera (Figure 1). Here we report our first steps in this study. Figure 1 shows an STa analog design including the 13 residue toxin domain (compound **2**), in which Cys6 and Cys17 are replaced with two Ala residues.

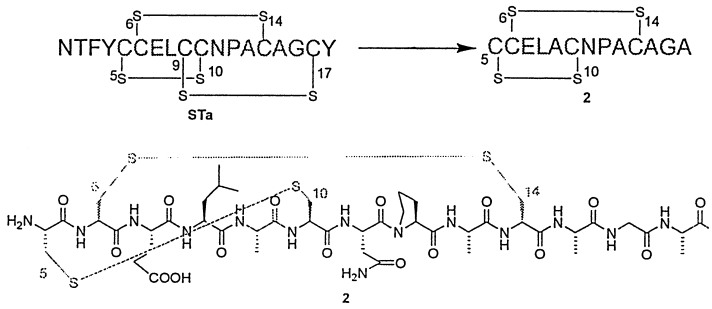
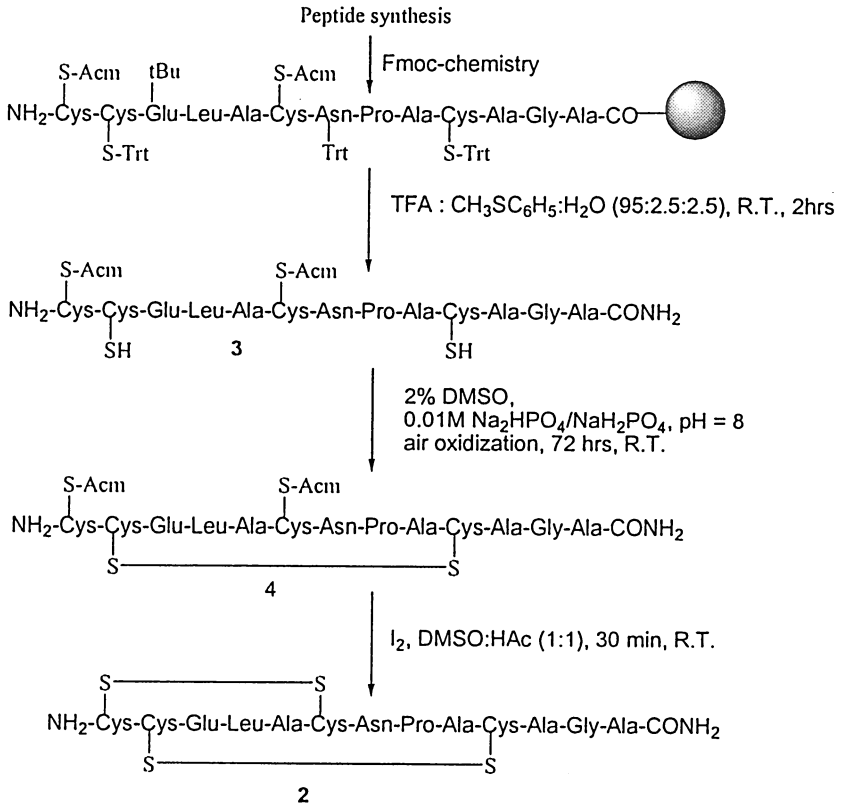


FIGURE 1 Design of an STa analog.

RESULTS AND DISCUSSION

Synthesis of the STa Analog

We tried to cyclize the small loop (Cys5 and Cys10) first by air oxidation, assuming that the smaller loop would be easier to form. Five μmol (6.48 mg) of STa



SCHEME 2 Synthesis of STa analog.

with deprotected Cys5 and Cys10 were dissolved in 10 mL of 0.01 M Na_2HPO_4, pH 8.0 plus 200 μL Me_2SO. However, RP-HPLC and MALDI-TOF analysis did not detect any new compound formed after the solution was stirred for 24 h at room temperature. Then we decided to cyclize the large loop (Cys6 and Cys14) first (Scheme 2). Under the same conditions, 27.3 mg (21.07 μmol) crude peptide solution was stirred for 24 h, a new main chromatographic peak having longer retention time than compound **3** appeared. After 72 h, the starting material chromatographic peak disappeared. At this point, the Ellman test detected no free thiols in the reaction mixture. The reaction mixture was concentrated and purified by HPLC, and then lyophilized to give pure compound **4** as a white powder in a yield of 48.1%. MALDI-TOF indicated the cyclized peptide **4** with mass 1364.4 Da (calculated 1364.6 Da) and M + Na^+ (1386.1 Da). Thirteen mg of purified peptide **4** (9.52 μmol) were dissolved in 6.3 mL Me_2SO:HOAc (1:1), then 12.1 mg I_2 (47.6 μmol) was added to the solution, which was then stirred for 30 min. Saturated aqueous ascorbic acid was dropped into the solution to remove excessive I_2. Purification by RP-HPLC gave 7.4 mg white powder (compound **2**), a yield of 63.4%. MALDI-TOF showed a molecular ion peak of 1220.4 Da (calculated 1220.46 Da).

The measured binding constant K_i of the STa analog **2** to the GC-C receptor on rat intestinal mucosal cells was 6 nM, compared with 1 nM for natural STa. This indicated that the analog bound the receptor with comparable affinity as the natural ligand sufficiently for our hybridization probe to be internalized preferentially by the target colon cancer cells. The synthesis of chelator-PNA-ST peptide is underway.

Human Breast Cancer Xenograft Imaging and Tissue Distribution

Briefly, breast cancer probes were labeled with ^{99m}Tc at room temperature essentially as described previously.[11] In order to assess the conjugate distribution

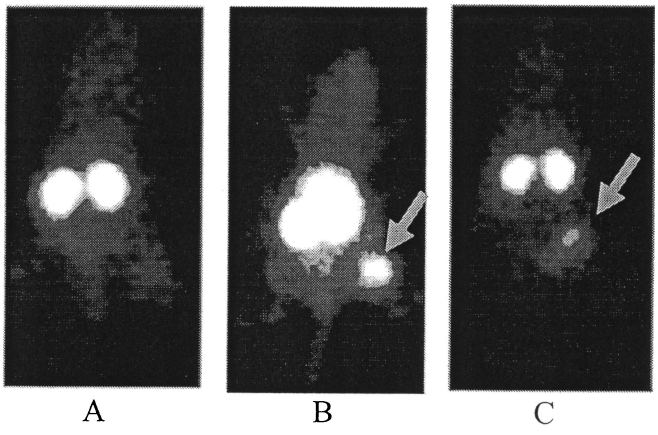


FIGURE 2 Images of MCF7:IGF1R human breast tumor xenografts in nude mice at 4 h. (A), 12 h (B), and 24 h. (C) after injection of [^{99m}Tc]PNA-peptide antisense probe, compound **1**.[12] The green arrows indicate tumors.

and imaging, 0.2–0.3 mCi of the *CCND1* [^{99m}Tc]peptide-PNA-peptide in 0.2 mL of sterile 0.1 M Na_2HPO_4, pH 7, was administered to groups of five mice each through a lateral tail vein using a sterile 27-gauge needle.[12] For the 24 h distribution, 0.8–0.9 mCi of probe was administered. At 4, 12, and 24 h post-injection, mice were lightly anesthetized, then imaged using a Starcam (GE Medical, Milwaukee, WI) gamma camera equipped with a parallel hole collimator. For each image, 300,000 counts were collected (Figure 2).

Mice were then euthanized, and tissues were dissected. These were washed free of blood, blotted dry, weighed, and radioactivity associated with each tissue was counted in an automatic gamma counter, together with a standard radioactive solution of a known quantity prepared at the time of injection. Results were expressed as percent of injected dose per gram of tissue (% I.D/g) (Table 1).[12]

The synthetic strategy described successfully demonstrates that the peptide-PNA-peptide conjugates can be produced in large scale in one run using a peptide synthesizer, in which the various conditions for various amino acid monomers and four PNA monomers were optimized respectively based on our previous work. The efficiency of each coupling was checked by a standard ninhydrin assay. This development also makes it possible to synthesize much longer peptide-PNA-STa analog conjugates by reducing the accumulation of truncated oligomers as much as possible, which result in difficult purifications and low overall yield. To target the GC-C receptor on the surface of colorectal cancer cells, a relatively simple analog of natural STa was successfully designed and synthesized, which retains high affinity for GC-C. In vivo studies performed in human breast cancer xenografts demonstrated that non-invasive mRNA imaging in solid tumors with receptor-targeted peptide-PNA-peptide is both receptor-specific and sequence-specific. This might provide sensitive, oncogene-specific probes for early detection of tumors by non-invasive molecular imaging.

TABLE 1 Tissue Distribution (% Injected Dose/g) of the PNA Antisense Probe, Compound **1**, at 4, 12, and 24 h after Systemic Administiation (n = 5)

Tissues	4 h	12 h	24 h
Muscle	0.12 ± 0.03	0.10 ± 0.05	0.05 ± 0.02
Intestine	0.12 ± 0.01	0.09 ± 0.01	0.05 ± 0.01
Heart	0.11 ± 0.01	0.07 ± 0.02	0.05 ± 0.01
Lungs	0.29 ± 0.03	0.19 ± 0.03	0.09 ± 0.02
Blood	0.23 ± 0.02	0.11 ± 0.02	0.05 ± 0.01
Spleen	0.17 ± 0.02	0.17 ± 0.02	0.12 ± 0.02
Kidneys	21.55 ± 2.90	19.10 ± 3.94	11.33 ± 2.74
Liver	0.52 ± 0.04	0.81 ± 0.10	0.39 ± 0.09
Tumor	0.20 ± 0.06	0.17 ± 0.06	0.11 ± 0.05
T/M Ratio	1.78 ± 0.53	1.85 ± 0.57	2.01 ± 0.29
T/B Ratio	0.88 ± 0.20	1.49 ± 0.34	1.92 ± 0.58

(From Ref. [12]).

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