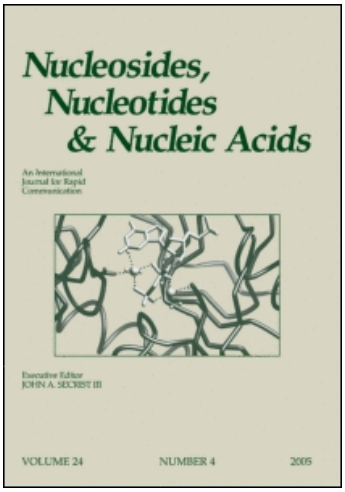

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Synthesis of Novel Peptide Nucleic Acid-Peptide Chimera for Non-Invasive Imaging of Cancer

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SYNTHESIS OF NOVEL PEPTIDE NUCLEIC ACID-PEPTIDE CHIMERA FOR NON-INVASIVE IMAGING OF CANCER

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▫ *A chelator-peptide-PNA-peptide chimera specific for KRAS has been prepared by continuous solid phase coupling with a C-terminal insulin-like growth factor 1 (IGF1) ligand,* D*(cys-ser-lys-cys), and N-terminal bis(*S*-benzoyl thioglycoloyl) diaminopropanoate chelator for radionuclide labeling. The probe was purified by RP-HPLC and characterized by MALDI-TOF mass spectroscopy. The probe was labeled with* $^{99\,m}$*Tc and* ^{64}*Cu. Both labeled probes accumulated in human pancreatic cancer xenografts in immunocompromised mice. Control experiments with mismatch chimeras and control xenografts will be necessary to determine the specificity of this molecular diagnostic strategy.*

Keywords Antisense, Imaging, *KRAS*, Oncogene, Peptide Nucleic Acid, Positron, Radionuclide

Out of 30 thousand people who die from pancreatic cancer every year, ~90% carry 12th codon activating mutations in their *KRAS* oncogenes.[1] Physical examinations, X-rays, computerized tomography (CT) scans, resonance imaging (MRI), blood analyses, and urinalyses, the only generally accepted screening tools available, miss many early cancers. CT and MRI provide precise structural details

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but nothing about function, let alone molecular signature. Imaging gene expression noninvasively with high sensitivity and specificity could identify cell malignancy at an early stage and provide a more powerful diagnostic tool than any available currently. We have observed previously that Tc-99 m-peptides can delineate tumors.[2]

Previous studies showed anti-proliferative activity of *KRAS* phosphorothioate oligonucleotides in vitro against pancreatic cancer cell lines, and in vivo against AsPC1 pancreatic cancer xenografts in nude mice.[3] But phosphorothioates are pleiotropic agents, capable of multiple mechanisms of antiproliferation, and multiple modes of toxicity.[4] Nevertheless, specific tumor uptake of [^{111}In]*MYC* phosphorothioate[5] and [^{68} Ga]*KRAS* phosphorothioate[6] have been asserted, despite their RNase H mechanism.

PNA analogs, on the other hand, are uncharged and do not exhibit the nonspecific interactions or RNase H activation of phosphorothioates.[7] Hence, they do not catalyze degradation of bound mRNA but provide specificity for inhibiting mRNA translation by hybridization arrest alone. These characteristics enable a diagnostic application. Although PNAs are generally poorly internalized by cells,[7,8] PNA assembled with a reversed, inverted D-peptide analog of IGF1, D(Cys-Ser-Lys-Cys), was efficiently internalized by cells expressing IGF1 receptor, a characteristic marker of malignant cells.[9] Peptide-*CCND1* PNA-peptides were taken up similarly by breast cancer cells that overexpress IGF1 receptor.[10] Here we report preliminary results on preparation and accumulation in pancreatic cancer xenografts of a chelator-*KRAS* PNA-peptide chimera labeled with $^{99\ m}$Tc (Figure 1) and ^{64}Cu.

The IGF1 peptide analog D(cys-ser-lys-cys) was extended from C to N by Fmoc coupling[11] of Fmoc-D-amino acids (Novabiochem) on Novasyn TG Sieber resin (9-Fmoc-amino-xanthen-3-yloxy TG resin) (Novabiochem), activated by *O*-(7-azabenzo-triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (Applied Biosystems) using a PS3 peptide synthesizer (Protein Technologies). A spacer Fmoc-aminoethoxyethoxyacetate (AEEA) was coupled, then Fmoc-PNA monomers (Applied Biosystems) were added to create the *KRAS* V12 mutant sequence N-GCCACCAGCTCC-C, followed by two AEEA spacers, then di-

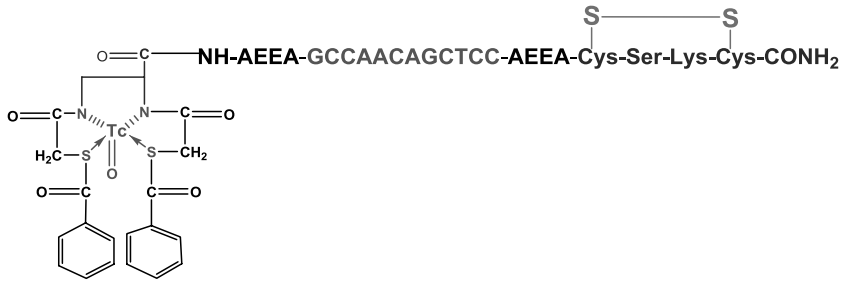

FIGURE 1 N_2S_2 chelator-*KRAS* PNA-peptide chimera.

aminopropanoate (DAP). The chelator moiety S-benzoyl thioglycolic acid (SBTG) was synthesized, re-crystallized from ethyl acetate, and characterized by melting point analysis.[12] SBTG was then coupled on solid phase to both amines of deprotected DAP to yield a *bis*(S-benzoyl thioglycolate) N_2S_2 chelator.[13]

The cysteine thiols of D(cys-ser-lys-cys) were cyclized by iodine in Me_2CHO on the solid support after complete extension, before cleavage with CF_3CO_2H containing 5% m-cresol as radical scavenger.[11] The final product was precipitated from chilled ether, then purified by HPLC on a 10 × 250-mm Varian C_{18} column with a gradient from 5% to 50% CH_3CN in aqueous 0.1% CF_3CO_2H at 1 mL/min, at 50°C. The chimera was characterized by MALDI-TOF mass spectroscopy on a Ciphergen SELDI spectrometer.

For Tc-99 m labeling, 10 μg of the purified SBTG$_2$-DAP-AEEA-GCCAA-CAGCTCC-AEEA-D(cys-ser-lys-cys) in 100 μL of H_2O was added to a mixture of 200 μL of freshly eluted Na[$^{99\,m}$Tc]O_4 (7.50 mCi) with 300 μL 0.05 M Na_3PO_4, pH 12, 0.1% Tween 80, and 10 μg of $SnCl_2 \cdot 2H_2O$ in 15 μL of 0.05 M HCl, then vortexed and incubated for 15 min at room temperature. At the end of the labeling reaction, the pH was adjusted to ≈7 by the addition of 1 mL of 0.05 M NaH_2PO_4, pH 4.5.

Unchelated free $^{99\,m}$Tc (R_f 1.0) of 14.1% was determined by instant thin-layer chromatography on silica gel (ITLC-SG, Gelman Sciences, Ann Arbor, MI) developed with methylethyl ketone. Colloid formation (R_f 0.0) of 4.4% was determined on ITLC-SG developed with pyridine/HOAc/H_2O (3:5:1.5).

For ^{64}Cu labeling, 20 μg of N_2S_2 chelator-*KRAS* PNA-peptide chelator dissolved in 15 μL of water was added to 200 μL of 0.1 M ammonium acetate, pH 5.5, and vortexed. 2 μL of [^{64}Cu]Cl_2 (100 μCi) in 0.1 M HCl, was added to the mixture. The reaction mixture was incubated for 30 min at 90°C. Radiolabelled *KRAS* PNA-peptide probes were analyzed by HPLC on a 4.5 × 250-mm Varian C_{18} column with a gradient from 10% to 100% CH_3CN in aqueous 0.1% CF_3CO_2H at 1 mL/min, at 25°C.

HPLC analysis of the crude N_2S_2 chimera (Figure 1) yielded a main product peak (Figure 2A) with a mass of 4350.5 Da (calculated 4351.09 Da) (Figure 2B). The HPLC purified product was labeled with $^{99\,m}$Tc, eluting at 12 min (Figure 2C). The $^{99\,m}$Tc-probes were administered intravenously to cohorts of nude mice bearing

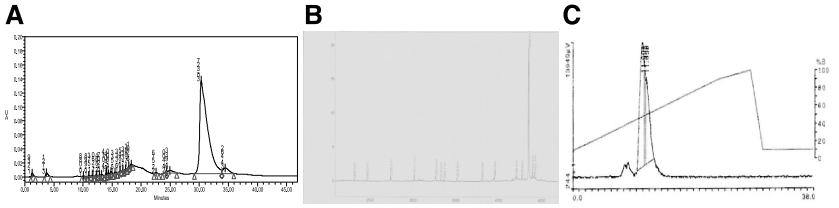

FIGURE 2 N-SBTG$_2$-DAP-AEEA-GCCAACAGCTCC-AEEA-D(cys-ser-lys-cys)-$CONH_2$. A, C_{18} HPLC purification profile; B, SELDI-TOF MS; C, C_{18} HPLC analytical profile of $^{99\,m}$Tc-labeled probe; Percentage of CH_3 CN is shown on the right y-axis, and radiometric gamma emmission is shown on the left y-axis.

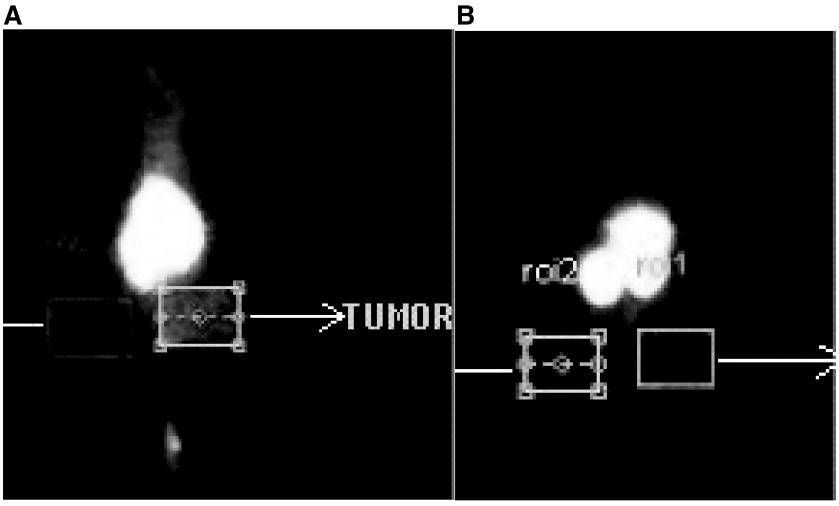

FIGURE 3 Scintigraphic imaging of Tc-99 m-N_2-S_2-*KRAS* PNA-peptide in AsPC1 pancreatic xenografts 4 h (C) and 24 h (D) after probe injection into the tail vein.

AsPC1 human pancreatic cancer xenografts to determine the sensitivity of scintigraphic imaging of the targeted oncogene mRNAs in the tumors, relative to the nonspecific signals expected in the liver, gall bladder, and kidneys 4 h (Figure 3A) and 24 h (Figure 3B) after injection of probe into the tail vein. Scintigraphic imaging of Tc-99 m-N_2S_2-*KRAS* PNA-peptide in AsPC1 pancreatic xenografts showed tumor to muscle image intensity ratios of 5.4 at 4 h (Figure 3A) and 2.7 at 24 h (Figure 3B) after injection of probe into the tail vein.

The N_2S_2 chelator-*KRAS* PNA-peptide chimera was also labeled with ^{64}Cu (Figure 4A) with only approximately 10% labeling efficiency in the first attempt; the labeled probe eluted at 6.8 min (Figure 4B). PET imaging of mice with the ^{64}Cu-N_2S_2-*KRAS* PNA-peptide showed tumor to muscle image intensity ratios of 2.0 at 4 h (Figure 5A), 3.0 at 24 h (Figure 5B), and 3 after injection.

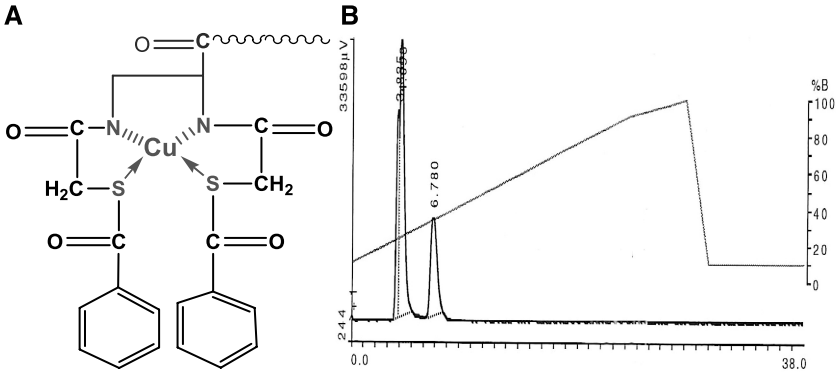

FIGURE 4 ^{64}Cu chelation. A, N_2S_2 chelator structure. B, C_{18} HPLC of ^{64}Cu-N_2S_2-*KRAS* PNA-peptide.

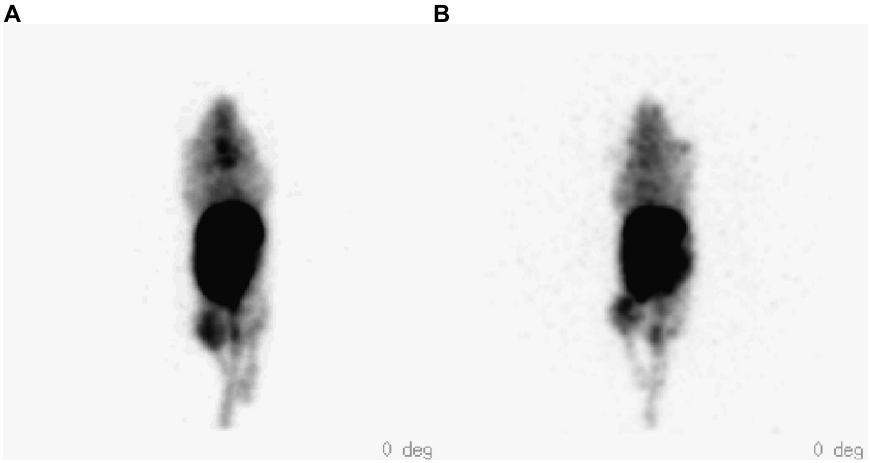

FIGURE 5 PET imaging of ^{64}Cu-N_2S_2-*KRAS* PNA-peptide 4 h (A) and 24 h (B) after injection of the probe into the tail vein.

Necessary control studies with one-mismatch and three-mismatch controls, cellular uptake of probes, probe:RNA hybrid Tm measurements, and reverse transcription of PNA-blocked RNA are underway. These experiments show that the *KRAS* radioprobes can be synthesized and administered to tumor-bearing mice, with subsequent tumor imaging by gamma or PET scanning.

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