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In Vitro Assessment of a Peptide Nucleic Acid (PNA) - Peptide Conjugate Labeled with an Auger-Emitting Radionuclide for Prostate Cell Killing

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The ultimate goal of the research is to develop new treatment reagents for metastatic prostate cancer. Unfortunately, treatment of metastatic prostate cancer is problematic as the cells can become resistant to the therapy being used. Therefore, this exploratory research was directed at developing new therapy reagents that will not be affected by changes within cells. The therapy reagents use a radioactive material, Auger electron emitting radionuclides, to help solve the problem. Auger emission is quite different from emission from other radioactive materials as they have short-range electron emissions that are very toxic to cells and are not affected by other biological factors. Importantly, the very short particle range can greatly decrease the toxicity outside of the cells that it is targeted to. The studies focused primarily on the synthesis of a peptide nucleic acid (PNA) that has an Auger-emitter (I-125) incorporated. By design the PNA will bind with mRNA and DNA associated with insulin-like growth factor receptor that is upregulated on PC-3 prostate cancer cells. The first objective was to obtain a thymine analog, an iodouracil-amino acid for incorporating into a PNA. This was accomplished in 8 synthetic steps. However, the tri-n-butylstannyluracil-amino acid required to couple with the PNA could not be prepared in the same manner. Therefore, a second synthetic route was undertaken which proved to be successful. The iodouracilamino acid derivative and tri-n-butylstannyluracil amino acid derivative were coupled with a free amine on the PNA to form new PNA molecules (iodouracil conjugate was used as standard for radioiodinated derivative). The stannyl-PNA was readily radioiodinated to give the desired [125I]odo-PNA. To obtain selective targeting to PC-3 prostate cancer cells and cause internalization of the PNA, the peptide bombesin was coupled to the PNA. Because the PNA have very low solubility in water, a polyethylene glycol (PEG) linker was used between the PNA and bombesin. Evaluation of the cell killing abilities of the radioiodinated PNA are underway.
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I. Introduction:

These studies have been conducted as a preliminary step in the development of new therapeutic agents that are designed to improve the survival rates for prostate cancer patients with metastatic disease. Survival rates for prostate cancer drop from nearly 100% for cancers localized in the prostate to only 34% when metastases are present in other tissues (1). Unfortunately, treatment of metastatic prostate cancer is problematic as the cells can become resistant to the therapy being used. Therefore, new therapeutic agents that are not affected by changes in the cells are needed. In the studies, we used a specific type of radioactive material, an Auger electron-emitting radionuclide iodine-125 ($^{125}$I)(2). It is believed that Auger-emitting radionuclides targeted to nuclear material may hold the answer to the problem of cellular resistance. Auger emission is quite different from emissions from other radioactive materials as they have short-range electron emissions that are very toxic to cells when internalized, and are not affected by biological factors that cause resistance. Importantly, the very short particle range can greatly decrease the toxicity outside of the cells that it is targeted to. Our approach is to design a pharmaceutical that carries an Auger-emitting radionuclide and binds with the DNA in the cancer cell nucleus. Since Auger electrons have been shown to break DNA, such an agent could specifically kill the prostate cancer cell, or at a minimum cause damage to a gene (or gene products) that promote cancer growth.

In the exploratory studies, we have prepared a new type of radiopharmaceutical that is specifically designed to target prostate cancer cells, then be internalized and bind with messenger RNA in the cytoplasm and/or DNA in the nucleus. The radiopharmaceutical contains $^{125}$I, on a targeting agent composed of a peptide nucleic acid (PNA)-peptide conjugate. Targeting to, and internalization in, prostate cancer cells is accomplished by the peptide portion of the conjugate. The peptide bombesin, can bind with cell surface gastrin-releasing peptide receptors and be internalized (3). Binding with mRNA and nuclear DNA specific to the insulin-like growth factor type 1 receptor (IGF-1R) is accomplished by an “antisense” PNA portion of the conjugate (4). When bound with IGF-1R mRNA or DNA, the radioiodine is physically located in close proximity to the phosphate backbone of the oligonucleotides, and decay of the radionuclide can cause extensive breakage of the backbone(s).

The following research report uses to statement of work to point out the progress made.

II. Research Conducted:

There were three objectives stated in the proposal. Those objectives, and the steps outlined to accomplish them in the statement of work, are listed in the following paragraphs. The progress and difficulties encountered in the research are provided after each objective.

Objective 1. Synthesize a stannyluracil (thymidine) analog that can be incorporated into a PNA and subsequently be radioiodinated (Months 1-3).
   a. Order chemicals (including PNA and bombesin) and synthesize glycine-ethylamine precursor (compound 5), months 1 & 2.
   b. Prepare iodouracil adduct with glycine-ethylamine having N-tBoc and tetrafluorophenyl ester (compound 9), months 2 & 3.
   c. Prepare stannyluracil derivative (compound 10), month 3.
The first objective was to prepare a novel reagent, a stannyluracil containing activated amino acid derivative 10. The synthetic route proposed to prepare 10 is shown in Figure 1 (compounds 1-10). This synthesis began with the preparation of the base-reactive intermediate 5 using the procedure previously published (5). The synthesis of the requisite iodouracil amino acid derivative 9 was achieved by coupling 5 with iodouracil 6, followed by ethyl ester hydrolysis to form 8 and re-esterification. While this synthesis was successful, it took longer than projected due to difficulties with purification of some intermediates. Unfortunately, low yields of the stannyluracil amino acid derivative, 10, were obtained when using the route shown in Figure 1. Attempts were made to prepare 10 from 9, but the high temperature required in the reaction (i.e., refluxing toluene) caused 10 to cyclize, giving the unwanted 11 instead. Decreasing the time of reflux from 5 h to 2 h provided 10 in 29% yield, but this was not an optimal approach. Therefore, alternative routes to 10 were examined. One route explored was to prepare the stannyluracil derivative 12 from 8. While a small quantity of 12 was formed under the reaction conditions, there were many other side reaction products formed so that route was not further explored. Another synthetic route explored was conversion of iodouracil 7 into its stannyluracil derivative 13. Stannyluracil derivative 13 was readily prepared, but the stannyl group was not stable under the conditions for removing the ethyl ester. Other iodouracil derivatives, 14 and 15, were prepared, and

**Figure 1: Initial synthetic route to targeted stannyluracil-amino acid derivative 10.**
these were converted to the corresponding stannyluracil derivatives, but again there were problems with removing the carboxylate protecting groups without also removing the stannyl group.

To circumvent the problem encountered with activated esters (i.e. TFP ester in 10), and in removing the carboxylate protecting groups after preparation of the stannyl functionality, another synthetic route was devised for preparation of the stannyluracil derivative 10. That synthesis is shown in Figure 2. Using reactions similar to those in Figure 1 to produce the ethyl esters 3 and 5, the base-reactive 18 was prepared. Reaction of 18 with 6 provided iodouracil amino acid derivative 19. The iodouracil derivative 19 was converted to the stannyluracil derivative 20 in two steps. The first step was preparation of the stannyl functionality and the second step was removal of the tert-butyl ester and tert-butyloxy carbonyl (tBoc) groups in neat TFA. To prepare 10 from 20, the free amine was reprotected as a N-tBoc group to give 21, then the tetrafluorophenyl ester was prepared.

**Objective 2.** Prepare $[^{125}\text{I}]$PNA-bombesin adduct that incorporates a water solubilizing PEG linker (Months 4-8). All compounds will be fully characterized by NMR and mass spectral analysis. HPLC standards for radioiodinated compounds will be prepared using stable iodide.

a. Conjugate stannyluracil derivative with PNA to prepare new PNA, which can be radioiodinated in high specific activity, month 4.

b. Develop conditions for radioiodination, purification, and removal of the tBoc protecting group to yield $[^{125}\text{I}]$PNA 23, month 5.

c. Prepare PEG derivative (compound 29) and conjugate with bombesin to obtain adduct, months 6 & 7.

d. Conjugate radioiodinated PNA, $[^{125}\text{I}]$PNA with bombesin-PEG adduct (compound 31) to prepare the $[^{125}\text{I}]$PNA-bombesin adduct (32), months 7 & 8.
A peptide nucleic acid (PNA), 22, having a sequence for binding to IGF-1 mRNA (H₂N-CGAAGAATCGCATC-CO₂NH₂) was prepared in the Biotechnology Laboratory at the University of British Columbia (Dr. John Hobbs). This material is quite difficult to prepare and purify. We obtained two runs (5 mg and 6 mg) of this material. Our initial difficulty with the highly insoluble PNA was development of HPLC conditions to determine purity. Conditions were obtained which used a reversed-phase separation on a heated column using acetonitrile and water containing a small amount of trifluoroacetic acid. Mass spectral data and HPLC chromatograms indicated that the PNA synthesized was of sufficient purity (>95%) to work with.

Reaction conditions were worked out to achieve coupling of the iodouracil derivative 9 with PNA 22 to prepare the new iodo-PNA 23, as shown in Figure 3. Similar conditions were used to conjugate the stannyluracil derivative 10 with PNA 22 to prepare the stannyl-PNA 24. Both new PNA were treated with trifluoroacetic acid to remove the tBoc protecting group. Deprotection of the amine was done to offset the very low solubility of the PNA in water. Higher solubility can be obtained when a free amine is present as that amine can be protonated with strong acid (e.g. TFA). Iodo-PNA 23 was prepared as a HPLC standard to be used when radioiodinating the stannyl-PNA 24. In an initial reaction, non-radioactive sodium iodide was reacted with chloramine-T to give the iodinated PNA 23. That material was identical to PNA 23 prepared from reaction of 9 with 22. High specific activity radioiodination of 24 provided [¹²⁵I]23 in 46% radiochemical yield.

Figure 3: Preparation and (radio)iodination of stannyluracil PNA conjugate 24
To offset the low solubility of the PNA in water, a new homobifunctional cross-linking polyethylene glycol (PEG) derivative, 29 was synthesized as shown in Figure 4. This molecule was prepared for use as a linker between the PNA and the cell targeting peptide bombesin. The bis-dPEG$_6$-acid 25 was obtained from Quanta BioDesign (Powell, Ohio) and the di-tetrafluorophenyl (TFP) activated ester 26 was prepared and purified. Reaction of 26 with two equivalents of an amino acid PEG derivative 27 (amino-dPEG$_{12}$-$t$-butyl ester; Quanta BioDesign) provided the new PEG derivative 28. To make the PEG derivative 28 useful in our studies, the $t$-butyl esters were cleaved with TFA and tetrafluorophenyl (TFP) activated esters were prepared from the free acid to form bis-TFP ester PEG compound 29. The next step in the synthesis was coupling the bis-TFP ester 29 with less than 1 equivalent of the peptide bombesin 30 to provide the adduct 31. The bombesin derivative 30 was synthesized by Celtek Bioscience, LLC (Nashville, TN). This reaction was facile, but an excess of 29 in the reaction mixture with 31 made it difficult to purify.

Figure 4: Synthesis and conjugation of a PEG-diacid derivative with bombesin
The penultimate step in the preparation of radioiodinated PNA-bombesin conjugate was coupling of the bombesin-PEG-TFP ester 31 with the iodoPNA derivative 23 and (separately) with stannyl-PNA derivative 24, as shown in Figure 5. Again, the iodoPNA-bombesin conjugate 32 was prepared as a HPLC standard for the radioiodinated PNA-bombesin conjugate $^{[125I]}32$. Based on the HPLC chromatogram, we have obtained the iodoPNA adduct 32. To confirm the identity of 32, we have isolated the peak from HPLC and have sent that material for mass spectral analysis. We have not yet received the mass spectral data. The stannylPNA 24 has also been coupled with the bombesin-PEG adduct, but we have not obtained the mass spectral data to confirm that coupling either. We anticipate obtaining the mass spectral data of these compounds within a week. The final radioiodination step has not been conducted. While the period of grant funding has expired, we plan to finish this project (we are very close) by conducting the radioiodination step and isolating the $^{[125I]}32$ for testing in vitro cell survival.

Figure 5: Synthesis of PNA-linker-Bombesin Conjugate
Objective 3. Measure cell survival and quantify IGF-1R mRNA in PC-3 and LNCaP human prostate cancer cells treated with $[^{125}\text{I}]\text{PNA}$ and $[^{125}\text{I}]\text{PNA}$-bombesin adduct (Months 9-12).

a. Evaluate the cell survival and quantify IGF-1R mRNA for PC-3 and LNCaP cells treated with four concentrations of $[^{125}\text{I}]\text{PNA}$, months 9 & 10.

b. Evaluate the cell survival and quantify IGF-1R mRNA for PC-3 and LNCaP cells are treated with four concentrations of $[^{125}\text{I}]\text{PNA}$-bombesin, months 11 & 12.

The synthesis and radioiodination of reagents required for this objective took much longer than anticipated, so the cell survival studies have just begun. Initial experiments were designed to determine optimal conditions for transferring the PNA oligos into the 2 cell lines. For the first experiment, 1 x 105 cells of each line were suspended in electroporation media (phosphate buffered saline supplemented with 10 mM glucose), incubated at 4°C for 10 min, and subjected to electroporation using a Bio-Rad (Hercules, CA) Gene Pulser II system. The cells were shocked in 0.4 cm gap cuvettes using settings 0.25 kV and 960 μF. Immediately following electroporation the cells were plated in 100 mm tissue culture dishes at 100 cells/plate. Cells that were not electroporated but otherwise identically treated were plated as well. Once the effect of electroporation on the cell lines plating efficiency has been quantified, we will repeat the electroporation using labeled and unlabeled PNA oligos. We should have that information shortly.

Assuming we do not have to modify electroporation conditions, we will then electroporate 1.2 and 12μCi of I-125 labeled PNA oligo into each cell line. An equal mass of unlabeled oligo will be electroporated into a duplicate set of cells. Following electroporation a portion of the cells will be immediately plated to measure plating efficiency and toxicity. The remaining cells will be plated and maintained in mass culture at 37°C for 24 h to allow oligos to migrate (in cytoplasm and into nucleus) and hybridize to appropriate target site. After 24 h, these cells will be cryogenically frozen and stored at -80°C for 6 months to allow for a sufficient number of I-125 decays to effect survival. At this point, we don’t know how much activity will be taken up, so we have arbitrarily chosen 6 months to examine. Future studies will make more careful measurements of activity and time experiments to examine effects of different numbers of disintegrations. We anticipate obtaining results from this experiment over the next 6 months.

III. Key Research Accomplishments:

1. Synthesis of an amine protected and carboxylate activated iodouracil amino acid, 9. This compound is important as a standard for preparation radioiodinated PNAs, but it has the potential to be used for more than that. The iodouracil base is sterically similar to a thymine base, but has different electronic properties. Therefore, it may be useful in preparing PNA that have tailored binding properties.

2. Synthesis of the amine protected and carboxylate activated stannyluracil amino acid, 10. As shown in this research, this base can be used to site-selectively introduce radioiodine into a PNA.

3. Synthesis of iodo- and stannylPNA derivatives 23 and 24. Using the synthetic methods developed with this funding, investigators can prepare these unique PNA, or similar ones, for other applications to cancer therapy.
4. Synthesis of a new PEG cross-linking reagent 29 that can be used for water solubilization and to provide a spacer between biomolecules being tested.

5. Synthesis of the bombesin-PEG adduct 31. This adduct can be used to couple with molecules other than PNA to carry them across cell membranes using the GRP receptor system.

6. Synthesis of bombesin-iodoPNA adduct 32 and bombesin-stannylPNA adduct 33. The stannyl derivative 33 can potentially be radioiodinated (or radiohalogenated) such that it can be used to break DNA or mRNA at specific locations in their sequence after binding. This reagent is of primary interest as a carrier of Auger electron emitter for therapy of metastatic cancer. Additional studies need to be conducted to prove its value.

IV. Reportable Outcomes:

1. This report
2. Abstract for preparation of radioiodinated PNA

V. Conclusions:

We have been successful in preparing a number of unique compounds (as listed above), and have begun cell survival testing of compounds of interest. We had several difficulties in the syntheses and those resulted in our progressing at a slower pace than anticipated. Our demonstration that a stannyluracil could be used to prepare high specific activity radioiodinated PNA is very important. This step was essential to the concept being investigated and in obtaining materials for testing. However this synthesis was accomplished late in the studies, resulting in our not obtaining testing of the radiolabeled reagents for cell killing at the time of this report. We are disappointed that we did not complete all of the studies outlined, particularly the in vitro testing. It is unfortunate that the manpower and time for the studies were limited. We believe that the studies are very important and plan to continue them to their completion. This should not take too much more effort as we have achieved most of what was planned.

VI. References

VII. Appendices

1. Abstract submitted to 12th International Symposium on Radiopharmaceutical Chemistry.

VIII. List of Personnel Receiving Pay for Research Effort

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Appendix Item: Abstract submitted to 16th International Symposium on Radiopharmaceutical Chemistry, Iowa City, Iowa, June 24-28, 2005

Synthesis and Radioiodination of a PNA Containing an $[^{125}\text{I}]$Iodouracil Moiety.

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Peptide nucleic acids (PNA) hold potential as agents for delivering radiation to specific gene sequences and mRNA. Previous investigators have shown that In-111-labeled PNA is effective at sequence-specific DNA cleavage [1]. We are interested in PNAs with Auger electron emitting radiohalogens directly incorporated into a base such that the radionuclide is held close to the DNA/RNA backbone. As a preliminary investigation, we have prepared a PNA that has an I-125 labeled uracil moiety in the place of a thymine moiety. The base sequence of the PNA was chosen as it has been shown to bind with the insulin-like growth factor type 1 receptor (IGF-1R) [2], which is upregulated in prostate cancer. The initial goal in the study was to produce uracil containing amino acid derivatives that could be incorporated into a PNA. The sequence of reactions is shown in the figure below. The first step was to prepare the base-reactive amino acid derivative 2. That synthesis was carried out in four steps as described in the literature [3]. Reaction of 2 with commercially available iodouracil, 1, was accomplished in 49% yield after purification. Hydrolysis of the ethyl ester in 1N NaOH at room temperature for 4 h gave the free acid 4 in 58% yield, and preparation of the TFP ester 5 was accomplished in 66% yield using tetrafluorophenyl trifluoroacetate (TFP-OTFA) and Et$_3$N in DMF at rt for 5 min. Unfortunately, low yields (e.g. <30%) of the desired stannyluracil 6 were prepared from 5 using the standard conditions, i.e. (nBu$_3$Sn)$_2$ / (Ph$_3$P)$_4$Pd in anhyd. toluene at reflux. An alternative pathway to 6 began with the preparation of 9 in a two-step synthesis. Reaction of 9 with 1 provided 10 in 44% yield. Conversion of 10 to the stannyluracil derivative 11 was readily accomplished using the standard stannylation conditions. Deprotection of 11 was accomplished in neat TFA at rt for 5 min, and reformation of the N-Boc to form 12 was accomplished with NaHCO$_3$/Boc$_2$O in acetone/H$_2$O for 3 h at rt. This was followed by preparation of the TFP ester using TFP-OTFA to give 6. Once prepared, uracil amino acid derivatives 5 and 6 were reacted with PNA 7 to form PNA 8 (calc. 4181 Da, found 4185 Da; M+4H) and PNA 9 (calc. 4445 Da, found 4484 Da; M+K). Radioiodination of 9 was facile using Na$[^{125}\text{I}]$I and chloramine-T in H$_2$O/5% HOAc at rt for 5 min, providing a 46% (HPLC) isolated yield of $[^{125}\text{I}]$8.

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2. T. Nickerson, F. Chang et al., Cancer Res. 61, 6276-6280, 2001.