IMAGING ONCOGENE MRNA WITH TC-99M-PNA-PEPTIDE CHIMERAS

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Rationale:

Cancer is a proliferative disease that stems from genomic modulations at the cellular level and results in overexpression of characteristic proteins on the cell surface. Targeting oncogene mRNA *in vivo* with a Tc-99m-labeled specific biomolecule could provide a powerful tool for imaging malignant lesions scintigraphically. Peptide Nucleic Acids (PNAs) display ruggedness and facilitate hybridization better than corresponding phosphorothioate and morpholino phosphoamidate oligonucleotides.

Aims:

Our goals were to 1) synthesize PNAs specific for cMYC and cyclinD-1 specific chimeras with a conjugated chelating moiety, 2) examine their hybridization properties, 3) label them with Tc-99m, and 4) perform feasibility studies to image human breast tumors in nude mice chimeras.

Methods:

Using a solid support and 9-fluorenylmethyloxycarbonyl (Fmoc) coupling technique, PNA dodecamers were extended from the N terminus to a G(D)AGG chelating moiety. This hybrid molecule was further extended from its N terminus with a disulfide bridged peptide. This peptide has a high affinity to insulin-like growth factor (IGF)-1 receptor which is expressed abundantly on breast cancer cells. The hypothesis is that the S-S bridged peptide will enhance PNA specificity for malignant cells. The chimera was purified, characterized using a MALDI mass spectrometer and labeled with Tc-99m. Mismatch PNA chimeras similarly synthesized and characterized served as controls. Hybridization studies were performed using RT-PCR. Tissue distribution and tumor imaging studies at 4 and 24 hrs post-injection were carried out.

Results:

The specific chimera and corresponding mismatch PNA synthesis yields varied from 27.2 to 34%. As compared to the control chimeras, PNA sequences displayed significant gene inhibition as examined by RT-PCR. Radiolabeling efficiencies were >95%. SDS polyacrylamide gel electrophoresis analysis of the radiolabeled product demonstrated a M.W. consistent with the M.W. determined by MALDI analysis. Breast tumors in mice were visualized with a cyclinD-1 chimera but not with the corresponding mismatch PNA chimera or the chimera without the bridged disulfide peptide specific for IGF-1R.

Conclusion:

These results demonstrate that 1) complex PNA-peptide chimeras have been successfully synthesized, 2) These constructs retain their biochemical activity and hybridize with oncogene mRNA, 3) these can be radiolabeled efficiently, and 4) used for scintigraphic localization of malignant tumors *in vivo.* The approach is worthy of further investigation.

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[^{18}F]FEAU AS A NOVEL RADIOTRACER FOR HERPES SIMPLEX VIRUS THYMIDINE KINASE (HSVtk) GENE EXPRESSION

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Previously, we have shown that HSVtk gene expression can be monitored with 9-[(3-[^{18}F]fluoro-1-hydroxy-2-propoxy)methyl]guanine ([^{18}F]FHPG) and PET. Although [^{18}F]FHPG selectively accumulates in HSVtk expressing tissues, its overall uptake is rather low (SUV 1.2). In search for a better tracer for HSVtk expression, we synthesized the fluorinated thymidine derivatives [^{18}F]FMAU and [^{18}F]FEAU (figure 1). In this study the cellular uptake of these tracers was compared with the cellular uptake of [^{18}F]FHPG.

[^{18}F]FMAU and [^{18}F]FEAU were synthesized in 4 steps, as is shown in figure 1 (1). Both [^{18}F]FMAU and [^{18}F]FEAU were obtained in approximately 2% decay corrected overall yield. Optimization of the synthetic procedure is still in progress.

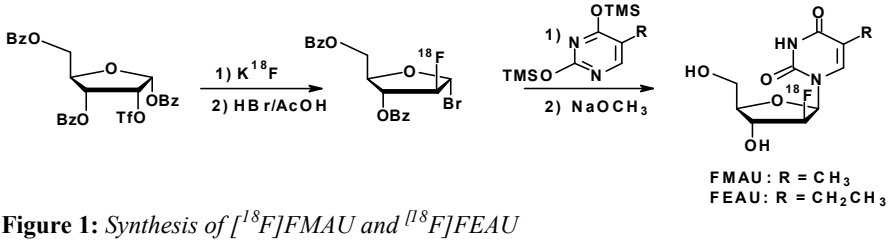

Figure 1: *Synthesis of [^{18}F]FMAU and [^{18}F]FEAU*

To determine the cellular uptake of the tracers, monolayers of either C6 rat glioma cells (control cells) or HSVtk expressing C6Tk cells were incubated with 0.3-1.7 MBq of [^{18}F]FHPG, [^{18}F]FMAU or [^{18}F]FEAU. After 15, 30, 45, 60, 90 and 120 min, cells were washed, trypsinized and cellular uptake of radioactivity was measured and normalized to the number of viable cells. The initial rate of tracer uptake was determined from the slope of the plot of tracer uptake versus incubation time.

The initial tracer uptake rate (expressed as [%dose]/10^6 cells*min] in C6 and C6Tk cells was 0.0003 0.0002 and 0.0021 0.0003 for [^{18}F]FHPG, 0.039 0.002 and 0.54 0.14 for [^{18}F]FMAU and 0.0002 0.0002 and 0.23 0.05 for [^{18}F]FEAU, respectively. After 2 h, normalized uptake of [^{18}F]FMAU and [^{18}F]FEAU in C6Tk cells was 52 and 29-fold higher than [^{18}F]FHPG uptake. After 2 h of tracer incubation, the C6tk/C6 accumulation ratio was 3.0 for [^{18}F]FHPG, 4.4 for [^{18}F]FMAU and 84.6 for [^{18}F]FEAU.

This study shows that the thymidine derived tracers [^{18}F]FMAU and [^{18}F]FEAU exhibit much higher uptake rates in HSVtk expressing C6Tk cells than the guanosine derivative [^{18}F]FHPG. However, as the selectivity for HSVtk is concerned, the C6tk/C6 accumulation ratio of [^{18}F]FMAU is rather low. In contrast, [^{18}F]FEAU rapidly accumulates in C6Tk cells, but not in C6 control cells. Taken together, the combination of high uptake rate and high selectivity for HSVtk makes the thymidine derived tracer [^{18}F]FEAU an excellent candidate as PET tracer for HSVtk gene expression.

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A FLUORINE-18 LABELED OLIGONUCLEOTIDE AS PET TRACER FOR iNOS mRNA.

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Keywords: Oligonucleotide, Fluorine-18, PET, inducible NO synthase, Inflammation

In inflammatory bowel diseases like colitis ulcerosa and Crohn's disease, inducible NO synthase (iNOS) is expressed in inflamed gut epithelium, where it produces high levels of harmful NO radicals. Experimental therapies to block iNOS expression with antisense oligonucleotides (ODN) are under investigation. To monitor disease activity and efficacy of antisense therapy, we aim to develop a non-invasive method to assay iNOS mRNA with labeled antisense ODN and PET.

Eight antisense ODNs against iNOS mRNA were retrieved from the literature. Hybridization of these ODNs to iNOS mRNA was tested in a standard RT-PCR assay, using a fixed sense primer. The ODN with the sequence 5'-GTC-CAT-GAT-GGT-CAC-ATT-CTG-CTT-3' was selected for labeling on basis of PCR product yield. Fluorine-18 labeling of this ODN, modified with a thiophosphate backbone and a hexyl thiol spacer at the 5'-terminus, was performed with *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide at 120 ^{o}C for 30 min. When 100 nmol of ODN precursor was used, the decay corrected radiochemical yield was 31±16% (based on the bromide). The fluorine-18 labeled ODN was evaluated in DLD-1 colon carcinoma cells that were stimulated with a cytokine mixture composed of human recombinant interleukin-1-β (10 ng/ml), human recombinant TNF-α (10 ng/ml) and human recombinant interferon-γ (1000 U/ml) for 8 hours. This cytokine mixture and exposure time resulted in a strong induction of iNOS mRNA, as was detected by RT-PCR.

To study the ODN uptake, stimulated and control DLD-1 cells were incubated with 60±4 pmol of the labeled ODN for 15-120 min. Labeled ODN was accumulated in both stimulated and control DLD-1 cells. The lipofectant fugene (3 µl/µg ODN) significantly increased the ODN uptake by approximately 2.5-fold after 2 h of incubation at 37 ^{o}C (Figure 1, left panel). In the presence of fugene, uptake of labeled ODN strongly depended on the temperature, as ca. 4 times more tracer accumulated at 37 ^{o}C than at 0 ^{o}C (Figure 1, middle panel). In none of the uptake experiments, any significant differences between ODN accumulation in stimulated and control cells were observed.

To investigate ODN efflux, stimulated and control DLD-1 cells were loaded with labeled ODN in the presence of fugene. After the cells were washed, fresh medium was added and the radioactivity that was retained in the cells was measured after 0, 60 and 120 min. Within 60 min, 70-80% of the activity was cleared from the cells. From 60 to 120 min, efflux of activity was negligible. No significant differences in efflux between stimulated and control cells were found.

In conclusion, an antisense ODN against iNOS mRNA was reproducibly labeled with fluorine-18. Uptake and efflux of the [^{18}F]-labeled ODN were rapid. Further studies are needed to establish specific hybridization of the ODN in iNOS expressing intestinal epithelial cells.

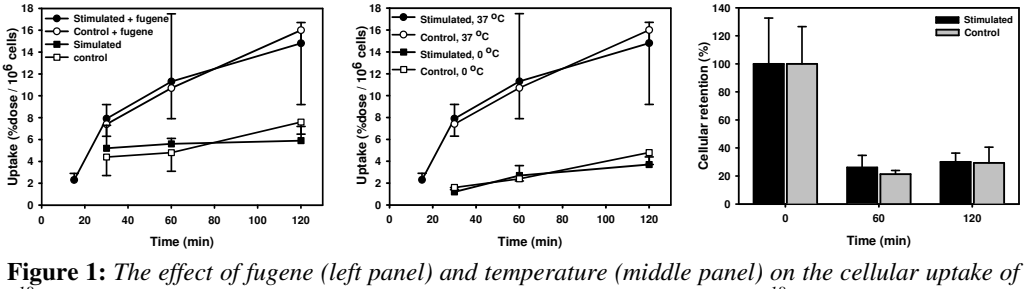

Figure 1: *The effect of fugene (left panel) and temperature (middle panel) on the cellular uptake of [^{18}F]-labeled ODN in stimulated and control DLD-1 cells. Efflux of [^{18}F]-labeled ODN from stimulated and control DLD-1 cells (right panel).*

LYMPHOMA CELL UPTAKE OF RADIOMETAL- AND FLUORESCENT-LABELLED *BCL-2* ANTISENSE PNA CONJUGATES IS MEDIATED BY A *RETRO-INVERSO* DELIVERY PEPTIDE

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The *B-cell lymphoma/leukemia-2* (*bcl-2*) proto-oncogene is associated with inhibition of tumour cell apoptosis and resistance to radiation and chemotherapy. In patients with aggressive non-Hodgkin's lymphoma (NHL), *bcl-2* expression correlated strongly with increased relapse rate, reduced disease-free survival, and poor overall survival (1, 2). Previously, we prepared radiometal-labelled antisense peptide nucleic acid (PNA) conjugates, complementary to the first 6 codons of *bcl-2* mRNA and coupled to a cell-permeating peptide (PTD-4), as potential agents to target *bcl-2* for prognostic imaging and therapeutic applications (3).

In the present studies, we compared uptake, retention, and internalisation of ^{111}In- and tetramethylrhodamine (TMR)-labelled anti-*bcl-2* PNA, conjugated to PTD-4 (**1** = ^{111}In) and its *retro-inverso* analogue (*ri*-PTD-4; **2** = ^{111}In, **3** = TMR), in NHL cells. Raji cells express high (4) and U937 cells express low (5) levels of *bcl-2* mRNA. Plasma stability studies showed that **2** was 100% stable at 37 °C for 168 h, while ~9% of **1** remained intact under the same conditions. Cell uptake studies were performed during continuous radiopharmaceutical exposure at 37 °C and 5% CO_2, while retention of ^{111}In was measured after removal of the radiolabelled conjugate following 30 min of exposure. By 4 h, uptake of **2** was >4 times higher ($p = 0.001$) in Raji than in U937 cells, compared to a 2.5-fold difference ($p = 0.001$) for **1** (Table 1). At 4 h, retention of **1** in Raji cells (5.02%, ~2320 molecules per cell) was 16-fold higher ($p = 0.008$) than in U937 cells (0.305%, ~141 molecules per cell). Retention of **2** was 1.8- to 6-fold lower ($p < 0.023$) than **1** in both cell lines. However, retention of **2** was still >11 times higher ($p = 0.003$) in Raji than in U937 cells at 4 h. Like compound **1**, conjugate **2** showed strong selectivity for the high *bcl-2*-expressing Raji cells.

Table 1. Uptake of ^{111}In-labelled anti-*bcl-2* PNA conjugates **1** and **2** in Raji and U937 cells. Data are presented as the % of the total radioactivity added ± s.d. (n = 3 for each group).

	Raji		U937	
Time	**1**	**2**	**1**	**2**
1 min	23.1±2.13	33.2±0.93	30.0±1.79	24.3±1.62
15 min	11.4±2.66	21.5±1.93	8.60±1.33	13.3±1.92
1 h	11.0±1.73	17.7±0.76	4.24±0.94	11.5±0.79
4 h	11.1±0.56	16.7±2.36	4.38±0.12	4.06±1.76

Fluorescence microscopy studies of TMR-*ri*-PTD-4-anti-*bcl-2*-PNA (**3**) and TMR-anti-*bcl-2*-PNA (**4**) were performed in Raji cells. Widespread, intense fluorescence was observed in the cytoplasm of cells treated with **3** for 2 h, while relatively weak fluorescence from **4** was confined to the plasma membrane. These studies confirmed that *ri*-PTD-4 was active in membrane transduction and mediated cellular internalisation of the anti-*bcl-2* PNA conjugate.

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BASIS OF NON-INVASIVE VISUALIZATION OF TRANSPLANTED LIVING FUNCTIONAL CELLS BY USING PET TRACER

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Key words: Molecular Imaging, PET, Regenerative Medicine, ES cell, Tamoxifen

Regenerative medical science employs newly invented methods, such as to provide conditions for the human body to be regenerated or to stimulate its regeneration in order to assist tissues or organs to regain their lost functions. Transplantation of functional cells into damaged tissue is now on going clinically but in the present, the population of surviving cells could not be examined none-invasively.

Embryonic stem cells (ES cells) are pluripotent cells directly derived from early stage embryos that retain the ability to differentiate into all cell types. This unique feature suggests ES cells are attractive genetic source for cell therapy in regenerative medicine. The aim of our research is to visualize life and death of transplanted functional cells by using non-invasive method. In general, target product used in molecular imaging requires the following features; Reporter should be non-toxic, non-immunogenic, no endogenous protein in the expressed area, non-secretory and small in size; Tracer should be safe for use in human and able to cross cell membrane (preferably blood brain barrier : BBB). So, we chose the mutated estrogen receptor (Mer : tamoxifen receptor) ligand binding domain as a reporter, which has altered ligand specificity (tamoxifen >> estrogen); this allows us to ignore endogenous estrogen level, and to avoid no transcriptional effects on genes regulated by estrogen. F-18 labeled tamoxifen is an established positron emission tomography (PET) tracer with known labeling procedure, known biodistribution in human, and high BBB permeability. The antiestrogenic drug tamoxifen is also widely used drug for the therapy of breast cancer.

We used mouse ES cell line for recipient cells as a model for non-invasive visualization. To obtain ES cells expressing tamoxifen receptor stably or inducibly, we transfected three types of expression vectors into EB5 parental ES cell line by using lipofection. We obtained about twenty clones in each of three types transfectants. To examine the expression level of tamoxifen receptor, dot blot analysis and western blotting analysis were performed using PCR labeled fragment of Mer gene as hybridization probe and anti-estrogen receptor alpha antibody, respectively. Results indicated that some of clones overexpressed about 100 folds more Mer mRNA than control EB5 cells and Mer protein was also observed to be expressed much higher than in control. These clones also expressed equal level of Oct-3/4 gene, which is one of the marker of pluripotency, in comparison with EB5 parental cells. Biodistribution of 4-hydroxy[3H]-tamoxifen in the nude mice transplanted with control cells and Mer transfectants are under progress. To get further information, we are also planning other experiments.