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New Gene Based Probes for Imaging Breast Cancer with PET

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We are developing methods to image her-2-neu oncogene over-expression in breast cancer using positron emission tomography (PET). Small oligodeoxynucleotides (ODNs) that are complementary to the Her-2-neu messenger RNA (mRNA) are being investigated as potential imaging probes. Fluorine-18 (2 hour half-life positron emitter) has been used to label 15-18 mer ODN probes. Furthermore, several breast and ovarian cancer cell lines which over-express ODNs have been used to study trapping of 18F-ODNs in cell culture. We have also preliminary studied the biodistribution of ODNs in 2 living nude mice using microPET in order to understand the limitations of imaging in vivo with the antisense approach. Currently the yield of radiolabeled ODNs is very low (<20 μCi), and therefore more work will have to be performed to optimize yields of the ODNs prior to proceeding further. Once ODN yields can be brought into the 150-300 μCi range, then more cell culture and in vivo experiments will be able to be performed to optimize an antisense based approach for imaging breast cancer.

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Introduction

Improved methods to image breast cancer are critically needed in order to lead to earlier initial detection, earlier detection for recurrence, and better management of patients undergoing treatment. Most approaches to date have focused on anatomical changes due to tumor growth (e.g., mammography, computerized tomography, magnetic resonance imaging) or metabolic changes in the tumor (e.g., FDG Positron emission tomography). As molecular oncology continues to shed insight into the molecular basis for breast cancer, methods are needed to directly image molecular aberrations in breast cancer cells. We are developing methods using radiolabeled antisense oligodeoxynucleotides (RASONs) which can be injected via the bloodstream and then accumulate in cells that have sufficient levels of a particular target messenger RNA (mRNA). Normal cells (breast and other tissues) which don’t have high level of target mRNA would not lead to intracellular trapping of the RASONs. One known molecular abnormality in about 25% of breast cancer patients is the over-expression of the Her-2-neu (c-erb-B2) oncogene. We have selected this gene as our first target using RASONs labeled with fluorine-18 (a positron emitter). We seek to develop RASONs that can be validated using nude mice carrying human breast cancer tumor xenografts imaged using microPET technology. With pre-clinical proof of their ability to home to breast cancer tumors over-expressing Her-2-neu we hope to have sufficient proof to eventually transition to human applications. It is hoped that this approach will lead to more specific and sensitive detection of breast cancer with over-expression of Her-2-neu and set the foundation for a new antisense based imaging approach which could potentially be applied to many different oncogenes.
Aim 1: The development of $^{18}$F (a positron emitter, half-life of 110 minutes) labeled oligodeoxynucleotides. The bulk of the effort to date has been towards this goal. Because all other goals are dependent on the successful completion of this goal, most of our efforts have been towards radiolabeling with Fluorine-18 our oligodeoxynucleotide probes.

The details of the chemistry developed to date is described next.

Scheme 1

Reagents: a. (1) TBDMCl, imidazole. (2) DMTCl; b. (1) POCl$_3$, Et$_3$N, 1,2,4-triazol. (2) CH$_3$OH, DBU. (3) Bu$_4$NF; c. TsCl; d. KF (or [F-18]KF)/Kryptofix[2,2,2]; e. TCA

6a, R=$^{19}$F
6b, R=$^{18}$F
Our synthetic strategy is comprised of two key steps: synthesis of a 5'-deoxy-5'-fluoro-nucleoside followed by its incorporation into a CPG-bound ODN by reverse-activated phosphoramidite chemistry.\textsuperscript{1} In our earlier communication,\textsuperscript{2} we reported nucleophilic fluorination of 3'-acetyloxy-5'-p-methylphenylsulfonyl-4-O-methyl thymidine followed by deacetylation in hot NH\textsubscript{4}OH to produce 5'-deoxy-5'-fluoro-4-O-methyl thymidine 6a. But it is a tedious practice to heat a sealed reaction vessel with conc. NH\textsubscript{4}OH and F-18 labeled chemicals at 100 °C. We further simplified the deprotection by using the 4,4'-dimethoxytrityl group to protect 3'-OH of the nucleoside analog, which can be removed by acid treatment. The new precursor, 5'-O-tosyl-3'-(4,4'-dimethoxytrityl)-O\textsuperscript{4}-methylthymidine 4, was synthesized from thymidine 1 in a total chemical yield of 22% (Scheme 1). The 4 was then subjected to nucleophilic [F-18]fluorination at 98~100 °C for 5 min., followed by deprotection with trichloroacetic acid (TCA). Purification of the reaction mixture by normal-phase HPLC afforded 5'-deoxy-5'-[F-18]fluoro-O\textsuperscript{4}-methylthymidine 6b in 15 ~ 20% (decay corrected) of radiochemical yield. The chemical identity and radiochemical purity of F-18 labeled nucleoside 6b was confirmed by HPLC co-injection with 6a (Figure 1).
Coupling of 3b to the CPG-bound ODN 7 was carried out by the reverse-activation protocol (Scheme 2). Phosphitylation of 7 was conducted by treatment with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. The resulting phosphoramidite 8 was then reacted with 6b (50 ~ 100 mCi) in MeCN containing 1H-tetrazole at RT for 10 min. After oxidation with I₂, the F-18 labeled ODN was cleaved from CPG beads by NH₄OH/CH₃NH₂ and deprotected at 65 °C for 10 min. The crude mixture was purified by anion-exchange HPLC (POROS 20 HQ) to yield the desired 5'-[F-18]fluorinated ODN 9b (10-70 µCi) (Figure 2).

Both duplexes of fully deprotected ODN of 7 and fluorine modified 9a against complementary RNA 25-mer, 5'-CGA CGA UGC CCC UCA ACG UUA GCU U, showed identical Tₘ values, ~ 48 °C. It indicates that a nucleoside tag group attached to 5'-end of the 15-mer ODN doesn’t significantly change its hybridization affinity.

The key issues that remain in order to complete Aim 1 are to improve yields of the final product. Although we can get on average 20 µCi of the radiolabeled ODN, we will need ten times more in order to do routine cell culture testing as well as in vivo imaging in our mice models. Current work is attempting to optimize the yields further. In another 3-5 months we hope to have sufficient yields to proceed further.
**Aim 2:** The development of (15-20)-mer oligodeoxynucleotides for targeting the Her-2-neu (c-erbB-2) proto-oncogene mRNA. We have studied several candidate sequences for targeting the Her-2-neu mRNA. Through structural analysis we have now defined several optimal sequence that we feel should be accessible by our RASON probes. We have also started synthesis of modified-backbone ODNs in order to improve their plasma stability. We find that 2’ O-methyl modified ODNs may be optimal for eventual use *in vivo*.

**Aim 3:** Tissue culture testing of the developed probes to determine the specificity and kinetics of the probe for the c-erbB-2 mRNA. We have studied 4 cell lines for their levels of Her-2-neu expression. These include a MCF-7 control cell line, a MCF-7 over expressing Her-2-neu, SK-BR-3, and SK-OV-3. Utilizing both Westerns and PCR we have shown that there is Her-2-neu over-expression in 3 of 4 of these cell lines. Furthermore, we have performed ODN uptake studies (using pharmacological levels of ODN) in order to show that a particular antisense sequence is capable of reducing levels of Her-2-neu protein in a cell culture assay. The best antisense sequence to date is a 18-mer with the sequence 5’ gca caa ggc ggc cag ctc 3’. This sequence has been compared to several control and mis-match sequences in order to show that it is effective in reducing levels of Her-2-neu in cell culture. Furthermore, the control sequence does not reduce levels of Her-2-neu as expected. We now await the labeling of this ODN sequence with Fluorine-18 after Aim 1 leads to improved yields for our RASON probes. Then cell culture uptake and efflux studies will be performed with the RASON probes (antisense and control probes).
Aim 4: To study the targeting properties of $^{18}$F-labeled antisense-oligodeoxynucleotides in vivo in a mouse animal model using PET.

Towards Aim 4 we have performed some very preliminary studies in two control nude mice in order to understand the limitations of injecting our 18F-ODN probes into mice and imaging with a microPET. Because the yields of 18F-ODN are still very low (see also Aim 1), we have not been able to get satisfactory images of biodistribution of the tracer. We will be able to better characterize the biodistribution when more tracer is routinely available. We have also been able to grow xenografted tumors in mice (e.g., MCF-7) in order to eventually use these tumor models to image with microPET and our 18F-ODN probes.
Key Research Accomplishments

- Synthesis of 18F-oligodeoxynucleotide (ODN) probes in low yields
- Purification of 18F-ODN probes for cell culture testing and in vivo testing
- Assessment of hybridization potential of 18F-ODN with target mRNA through $T_m$ measurements
- Synthesis of 2’-o-methyl modified ODNs for improved plasma stability
- Specific Activity measurements of 18F-ODN probes
- Isolation of an 18-mer antisense sequence that should have optimal targeting properties for Her-2-neu
- Study of cell lines for levels of Her-2-neu over-expression
- Demonstration that pharmacological levels of antisense probe targeted towards Her-2-neu leads to decrease in levels of Her-2-neu protein in cell culture
- Preliminary biodistribution studies of 18F-ODN probes in control mice using microPET
Reportable Outcomes

Presentation of 18F radiolabeling approach utilizing newly developed chemistry. Presented by Dr. Dongfen Pan at Annual meeting of the Society of Nuclear Medicine in June, 1999.


Conclusions

The results to date demonstrate that it is possible to label oligodeoxynucleotide molecules with Fluorine-18 (a positron emitter). This is a key step which must take place prior to the use of these probes as imaging agents for breast cancer. With further improvements in yield and specific activity of these probes many additional experiments can be undertaken. These include study of cell culture models and in vivo animal tumor models using microPET imaging technology. The groundwork has also been set for further study in cell culture models, and in vivo animal models. With continued funding of this work it should be possible to understand the feasibility of using RASON probes to image Her-2-neu over-expression in breast cancer with PET imaging.
References


Appendices
A SUPPLEMENT TO

The Journal of Nuclear Medicine

JNM

ABSTRACT BOOK

Scientific Abstracts of the 46th Annual Meeting of the Society of Nuclear Medicine
Los Angeles, California
June 6-10, 1999

Objective: Radiolabeled-labeled Mabs directed against tumor-associated antigens have been investigated as immunotherapeutic agents in human cancer. The a-emitter $^{211}$At (t$_{1/2}$ = 7.21 h) is a particularly promising candidate for radioimmunotherapeutic applications. A pivotal issue to consider in designing an optimal radioimmunotherapeutic agent is the choice of linker to couple the radionuclide to the Mab. Methods: The linkers N-hydroxy succinimidyli 4-[2-A]tagostato benzoxazole (1), N-hydroxy succinimidyli 3-[2-A]tagostato-4-methylbenzoate (2), A-hydroxy succinimidyli 4-[2-A]tagostato-3-methylbenzoate (3) and N-hydroxy succinimidyli N-[1-4-[2-A]tagostato phenethyl] succinimidyl (4) were prepared and employed for $^{211}$At labeling of the antibody. The anti-Tac (anti-IL-2Ra) antibody reacts with select leukemia cells but not resting normal cells was utilized for this study. The plasma survival of these compounds in nude mice was studied. Results: $^{211}$At-labeled humanized anti-Tac. Results: The comparison of the blood clearance curves of the $^{211}$At and $^{177}$Lu-labeled anti-Tac and free $^{211}$At indicating the stability of compounds 1-4 was in the following order: $^{177}$Lu $>$ $^{211}$At $>$ anti-Tac $>$ free $^{211}$At. Conclusion: This study showed that the stability of the compound prepared to date for $^{211}$At-labeling of humanized anti-Tac and its plasma survival appeared to be essentially equivalent to that of directly labeled $^{211}$At antibody. These results also suggest that humanized anti-Tac can be successfully labeled with $^{211}$At using linkers 4 and should be further evaluated for therapeutic applications.

No. 330
NON ENZYMATIC REDUCTION AS A POSSIBLE RETENTION MECHANISM OF $^{213}$HML-91 IN HYPOXIC TISSUES. Y. Fujibayashi, M. Ohno, A. Waki, K. S. Horiiuchi, Y. Yonekura, Fukui Medical University, Fukui, Japan; Kyoto University, Kyoto, Japan. (210)

A novel hypoxia imaging agent, $^{213}$Hm-4,9-diaza-3,10,10-tetramethyldecane-2,11-dione dioxime ($^{213}$Hm-91), shows high uptake in myocardium as well as tumors, but its retention mechanism has not been clarified. In our previous work, it was found that Cu-diethyl-his(N4-methylthiosemicarbazone) (Cu-ATSM) showed enzymatic and NADH-dependent reduction in hypoxic non-tumor tissues. Thus, in the present work, metabolic analysis was performed to clarify the reduction mechanism of $^{213}$Hm-91 in vivo system. Methods: Oxidation of $^{213}$Hm-91-LDL and Cu-ATSM was comparatively evaluated using reversed-phase HPLC system. Each sample was incubated with biologically relevant, glutathione reduced form or NADH, then analyzed. For controls, oxidized forms of the reductants were used. To evaluate the possibility of enzymatic system, subcellular fractions obtained from Ehrlich ascites tumor cells were added to the incubation medium. The effect of NADH on the enzymatic reduction of each sample was also studied. Results: The reduction of Cu-ATSM required microsomal enzymes and was NADH/NADPH dependent in tumor cells. Without enzymes, no reduction could be found. On the other hand, $^{213}$Hm-91-LHb showed chemical reduction when only NADH or glutathione reduced form was added to the incubation medium. This reduction was dose-dependent, but there seemed to be threshold levels of reductant concentration. More interestingly, enzyme system inhibited the reductive metabolism of $^{213}$Hm-91, but electron transport inhibitors receded the reduction of $^{213}$Hm-91 in the medium containing the microsomal enzymes. Conclusion: Cu-ATSM could be considered as a marker of reversible hypoxia, because it required biological reductants as well as intact enzyme system(s). On the other hand, $^{213}$Hm-91 only required abnormally high concentration of biological reductants, indicating it as a hypoxia imaging agent with wider spectrum, rather than Cu-ATSM. This finding will bring us a new sight of hypoxia diagnosis using $^{213}$Hm-91 as well as Cu-ATSM in clinical level.

No. 331
TARGETING HYPOXIA IN TUMOURS USING 2-NITROIMIDAZOLES WITH PEPTIDIC CHELATORS FOR TECNETIUM-99M: EFFECTS OF LIPOPOLYHYLITICITY. X. Zhang, Z. F. Su, J. R. Ballinger, A. M. Rauth, A. Pollak, J. R. Thornback, Ontario Cancer Institute/University of Toronto, Toronto, ON, Canada; Resolution Pharmaceuticals, Mississauga, ON, Canada. (100536)

Objective: Hypoxia in tumors is an important prognostic factor for response. Radiolabeled 2-nitroimidazoles (2-NI) have been used for imaging hypoxia and partition coefficient (P) appears to play a crucial role in suitability for imaging. We developed a series of eleven 2-NI containing a peptidic chelator for $^{99m}$Tc with divergent $\mathrm{pK}_a$ and evaluated them in an in vitro system. Methods: Two classes of N,S chelators were used: di-alkyl-Gly-Ser-Cys-linker-2-NI and di-alkyl-Gly-Lys(2)-2-NI-Cys. Prepared by automated solid-phase peptide synthesis, the chelators were labelled by transchelation from $^{99m}$Tc-gluconate at temperatures between 20 and 100°C. The reaction mixtures were analysed by HPLC. The accumulation of each complex in suspension cultures of Chinese hamster ovary cells incubated under aerobic or extremely hypoxic conditions was determined. Results: Radiochemical yields ranged from 5% to 80% for the 11 compounds. HPLC showed that some compounds formed two complexes with $^{99m}$Tc, possibly syn- and anti-conformations with respect to the Tc=O bond. In general, the Gly-Ser-Cys chelator labelled more readily than the Gly-Lys-Cys chelator. The $P$ values varied from 0.001 to 5, and were generally in accordance with predictions based on structure. There were differences in $P$ as a function of pH; the free acids had a lower $P$ at pH 7.4 than at pH 2.0 due to ionisation, whereas the amides did not show this effect. Accumulation levels in cells related to $P$ but varied over a narrower range. Six of the 11 compounds showed selective localisation in hypoxic cells, with 1.8- to 3.6-fold higher accumulation in hypoxic vs aerobic cells. Conclusion: The peptide class of 2-NI, with flexible and convenient solid-phase synthesis, deserves further study as agents for imaging hypoxia in tumors.

No. 332
SYNTHESIS OF A FLUORINE-18 LABELED ANTISENSE OLIGODEOXYNUCLEOTIDE AS A PROBE FOR IMAGING GENE EXPRESSION. D. Pan, T. Toyokuni, J. R. Barron, N. Satymurthy, M. E. Phelps, S. S. Gambhir, University of California at Los Angeles School of Medicine, Los Angeles, CA. (500498)

We are developing methods to image gene expression in vivo by positron emission tomography (PET) Antisense oligodeoxynucleotides (ODN) and their derivatives complementary towards a small region of mRNA are being studied for targeting the mRNA of various amplified oncogenes. Here, we describe the synthesis of a PET ODN probe in which 5'-OH group of the ODN is replaced by [F-18]fluoride. The synthesis involves radiofluorination of a modified nucleotide followed by its coupling to a fully protected CPG-bound ODN, The key precursor, 5'-O-tosyl-3-O-di(p-methoxyphenyl)phenylmethyl-4'-O-methyl-thymidine, was prepared in six steps from thymidine in a 22% overall yield. Nucleophilic fluorination of the precursor with [F-18]fluoride ion in the presence of K+/Kryptofix and subsequent deprotection gave 5'-deoxy-5'-[F-18]fluoro-4-O-methyl-thymidine. Coupling to the thymidine-activated CPG-bound ODN, simultaneous cleavage from the CPG and de-protection, and HPLC purification furnished the target 5'-deoxy-5'-[F-18]fluoro-OZN probe. The HPLC spectrum was identical to that of the

Gene transfer, especially herpes simplex virus thymidine kinase gene transfer has shown significant potential in treating several common cancers. The principal obstacle to successful gene therapy has been the delivery of genetic vectors capable of achieving efficient gene transfer and the methods of assessing their transfers in vivo non-invasively. [F-18]FHPG(1) has been synthesized and suggested as a potential agent for monitoring the efficiency of gene therapy. The purpose of this study was to improve and simplify the synthesis of [F-18]FHPG. Methods: [F-18]FHPG was synthesized by nucelophilic substitution of N2-(p-amidinophenethyl)-9-[[1-(p-amidinophenethylmethylene)-3-toluene-sulfonyl-2-propoxy][methyl]guanine with [F-18]fluoride at different temperatures. The resulting intermediate was deprotected in 1N HCl at different temperatures and the product was isolated with HPLC (Altech, 18x100mm, CH3CN/H2O:5:95; 2 mL/min) or silica Sep-Pak. The by-product was washed out first with CH2Cl2/MeOH, 9/1 and the product was isolated with CH3CN/H2O, 8/2. For stability studies, it was dissolved in 1N HCl and heated at 90°C and 120°C, respectively, for different time intervals and monitored with TLC. In vitro activity of 1 synthesized and purified by HPLC and TLC was evaluated with 9L (glioma) cells. Results: The yield of 1 decreases as the reaction temperature increases. At 120°C and 90°C, and the product was purified with HPLC, the yield of 1 was 2 and 5-10%, respectively. The synthesis time was 90 min. from EOB. The yield of 1 increased to 10-15% when the reaction temperature was 90°C and the product was purified with silica Sep-Pak. The synthesis time was 60 min from EOB. [F-18]FHPG was unstable in 1N HCl at high temperature. At 120°C, 50% of 1 was decomposed in 10 min while 90% of 1 remained intact at 90°C. [F-18]FHPG purified either by HPLC or silica Sep-Pak has the same in vitro activity. Conclusions: The yield of [F-18]FHPG can be improved by carrying out the reaction at lower temperature (90°C instead of 120°C) and purified with silica Sep-Pak. The same procedure probably can be applied to prepare similar radiotracers (e.g. pencillio).