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TITLE: Genetically Targeted Radiotherapy Utilizing the Human Sodium Iodide Symporter in Human Breast Carcinoma Cells

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14. ABSTRACT

The purpose of this proposal was to examine the efficiency of NIS mediated genetically targeted radiotherapy as a possible non-invasive therapeutic treatment in human breast carcinoma. SK-Br-3 cells were transfected with hNIS plasmid to develop stable NIS expressing cells. Clones were grown and are currently being screen for radioactive accumulation. The stable NIS-expressing cells will then be utilized in determining the level of NIS expression necessary to elicit a bystander effect. In addition to creating stable NIS-expressing cells, treatments with various histone deacetylase inhibitors or retinoic acid were used to increase the endogenous NIS expression in the breast carcinomas cells. Real time RT-PCR was utilized to analysis the NIS mRNA expression following the treatments. Radioiodide accumulation assays were then performed to examine the ability of the cells to uptake radioactivity. Ad-NIS treatment coupled with increases in endogenous NIS may help to improve the therapeutic outcome.

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Introduction

Delivered intra-tumorally, genetically targeted radiotherapy utilizes gene transfer of therapeutic genes to directly target malignant tissue. The sodium iodide symporter (hNIS) can confer the ability to accumulate radionuclides in transduced cells. The result is an increase in radioactivity, leading to cell death not only of the NIS expressing cells but also surrounding cells through the bystander effect.

hNIS is responsible for the physiologic accumulation of iodide in the human thyroid. Once iodide is transported across the basolateral membrane and into the cells, it is catalyzed by thyroperoxidase (TPO). The organification retains the iodide in the thyroid until stimulated to release thyroid hormone. In addition to iodide, NIS has an affinity for several halides and pseudohalides, enabling imaging and potential therapies. Clinicians have used the ability of hNIS to concentrate iodide for years in treatment for well-differentiated thyroid cancer. I-131 therapy is partially responsible for the excellent 10 year survival rates found in thyroid cancer patients.

The accumulation and retention of radioiodide in breast cancer patients can enable imaging and targeted therapy. Non-invasive imaging can provide real time assessments of this novel therapy while sparing the patient undo stress and pain. Transduction of hNIS coupled with increasing endogenous NIS could deliver a necessary lethal dose of radiation dose to the tumor yet spare surrounding normal tissue. This tumor targeted therapy could help to minimize the current side effects and morbidity associated with the current therapies.

This report will examine the current data gathered from each experiment. Current problems encountered and how new technology is being utilized to analyze new data will also be discussed.

Body

The aim of task 2 was to create stable NIS-expressing SK-Br-3 clones and empty vector control cells. Cells were transfected with 2 μg of plasmid DNA containing full-length sodium iodide symporter. Transfection was performed utilizing Effectene Transfection Kit (Qiagen Inc, Valencia CA) and cells were selected for neo resistance. Resultant clones were, and are currently, being screened for radioactive iodide accumulation.

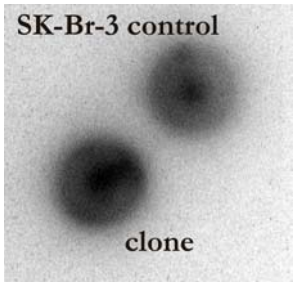
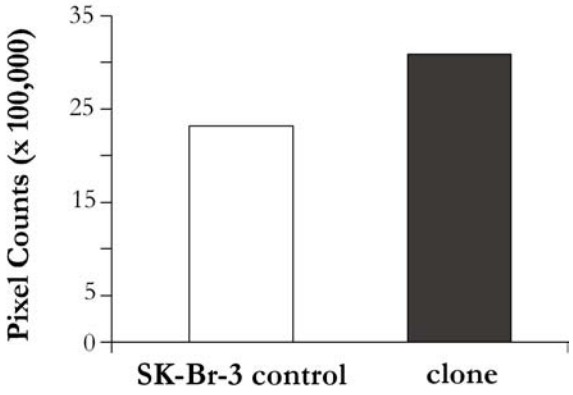


Figure 1. *In vitro* imaging with radioiodide. The clones were treated with 3 μCi of I-125 and visualized using autoradiography. The NIS-transfected clone accumulated iodide at slightly levels than the parent cells.

The NIS-expressing clone shown (Figure 1) accumulated approximately 1.5 times more I-125 than the parent cell line (Graph 1). Several other clones were screened in addition to that shown (Figure 1) yet displayed no detectable radionuclide accumulation above that of parent cells (data not shown).



Graph 1. The Stable NIS expressing clone was able to accumulate iodide in contrast to the control cells.

The level of accumulation is only slightly higher than the parent SK-Br-3 cells. For this reason more clones are currently being screened to identify higher expressing clones. Those clones will then be utilized to determine the required percentage of transfected cells to elicit a desired therapeutic effect.

Several papers have described silencing of endogenous NIS expression. Following treatments with different histone deactylase inhibitors or retinoic acid (RA), NIS expression was restored. Recent experiments performed in our laboratory using head and neck squamous cell carcinomas have shown a limited number of cells were transfected. These findings, in addition to the recent papers describing NIS silencing, has led to testing of certain histone deactylase inhibitors in combination with retinoic acid (RA) or RA alone to reactivate the endogenous NIS gene. The increased expression of NIS throughout the tumor coupled with Ad-NIS treatments may increase the overall cytotoxic effect to the tumor.

Several histone deactylase inhibitors (HDACi), including sodium butyrate (SB), trichostatin A (TSA), oxamflatin, were tested using T47D breast carcinoma cells. The RNA expression levels were analyzed using SYBR green real-time RT-PCR (ABI 7000 sequence detector, Applied Bioscience, Foster City CA). The primers were designed to span the 8^{th} and 9^{th} exon juction as seen in figure 2 of human NIS (hNIS).

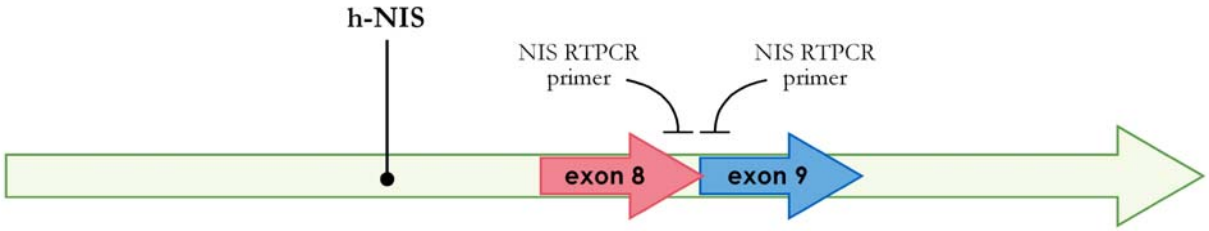


Figure 2. Schematic of hNIS depicts the location of the real time primers used to detect the RNA expression. The primers span the 8th and 9th exon junction.

T47D cells were treated with RA for 12 hours and then one of several HDAC inhibitors for 24 h. NIS re-expression was observed at the highest levels when the cells were treated with SB (Figure 3). The fold mRNA increases were determined by normalizing the samples to 18S control and comparing that to the untreated control cells utilizing the ΔΔCT method.

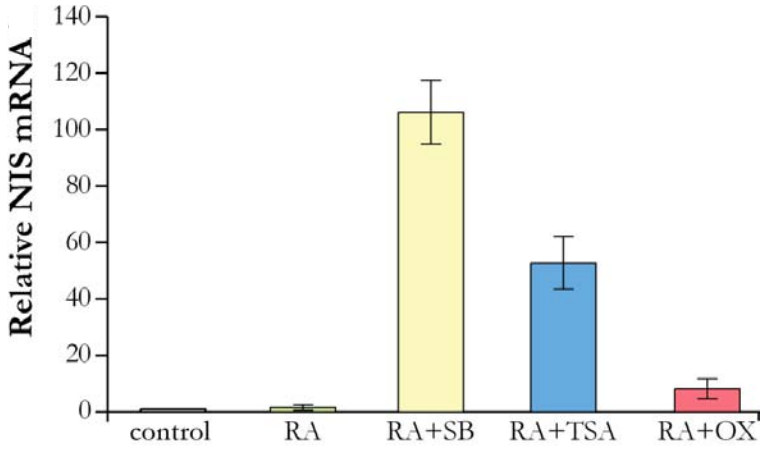


Figure 3. Quantitative real time RT-PCR analysis of NIS mRNA in T47D breast carcinoma cells. The cells were treated with HDAC inhibitors and RA. The SB induced the greatest expression of NIS mRNA. The cells receive 1 μM of RA, 3 mM of SB, 500 ng/ml TSA, or 2.5 mM of oxamflatin.

NIS expression was induced using the different HDAC inhibitors, suggesting NIS silencing may be due to chromatin condensation. To determine if NIS re-expression was sufficient to cause iodide accumulation, cells were treated 12 hours with 1 μM RA or 24 h with 3 mM of SB; then incubated with 3 μCi I-125 for 1 hour, and plates were visualized using phosphorimaging

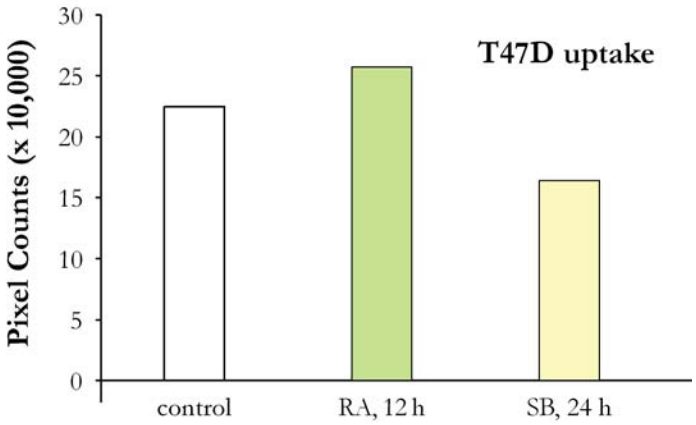


Figure 4. Radioactive iodide accumulation in T47D cells treated with RA or SB. The cells were unable to accumulate radioactivity following either treatment.

screens. No detectable signal was observed with RA or SB treated cells compared control cells (Figure 4).

Similar experiments were performed using SK-Br-3 breast carcinoma cells. These cells were treated with 3 mM of SB for 24 hours to determine if NIS mRNA could be induced. The treatment times used were based on earlier experiments (data not shown). mRNA was detected using quantitative real time RT-PCR and compared to 18S controls and untreated cells. The mRNA in SB treated cells was induced ~15-fold higher than the untreated SK-Br-3 cells (Figure 5).

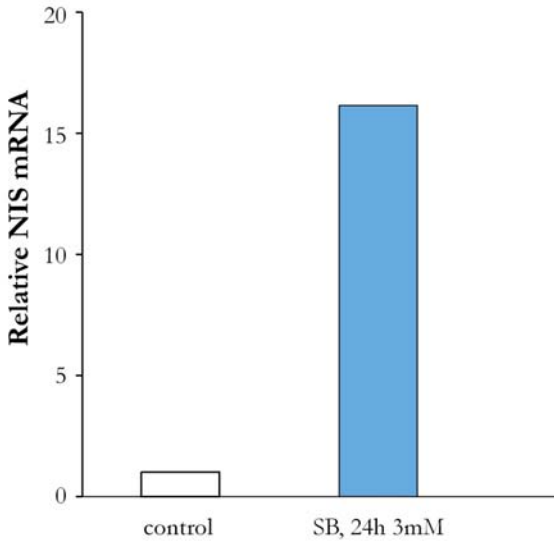


Figure 5. Real time analysis of NIS mRNA in SK-Br-3 cells. The mRNA was induced after treatment with 3 mM SB for 24hr.

An iodide accumulation assay was performed to determine if the increase in NIS mRNA could promote increases in iodide uptake compared to controls. As seen in the previous experiment, the mRNA was induced but it was not sufficient to confer radioactive accumulation. Figure 6 depicts SK-Br-3 either untreated or treated with RA or SB then incubated with radioiodide.

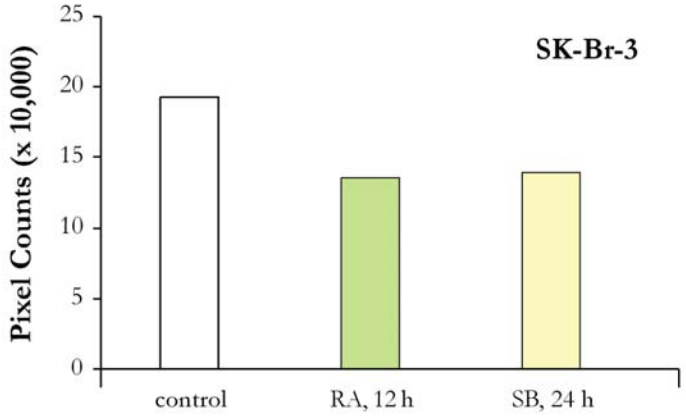


Figure 6. Graphic depiction of radioactive iodide accumulation. SK-Br-3 cells treated either RA or SB showed accumulation levels similar to untreated cells.

The MCF-7 breast carcinoma was shown to have lower radioiodide accumulation; thus, accumulation increases in this cell line may significantly improve therapeutic outcomes. These cells were used to determine if RA or SB treatment could enhance uptake. Cells were treated with 1 μM of RA or 3 mM of SB and then assayed for radioiodide accumulation. The MCF-7 cells treated with RA induced the mRNA expression significantly compared to the control cells. Cells were transfected with Ad-NIS to compare expression levels of cells that did accumulate radioactivity (Figure 7).

Real time RT-PCR was used to determine if mRNA in RA and Ad-NIS MCF-7 treated cells were similar. The cycle numbers were observed to be similar for the two different treatments, with cts about 24 or 25 cycles. Each experimental condition was performed in triplicate.

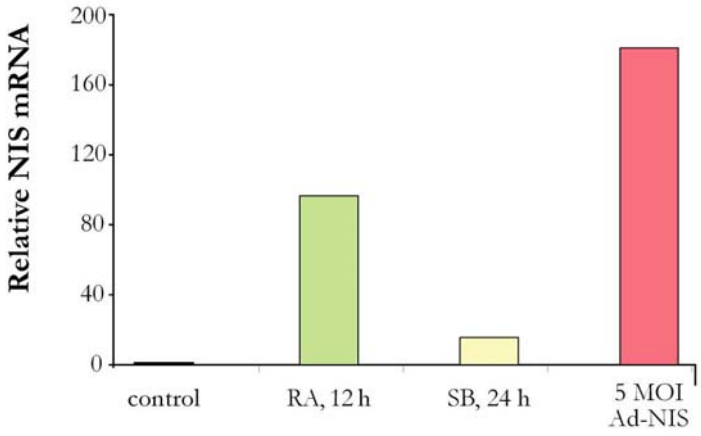


Figure 7. Real time RT-PCR analysis of endogenous NIS mRNA expression in MCF-7 cells. The two drug used increased the mRNA. RA treatment was significantly higher than control or SB.

Iodide uptake assays were performed to determine if increases in radioactive iodide could be observed. Cells were untreated or treated with RA, SB or Ad-NIS as in the real time RT-PCR assay and treated with I-125. The cells were then visualized using phosphorimaging screens.

MCF-7 cells given drugs were able to accumulate radioiodide at higher levels than the untreated cells. The levels of uptake observed were comparable to that seen with Ad-NIS transfected cells.

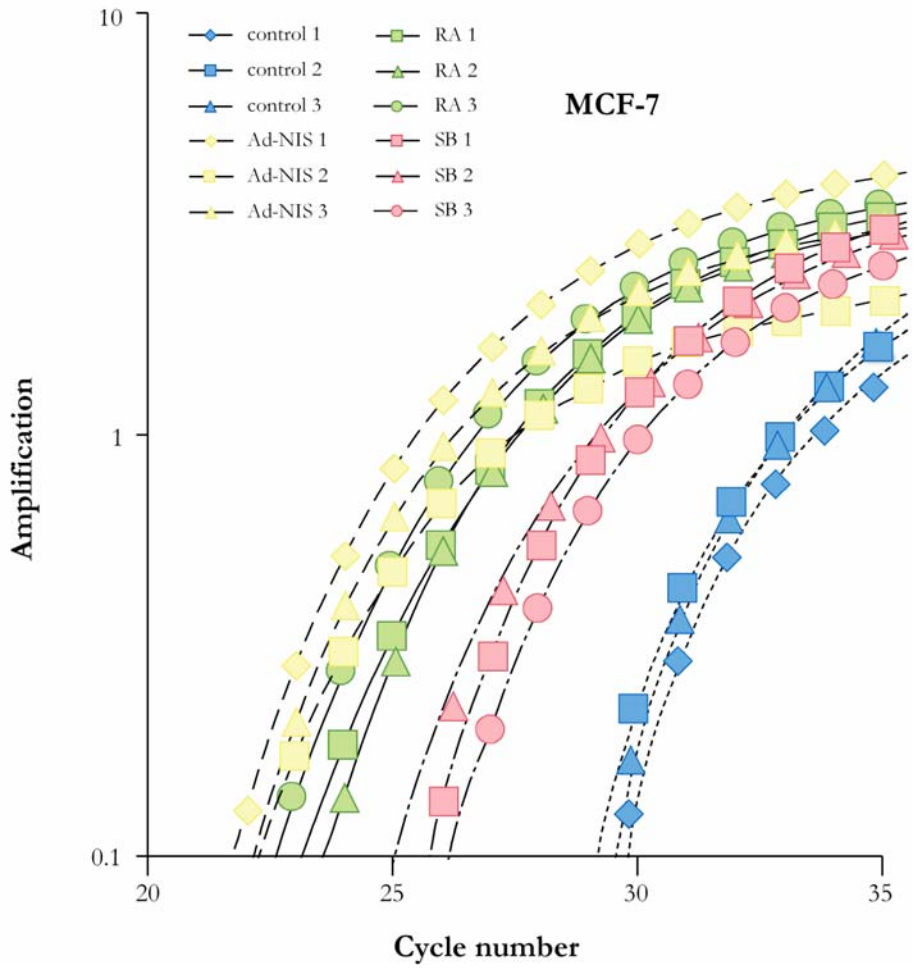


Figure 8. Amplification curves for MCF-7 cells untreated or treated with RA, SB or Ad-NIS. The experiment was performed in triplicate.

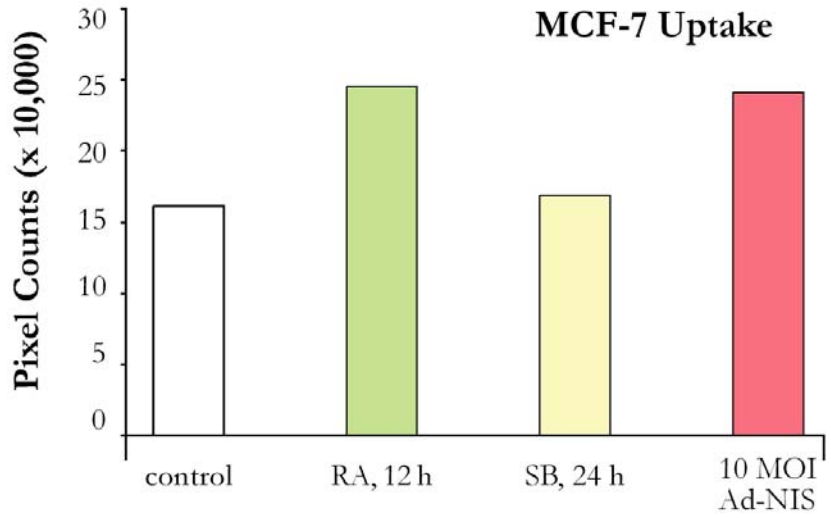


Figure 9. The iodide accumulation assay for MCF-7 treated cells. The cells treated with RA accumulated radioactivity at similar levels to Ad-NIS treated cells. The increase in radioactivity was higher than that of control or SB treated cells.

Further experiments are needed to determine if expression levels are insufficient to promote iodide accumulation in T47D and SK-Br-3 cells compared to MCF-7 cells or if the protein is not present at the surface of the membrane.

Key Research Accomplishments

Creation of stable NIS expressing clones derived from SK-Br-3 cells. Clones were created and currently being screened the ability to accumulate radioactivity compared to the control cells.

Retinoic acid and Sodium Butyrate. Experiments designed to increase endogenous NIS mRNA utilizing RA or SB resulted in the ability to increase the expression levels compared to untreated cells. The RA MCF-7 treated cells increased mRNA levels similar to lower MOI of Ad-NIS infected cells.

Iodide Accumulation in RA treated MCF-7 cells. Cells treated with RA were able to accumulate radioactivity. This treatment could increase cells within a tumor that could accumulate radioactivity. This coupled with Ad-NIS intratumoral injections could elicit a sufficient cytotoxic effect and a clinically relevant outcome.

Reportable Outcomes

1. Krager KJ, Gaut A, Madsen M*, et al.* Genetically Targeted Radiotherapy Using Sodium Iodide Symporter In Breast Cancer Cells. The 4th Era of Hope Department of Defense Breast Cancer Research Program Meeting. Philadelphia, PA; 2005.
2. Krager KJ., *et al*. Quantitative Dosimetry and Biodistribution of Radionuclide Concentrator Therapy in Ad-NIS Transduced Head and Neck Carcinoma. 52nd Annual Meeting of the Radiation Research Society, Denver, CO 2005.

The retinoic acid and HDAC inhibitor data is currently in preparation for a manuscript.

Conclusion

Stable NIS expressing clones are currently being screened for radionuclide accumulation. The cells will be utilized to determine the required percentage of transfected cells to elicit a desired therapeutic effect. The absorbed dose will be calculated using a gamma camera fitted with a pin-hole collimator to mimic the methods used in the clinic. Breast cancer cells treated with RA have been shown to accumulate radioactivity this could help increase the number of cells that will accumulate radioactivity in Ad-NIS infected tumors. MCF-7 infected cells were originally shown to have a low radioiodide accumulation treatment with RA in conjunction with Ad-NIS could increase the cytotoxic effect of this treatment. This treatment could again reduced the undesired effects seen with most current treatments.

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