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Doxorubicin Stimulates the Activity of the Na/I Symporter in Breast Cancer Cells

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Abstract

Background: The Sodium/Iodide Symporter (NIS) is a transmembrane glycoprotein with a molecular weight of 87 kDa and 13 transmembrane domains. NIS mediates the uptake of iodide into follicular cells of the thyroid gland and is the first step in the synthesis of thyroid hormone. This is achieved by transporting two sodium cations (Na+) for each iodide anion (I−) into the cell. Studies have shown that NIS expression in follicular cancer cells of the thyroid gland can be exploited in the treatment of thyroid cancer with 131I. Recent studies demonstrate that elevation of NIS expression may be augmented by treatment with the antibiotic doxorubicin and may not be limited to thyroid follicular cells.

Title: Therefore, this study was conducted in order to test the hypothesis that NIS expression and function can be augmented in the MDA-MB-231 breast cancer cells by doxorubicin treatment.

Method and Finding: This hypothesis were tested by treating MDA-MB-231 cells with doxorubicin at 0, 0.2, 2, and 20μM for 48hrs followed by measurement of NIS expression using Western blot and immunofluorescence assays and Iodine uptake using an uptake assay.

Conclusion: Our studies showed that NIS expression was augmented by doxorubicin treatment in MDA-MB-231 cells. Our studies also showed that increased expression of NIS corresponded to increased Iodine uptake. Thus, the use of the antibiotic doxorubicin in conjunction with radioiodine may provide a potential treatment for breast cancer.

Introduction

Cancer

What is Cancer?: Cancer is a genetic disorder that arises due to a defect in the regulation of cell division and differentiation [1]. These defects are caused by errors in DNA replication, chemical instability of certain DNA bases or from attack by free radicals generated during metabolism [2]. An error in the DNA is called a mutation; it involves a change of one or more bases. Mutations can be point mutation which changes the amino acid sequences and can result in a truncated protein product, or frameshift mutation which scrambles the amino acid sequences and affects the ability to make protein. Usually, when an error occurs during DNA replication, enzymes called proofreading and repair enzymes correct these errors, or the cell may cause itself to die due to these errors [1]. Sometimes, these errors are not fatal to the cell. An accumulation of such errors may cause the cell to lose its ability to perform normal cell functions, like regulating the progression through the cell cycle. This means that the cell will divide uncontrollably as compared to normal cells, and will eventually form a mass or tumor. Another cause of mutations is the generation of free radicals is due ionizing radiation. Ionizing radiation causes indirect damage to the DNA by radiolysis of water to produce free radicals or reactive oxygen species like superoxide, which react with the DNA, or direct damage to the DNA double-helix by causing single or double strand breaks [2]. For example, ultraviolet radiation fuses two adjacent Thymine bases together into a dimer, thereby disrupting normal DNA replication and base pairing. These can result in mutations if not repaired.

The mutated cells may occupy a mass, but still continue normal cell function, this is called a benign tumor. Alternatively, the cells may develop mutations that allow them act independently of surrounding cells and tissues. The mass formed by the latter type of cells is called a malignant tumor. The development of malignant cells takes years, sometimes, decades, as not all mutations result

in malignancy or neoplasia. The mutation of a gene involved in cell division, such as oncogenes and tumor suppressor genes, is required for tumorigenesis. This is because, the mutation of such genes may give the cell a selective advantage that will enable it divide faster than other cells [1]. Successive mutations in the mutated cells may give the cells the ability to divide even faster than previously, survive in the midst of normal cells or metastasize [3]. These successive mutations result i cell malignancy and eventually cancer. Malignant cells have the ability to invade other tissues and blood vessels, this enables them to form secondary malignant tumors. The spread of tumors around the body is what eventually kills the cancer patient.

Cancer Statistics in the U.S.: Cancer is the second leading (leading in 21 states) cause of death in the U.S. [4]. The National Cancer Institute (NCI) and its Surveillance, Epidemiology, End Results (SEER) Program collect and publish data on cancer incidents in the U.S. every year. An estimate of 1,685,210 cancer cases will be diagnosed in 2016, and 595,690 cases will be fatal [5]. Based on the 2008-2012 deaths, cancer has higher mortality among men than women, claiming 207.9 out of every 100,000 men and 145.4 out of every 100,000 women [5]. The number of cancer survivors is on the constant rise, and is expected to reach 19,000,000 by 2024, from 14,500,000 in 2014 [5].

Cancers of epithelial origin are the most common in humans. They are called Carcinomas, and include breast cancer, lung cancer and colon cancer. According to statistics, cancers of the digestive system will be the most common in 2016, with an estimate of 304,930 new cases, closely followed by cancers of the genital system, with an estimate of 297,530 new cases in 2016 [4]. With an estimate of 249,260 new cases in 2016, breast cancer is the third most common cancer in the U.S. Cancers of respiratory organs follow closely behind, with an estimate of 243,820 new cases in 2016 [4]. Other classifications of cancer include Lymphomas, Myelomas and Leukemia with estimates of 81,080, 30,330 and 60,140 new cases respectively [4]. Since it takes years for cancer to develop, the disease mostly affects the older population. In individuals above the age of 70, cancer will develop in every 1 in 3 men and every 1 in 4 women. For those younger than 50, cancer will develop in 1 out of 29 men, and 1 out of 19 women. The lifetime probability of getting cancer is 42 % for men, and 38 % for women. Breast cancer is more prevalent among women than men, with estimates of 246,660 and 2,600 new cases for women and men respectively. Breast cancer will develop in 1 out of every 8 women in her lifetime, and claim 40,450 female lives in 2016.

Breast Cancer: Breast cancers are classified under two major forms, noninvasive or in situ and invasive or infiltrating breast cancer [6]. Noninvasive breast cancers stay in a particular location, without spreading; while invasive breast cancers metastasize through lymph nodes and blood vessels to other parts of the body. Breast cancers are also classified based on their location of origin, for example, ductal carcinoma originates in the ducts and lobular carcinoma originates in the lobules [6]. Noninvasive breast tumors like Ductal Carcinoma *In Situ* (DCIS) are considered pre-cancers, as they sometimes become invasive and metastasize (American Cancer Society 2016). Invasive ductal carcinoma is the most common form of breast cancer [1]. Invasive cancers travel to axillary nodes. The presence of cancer cells in these nodes is important in determining the prognosis of the breast cancer [1]. Breast cancers are also classified based on the way they are treated. Examples include Estrogen Receptor (ER) positive, Progesterone Receptor (PR) positive and Human Epidermal Growth Factor Receptor 2 (HER2) positive breast cancers [6]. As indicated by their names, these cancers contain ER, PR and HER2 respectively, and can be treated using drugs that target these receptors [6]. Another example is triple-negative breast cancer. Triple-negative cancer cells developed mutations in the genes that code for ER, PR and HER2, making these receptors absent in the cells. Since this type of cancer is negative for all three receptors, it cannot be treated using drugs that target these receptors [6].

Treatments

Surgery: New advancements in oncological surgery have decreased the mortality of breast cancer patients as compared to twenty years ago [7]. Breast cancer surgery involves the removal of the tumor with or without other breast tissues. Surgery is usually the first treatment for breast cancer and can cure cancer in its early stage [7]. Surgery is also used as a prevention method, like in prophylactic mastectomy, which involves removal of the breast in high risk patients [8]. In some cases, surgery alone is not sufficient; a mixture of surgery with chemotherapy and radiation is used.

Chemotherapy: Chemotherapy involves using chemicals to target cancer cells in the entire body. These chemicals go through the bloodstream, so they can destroy cells that stay at their location of origin, as well as cells that metastasize [8]. Chemotherapy attacks processes like cell division in rapidly dividing cells, thereby reducing the number of cancer cells and size of the tumor. It is sometimes used to shrink tumors before they are surgically removed [8]. Chemotherapy is also used after surgery to destroy any cells left and reduce the chance of relapse [8]. Chemotherapy has side effects in some patients. It affects normal cells that rapidly divide like cells in the hair follicles, causing the patient's hair to fall off.

Radiation: Radiation therapy or radiotherapy involves the use of high-energy radiation, like X rays and gamma rays, to kill cancer cells and shrink tumors [9]. Unlike chemotherapy, radiotherapy targets a particular tumor. Radiotherapy can be external-beam radiation therapy, whereby cancers cells are targeted from outside the body, or internal radiation therapy, whereby a radioactive material that will target cancer cells is ingested by the patient [9]. The radiation damages the DNA of the cancer cells or creates free radicals that can damage the DNA, causing the cell to stop dividing or kill itself [9].

Radiation

Doxorubicin: Doxorubicin (DOX) is an anthracycline antibiotic.

It is produced by *Streptomyces peucetius* [10]. It is usually the first drug used against breast cancer [11]. During chemotherapy, DOX is administered intravenously. It enters cancer cells by diffusion, and then binds to the cytoplasmic proteasome forming a DOX-proteasome complex. A protease is a protein complex that degrades unneeded proteins. Via active transport, the DOX-proteasome complex is transported into the nucleus through nuclear pores [12]. Once in the nucleus, DOX binds to the DNA due to its higher affinity for DNA [13]. DOX induces programmed cell death in cancer cells by interfering with their DNA or membranes, inhibiting topoisomerase II activity, activating p53 activity and inducing cell apoptosis [13]. DOX also induces the activity of Sodium/Iodide Symporter (NIS) in cancer cells, could potentially mediate ^{131}I uptake during radiation treatment.

Na/Iodide Symporter: NIS is an iodide transport protein primarily located at the surface of thyroid follicular cells [14]. This forms the basis of radioiodine therapy for the treatment of thyroid cancer [14]. NIS allows the uptake of I^- in thyroid cells to synthesize thyroid hormone [15]. It is also expressed in lactating breasts and enables infants synthesize their own thyroid hormone [14]. Through NIS, cancer cells can accumulate therapeutic radionuclides like ^{131}I [16]. This provides the possibility of radionuclide gene therapy as cancer treatment [15,16].

1.1.1. **^{131}I:** ^{131}I or radioactive iodine is used in the treatment of thyroid cancers. It is easy to administer and highly effective [17]. It is administered after surgery to target remnant tumors, or before whole body scans to locate tumors [17,18]. Radioactive iodine works either by directly causing damage to the DNA (for example, double strand breaks), or by indirectly producing free radicals in the cells, which eventually cause cell damage [18]. Although DOX is effective in cancer treatment, some patients experience unfavorable side effects. DOX is highly dose-dependent, as it induces cardiotoxicity in patients [19,20]. Also, it is common for cancer cells to develop resistance to DOX [21,22]. Radiotherapy on the other hand, can affect normal cells and cause subsequent damages, if the cancer cells are not targeted properly. Therefore, a combination therapy of DOX, ^{131}I and external beam radiation is being proposed. The triple negative breast cancer cell line MDA-MB-231 will be treated with doxorubicin. This will increase the amount of NIS present in the cancer cells. The cells will then be treated with ^{131}I, which will be taken in through the NIS. Finally, the cells will be treated with radiation therapy targeting ^{131}I. As mentioned earlier, patients with triple negative breast cancer cannot be treated with the drugs that target ER, PR and HER2. Thus, this will serve as an alternative form of cancer treatment for cancers such as triple negative breast cancer that cannot be treatment with the regular therapies.

Materials and Methods

Protein Isolation and Western Blot Analysis of NIS

Cells were seeded in tissue culture treated 150mm dishes (Celltreat, Inc) at a density of 250,000 cells/plate and were treated with 0, 0.2, 2, or 20μM doxorubicin hydrochloride for 48hrs. Cells were washed in 1xPBS and lysed in lysis buffer (0.1% Triton X-100 in PBS, pH 7.4) by pipetting up and down. The lysates were then centrifuged at 13000G for 10 minutes and the supernatant was used for Western blot analysis using an antibody directed against the Sodium Iodide Symporter (NIS) protein as previously described. Specifically, 122ng of each protein extract was diluted with Lammli sample buffer, placed in a boiling water bath for 5 min and electrophoresed on 10% SDS-polyacrylamide gel followed by electro transferring for one hour at 100V. Subsequent to western blotting, the nitrocellulose membrane (Pierce) was rinsed in 10mL Phosphate Buffered Saline (PBS), pH 7.4. It was then blocked using 10 mL of Blotto (PBS/ 5% low-fat dried milk/0.1% Tween 20), at 4 deg. C with slow shaking for 3 hours. Following rinsing with PBS the membrane was slowly shaken overnight with a 1:2000 dilution of goat anti-NIS antibody (Santa Cruz Biotech, Inc.). The blot was rinsed twice with PBS/0.1% Tween 20 and washed three times for 5 minutes each using 100 mL volumes of PBS/0.1% Tween 20. The membrane was probed for 2hrs at 4 deg. C with a 1:1000 dilution of horseradish peroxidase (HRP) conjugated rabbit anti-goat IgG (Santa Cruz Biotech, Inc) in 10 mL Blotto. Visualization of the NIS protein was achieved by the use of an ECL kit for the detection of HRP-labeled antibodies on Western blots (Fisher, Inc). The blot was imaged using Gel Doc Imaging system (Biorad, Inc) and the relative intensities of the protein bands were assessed using ImageJ (NIH) software from NIH and the average intensities of two identical treatments plotted as a function of doxorubicin concentration.

Immunofluorescence

MDA-MB-231 cells were seeded at 1 x 10^5 cells per well for 48 hours in a 12 well plate containing rattail collagen coated coverslips. Seeded cells were treated with 0, 0.2, 2, or 20μM doxorubicin hydrochloride for 48hrs. Cells were washed in 1xPBS, fixed for 10 minutes with 4% paraformaldehyde (PFA), permeabilized for 20 minutes in 0.2% Triton X-100 in PBS and blocked with 3% BSA for 1 hour. Samples were then incubated overnight at 4°C with goat anti-NIS primary antibody (Santa Cruz Biotechnology) with a 1:1000 dilution, washed 3 times with 1X PBS, and incubated with mouse anti-goat Texas Red® labeled secondary antibody (Santa Cruz Biotechnology). Following rinses with 1X PBS, the coverslips were mounted on slides using Vectashield medium containing DAPI (Vector Labs, Ca) and sealed with nail polish. Fluorescence imaging was accomplished using a confocal microscope (inverted Nikon TE2000-U microscope equipped with a 60X apochromat oil-immersion TIRFM objective).

Iodine Uptake Assay in MDA-MB-231 Cancer Cells

On Day 1, 5,000 cells per well were plated in a 96 well plate. The cells were incubated for 24 hours. On Day 2, the 96 well plate was aspirated and treated with different concentrations of Dox. Columns 1-4 served as the control, columns 5 and 6 were

treated with 0.2 µM Dox, columns 7 and 8 were treated with 2 µM Dox and columns 9 and 10 were treated with 20 µM Dox and incubated for 48 hrs. On Day 4, two solutions were made. The first was a NaI uptake solution containing 9.5 mL Hank's Balanced Salt Solution (HBSS) and 0.5 mL 2 mM NaI. The second was an ammonium cerium sulfate/sodium arsenate solution containing 3.2 mL deionized water, 3.2 mL 1x ammonium cerium sulfate and 3.2 mL sodium arsenate. Both solutions were mixed and kept at room temperature. The 96 well plate from Day 2 was then aspirated and washed three times in 200 µL HBSS. 100 µL of the NaI uptake solution was added to each well, and the plate was incubated in the dark at room temperature for 1 hr. After 1 hr, the uptake solution was aspirated, and the cells were washed three times in 200 µL. 100 µL of ammonium cerium sulfate/sodium arsenate solution was added to each well. The cells were then incubated in the dark at room temperature for 30 mins. The cells in each well were then read at A_{690} and A_{420}. The optical density of NaI was calculated by subtracting the absorbance values at A_{690} from those at A_{420}.

Effect of Doxorubicin on MDA-MB-231 Cancer Cell Growth

In order to assess the effect of Dox on cell growth, MDA-MB-231 cells were plated in 24 well plates at 50,000 cells per well and incubated for 24 hrs. The growth media was replaced with fresh media and cells were treated with 0, 0.2, or 20 µM Dox and incubated for 48 hrs. The number of cells per well was counted using a hemacytometer. Briefly, cells were detached with 250µL of trypsin, neutralized with equal amount of growth media and transferred to 50ml tubes. After mixing the cell samples, 50µL was transferred to a microcentrifuge tube and mixed with 50 µL of Trypan blue dye. A 10 µL aliquot of each sample was added to each side of the hemocytometer by placing the tip on the wedge opening and injecting the sample. The hemocytometer was placed on the stage of a microscope and the four corner and a central square, each containing 16 squares, were identified. Cells in the five squares were counted. Cells touching the top and right border of each large square were counted while those touching the left and bottom borders were not. The total the number of cells in all five squares was used to calculate the cell concentration as follows: Cells/ml = (total number of cells in 5 squares x dilution factor x 10,000 cells/ml)/5. The number obtained was used to determine the effect of Dox on cell growth. The values obtained were used to normalize Iodine uptake in MDA-MB-231 cells.

Results and Discussion

Western Blot and Immunofluorescence

To determine the expression of NIS in MDA-MB-231 cells, the cells were treated with 0.2 µM and 2 µM and 20 µM Dox for 48 hrs followed by protein expression measurement using Western blot assay (Figure 1) and Immunofluorescence assay (Figure 2). The data demonstrate that NIS is not detected prior to Dox treatment but its expression is stimulated by Dox at 0.2, 2 µM and 20 µM for 48 hrs.

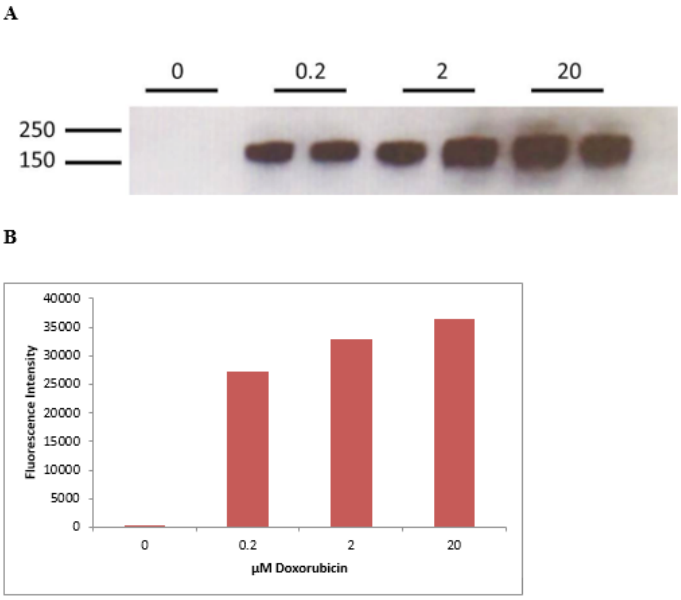

Figure 1: Doxorubicin stimulates NIS expression in MDA-MB-231 cells. MDA-MB231 cells were not treated (0) or treated with 0.2, 2, or 20µM of doxorubicin hydrochloride for 48 hours. Total protein was isolated, fractionated on SDS-polyacrylamide gel and subsequently transferred to nitrocellulose membrane. The NIS protein levels were determined by western blot using goat anti-NIS primary antibody and rabbit anti goat secondary antibody **(A)**. The relative intensities of each of the bands were quantified using Image J software and the average of each two identical treatments were plotted and compared with the control **(B)**. n=3; Means ± SEM; *p≤0.001.

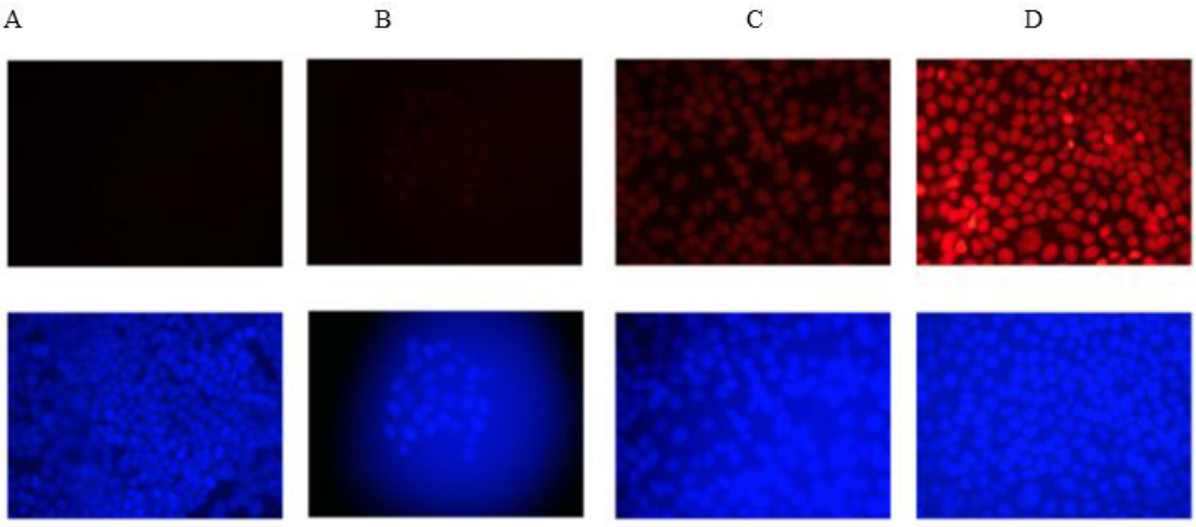

Figure 2: Doxorubicin stimulates NIS expression in MDA-MB-231 cells. MDA-MB231 cells were treated with 0 **(A)**, 0.2 **(B)**, 2 **(C)**, or 20µM **(D)** of doxorubicin hydrochloride for 48 hours. NIS protein expression was assessed by immunofluorescence as outlined under Methods using a goat anti NIS antibody followed by an anti-goat antibody conjugated to Texas Red. Images were acquired using a fluorescent microscope and levels of NIS expression was compared between treated and non-treated samples. Immunofluorescence images (top panels) and phase contrast images (bottom panels) are shown.

Cell Absorbance

To determine the amount of Iodide uptake in MDA-MB-231 cells, the absorbance of the cells in each well of a 96 well plate was measured using filters A690 and A420. The average of each concentration was taken and plotted in a graph shown in Figure 3.

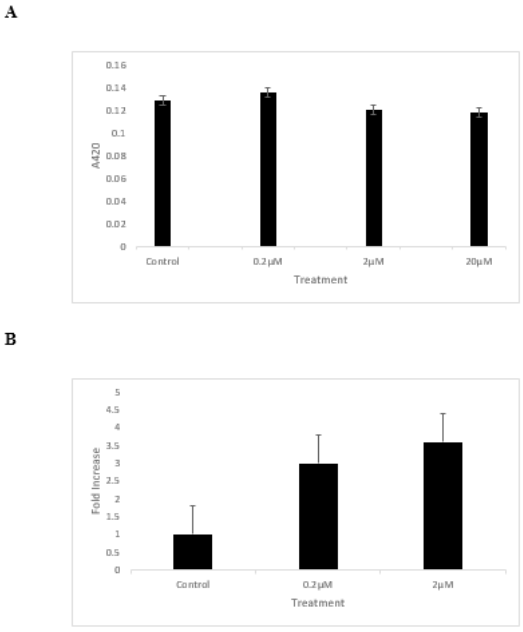

Figure 3: Doxorubicin stimulates Iodide uptake in MDA-MB-231 cells. Cells were treated with 0, 0.2 or 20µM of doxorubicin hydrochloride for 48 hours. Iodine uptake was assessed and expressed as Iodine uptake by plotting A_{420} versus Dox concentrations **(A)** or fold control by dividing Iodine uptake level in treated samples by non-treated samples **(B).** The effect of each treatment was determined by comparing fold control in treated and non-treated samples. n=16; Means ± SEM; *p≤ 0.05.

Fold Increase of Iodide Uptake in Cells with Different Dox Concentrations

To determine the amount of iodide uptake in each concentration of Dox, the effect of Dox on MDA-MB-231 cells was measured by counting treated cells under a hemocytometer after 48 hrs of treatment. The number of cells in each concentration were compared with the number of cells in the control, and the fold increase of Iodide uptake in each concentration was estimated and graphed in Figure 3b.

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