Aaron Kager
Day Lab
10 August 2017

DBZ and Notch-1 Therapy for Triple Negative Breast Cancer

Introduction

Triple Negative Breast Cancer (TNBC) accounts for 15-20% of all types of breast cancer.[1] TNBC cells lack expression of estrogen and progesterone receptors, and human epidermal growth factor receptor 2. Consequently, TNBC is unsusceptible to targeted therapy and hormonal treatment, so current treatment strategies primarily involve chemotherapy and radiation. These treatment methods are invasive, have many side effects and do not eliminate instances of recurrence, however.[1] Therefore, there is a need for new treatment methods to improve TNBC prognosis.

A promising approach for new therapeutic strategies involves targeting signaling pathways that are aberrantly active in cancer, particularly the Notch signaling pathway. The dysregulation of Notch signaling is associated with a variety of different cancers, as its overexpression activates downstream target genes associated with enhanced survival, proliferation and stem cell-like behavior (stemness).[2] At the cellular level, Notch signaling is induced by the binding of a Delta/Jagged ligand on one cell to a Notch receptor on a neighboring cell. Downstream in the target cell, the gamma secretase complex cleaves off Notch intracellular domain (NICD). NICD then enters the nucleus and acts to promote the expression of various oncogenes, driving disease progression.[2] Current therapies in development aim to inhibit Notch signaling, either through antibodies or gamma secretase inhibitors (GSIs). Antibody treatment can prevent ligand-induced expression, while GSIs block NICD cleavage. Non-targeted GSI treatment results in gastrointenstinal side effects, however, so new approaches are needed for delivery.[2]

I hypothesize that co-treating TNBC cells with a GSI, dibenzazepine (DBZ), and Notch-1 antibodies will be more effective than either treatment alone. DBZ and Notch-1 antibodies are potent therapies individually, but the combinatorial effects are unknown. My research will examine the potential for synergistic effects with a combination treatment of TNBC with Notch-1 antibodies and DBZ. Additionally, I hypothesize that encapsulating DBZ inside PLGA nanoparticles and functionalizing these particles with Notch-1 antibodies will help reduce the side effects observed with individual non-targeted therapy and increase efficacy of the individual and combined off-particle effects.

My approach, specifically, has involved the culture and treatment of the TNBC cell line MDA-MB-231. Notch-1 antibodies only, DBZ only, or combination treatments were administered to this cell line and the effects on survival (MTT assay), proliferation (EdU incorporation assay), and downstream protein levels (Western Blotting for the tumor suppressor

---

[1] Speiser, J., Foreman, K., Drinka, E., Godellas, C., Perez, C., Salhadar, A., . . . Rajan, P. (2012). Notch-1 and Notch-4 Biomarker Expression in Triple-Negative Breast Cancer.International Journal of Surgical Pathology,20(2), 137-143. doi:10.1177/1066896911427035

[2] Miguel Aste-Ame´zaga, Ningyan Zhang, Janet E. Lineberger, Beth A. Arnold, Timothy J. Toner, Mingcheng Gu… Hans E. Huber. (2010). Characterization of Notch1 Antibodies That Inhibit Signaling of Both Normal and Mutated Notch1 Receptors.

p53) were examined. I expected cells treated with DBZ and Notch-1 antibodies to show reduced viability and proliferation and increased p53 protein levels relative to cells exposed to no treatment, with combination treatment showing an enhanced effect over single therapies in MDA-MB-231 cells.

The overall goal of the project was to determine whether a combination of Notch-1 antibody and DBZ therapies can work synergistically to reduce TNBC cell viability and proliferation while increasing tumor suppressor p53 protein levels.[3] Additionally, this project is working towards creating a nanoparticle formulation that can be used to co-deliver these therapies in a clinical setting.

Results

DBZ and Notch-1 Antibodies Reduce Cell Viability

MDA-MB-231 cells treated with DBZ experienced a decrease in cell viability, as determined by an MTT assay 72 hours post-treatment (**Figure 1)**. A dose-dependent inhibition of cell viability was observed, which resulted in a calculated IC_{50} (half-maximal inhibitory concentration) of DBZ of approximately 10 nM.

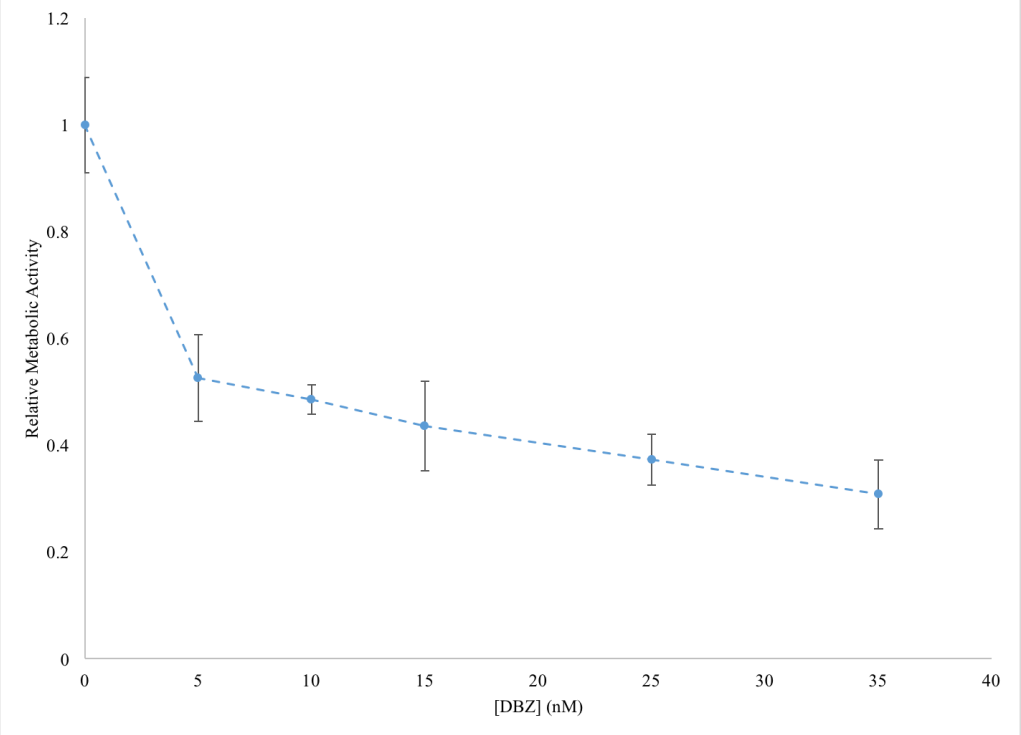

Figure 1: Effect of DBZ on MDA-MB-231 cell viability. The averages and standard deviations of normalized viability values of MDA-MB-231 cells are shown after treatment for 72 hours with a range of DBZ concentrations, 0 – 35 nM.

[3] National Center for Biotechnology Information (US). Genes and Disease [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 1998-. The p53 tumor suppressor protein. Available from: https://www.ncbi.nlm.nih.gov/books/NBK22268/

MDA-MB-231 cells treated with Notch-1 antibodies also showed a decrease in cell viability, as indicated in **Figure 2**. MDA-MB-231 cells were exposed to Notch-1 antibodies at concentrations of 0, 5, 10, 15, 20, and 25 μg/mL. 72 hours later, cell viability was determined by an MTT assay. A dose-dependent inhibition of cell viability was observed up until a threshold of 15 μg/mL, after which, the efficacy tapers off. The IC_{50} of Notch-1 was approximately 4 μg/mL (**Figure 2**).

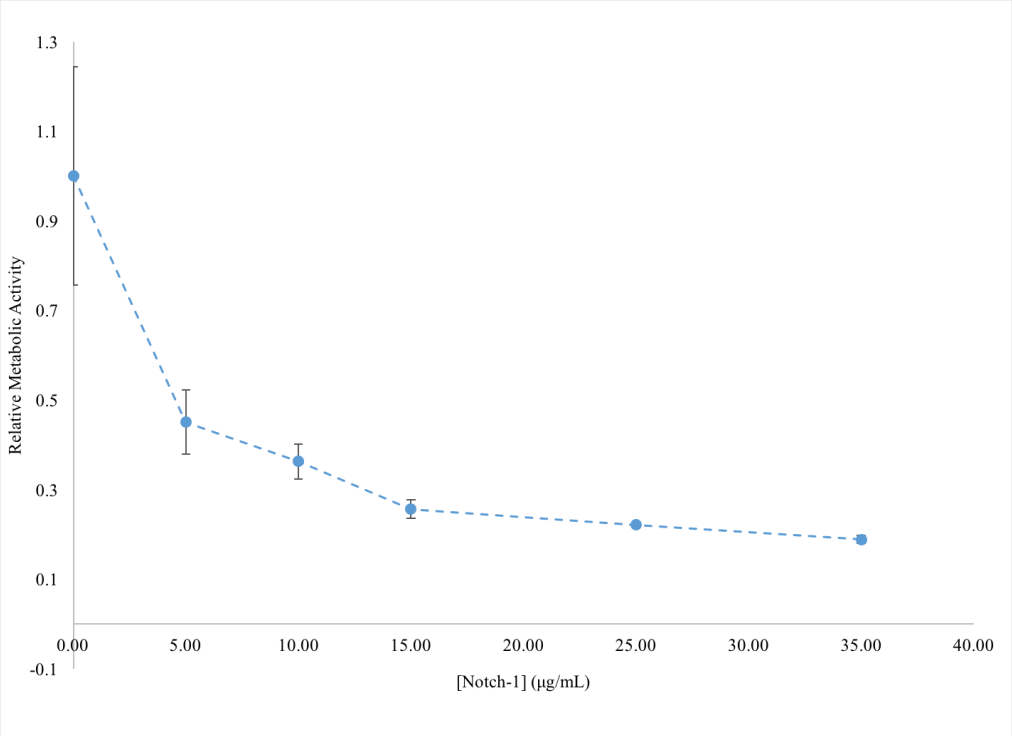

Figure 2: Effect of Notch-1 antibody therapy on MDA-MB-231 cell viability. The averages and standard deviations of normalized survival values of MDA-MB-231 cells are shown after treatment with a range of Notch-1 antibody concentrations, 0 – 25 μg/mL.

MDA-MB-231 cells were then treated with DBZ only, Notch-1 antibodies only, or a combination of DBZ and Notch-1 antibodies at the individual therapies' IC_{50} concentrations. Cells exposed to combination treatment exhibited lower viability than individual treatments, as expected (**Figure 3**). This suggests that DBZ and Notch-1 have an additive effect and further study into their combined delivery is warranted.

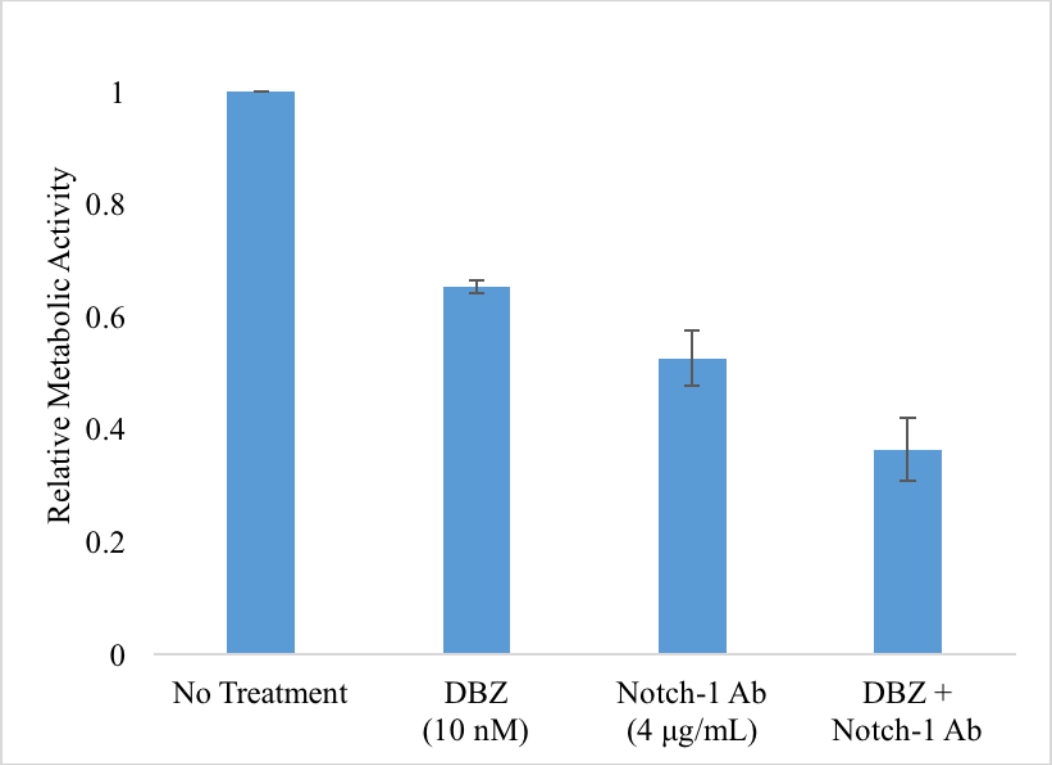

Figure 3: Effect of combined DBZ and Notch-1 antibody treatment on the relative metabolic activity of MDA-MB-231 cells. The averages and standard deviations of relative metabolic activity were measured by an MTT assay. Treatments included a control (untreated), DBZ at 10 nM, Notch-1 antibody at 4 µg/mL, and a combination of DBZ and Notch-1 antibody at these respective IC_{50} concentrations.

DBZ and Notch-1Antibodies Affect Cell Proliferation

A preliminary EdU incorporation assay showed that MDA-MB-231 cells treated with DBZ exhibited reduced proliferation in comparison to cells exposed to no treatment (**Figure 4a**). While Notch-1 antibodies alone did not appear to reduce cell proliferation (Figure 4a), they did appear to decrease the amount of cells present (**Figure 4b**). This assay needs to be repeated to validate the findings, but suggests that DBZ and Notch-1 antibody therapy may have different functional impacts on cells, warranting their combined application.

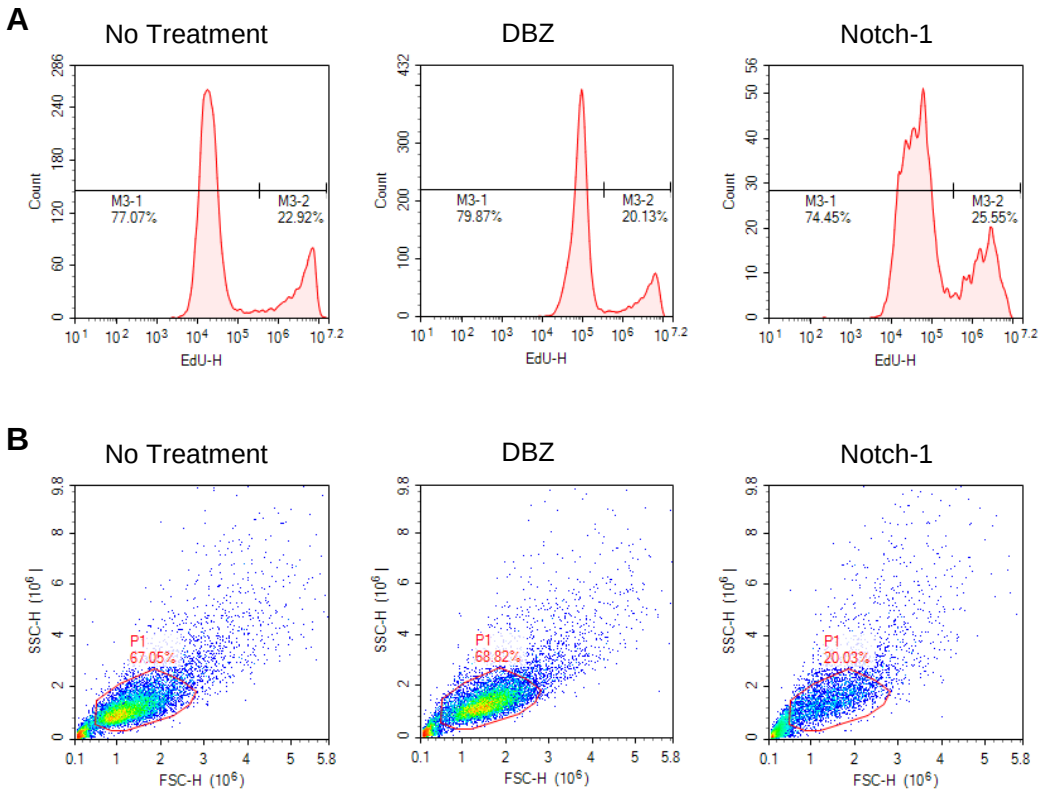

Figure 4: EdU incorporation measured via flow cytometry were used to indicate proliferative cells. (a) Histograms display cell proliferation for three different treatments. From left to right: No treatment, 10 nM DBZ, 4 µg/mL Notch-1 antibody. (b) Density plots of the same treatments described in (a).

DBZ Upregulates p53 Protein Levels

p53 is a known tumor suppressor protein, responsible for the regulation of target genes.[4] p53 is downregulated in cancer, which allows for the formation of tumors and rapid proliferation.[4] Western blot analysis of the lysate of MDA-MB-231 cells treated with DBZ, Notch-1 antibodies, or control IgM antibodies show that all three treatments upregulate p53 protein levels, indicating these therapies could promote tumor suppression (**Figure 5**). Here, it should be noted that the IgM and Notch-1 antibodies were not purified to remove azide groups before their application to the cells, so the amplified p53 observed with IgM is likely a result of the azide present in the sample, and the true effect of the Notch-1 antibodies is the amplification of p53 beyond that observed with IgM antibodies. Future studies will repeat this assay using purified antibody samples so that the effect of any residual azide is removed as a confounder. Regardless, this preliminary assay confirms that both DBZ and Notch-1 antibodies have the ability to amplify expression of the p53 tumor suppressor in MDA-MB-231 cells.

A

B

Treatment	p53 Normalized Intensities
No Treatment	1
DBZ	9.004
Notch-1	20.072
IgM	15.783

Figure 5: Effect of treatment groups on p53 protein levels in a Western Blotting analysis. (a) Western blot with lanes from left to right: Untreated, DBZ-treated, Notch-1-treated, and IgM-treated MDA-MB-231 lysate. Bands shown are p53 (53 kDa, top) and β-actin (42 kDa, bottom). (b) Quantification of Western blot band intensities showing DBZ and Notch-1 antibodies upregulate p53 protein levels in MDA-MB-231 cells.

DBZ and Notch-1 Can Be Incorporated into PLGA Nanoparticles

Poly(lactic-co-glycolic acid) (PLGA) nanoparticles have previously been explored for applications in drug delivery because they have biodegradable and biocompatible properties.[5] Functionalizing PLGA nanoparticles with targeting agents such as Notch-1 antibodies may increase their specificity for cancerous cells, improving the therapeutic efficacy of both the

[4] National Center for Biotechnology Information (US). Genes and Disease [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 1998-. The p53 tumor suppressor protein. Available from: https://www.ncbi.nlm.nih.gov/books/NBK22268/

[5] Makadia, H. K., & Siegel, S. J. (2011). Poly Lactic-*co*-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polymers*, *3*(3), 1377–1397. http://doi.org/10.3390/polym3031377

Notch-1 antibodies and any encapsulated treatment while decreasing systemic toxicity and side effects. Therefore, I explored whether DBZ could be encapsulated inside PLGA nanoparticles and whether Notch-1 antibodies could be conjugated to the surface of PLGA nanoparticles. The observed hydrodynamic diameter of the different particle types was indicative of successful functionalization or encapsulation. As expected, Notch-1 antibody conjugation to the surface of PLGA nanoparticles increased the hydrodynamic diameter by ~15 nm. When DBZ was encapsulated inside PLGA nanoparticles the hydrodynamic diameter decreased by about 17 nm (**Table 1**), which is consistent with the increase in hydrophobic interactions present in the particle core.

Table 1: Different particle types and their hydrodynamic diameter as measured by dynamic light scattering (DLS). Bare PLGA nanoparticles averaged 70.57 nanometers. DBZ loaded particles showed a smaller hydrodynamic diameter, while Notch-1 antibodies conjugated to the PLGA surface resulted in a larger diameter.

Particle Description	Hydrodynamic Diameter (nm)
PLGA NP	70.57
PLGA NP w/ Notch-1 mAbs	85.86
DBZ in PLGA NP	53.65

Limitations and Future Direction

While I used simple dynamic light scattering (DLS) measurements to compare the hydrodynamic diameters of antibody-coated and DBZ-loaded PLGA nanoparticles (NPs) to that of bare PLGA NPs in order to determine if the conjugation and/or encapsulation protocol had been successful, this simple technique does not actually quantify the amount of DBZ loaded in the nanoparticles or the amount of Notch-1 antibodies conjugated to their surface. In future studies, a modified enzyme-linked immunosorbent assay (ELISA) will be used as a method of quantifying Notch-1 antibodies conjugated to PLGA NPs. High-performance liquid chromatography (HPLC) will also be used to measure DBZ encapsulated in PLGA NPs.

Subsequent studies will also examine the efficacy of the nanoparticle therapy in MDA-MB-231 cells in comparison to freely delivered DBZ and/or antibodies. I will also compare the results observed in the TNBC cell line to a healthy mammary cell line (MCF-10A) in order to validate that the targeted nanoparticle therapy is specific for diseased cells.

Materials and Methods

Cell Culture. The human breast cancer cell line MDA-MB-231 was routinely cultured in complete medium (DMEM supplemented with 10% fetal bovine serum, 1% penicillin streptomycin) in a 5% CO_2 and 37°C incubator. During passaging, cells were washed prior to being trypsinized with PBS. Cells were then lifted using 0.25% Trypsin.

MTT Vitality Assay. To determine the therapeutic efficacy of different treatments *in vitro*, MDA-MB-231 cells were plated at 5,000 cells/well in a 96-well plate and incubated in DMEM with 10% fetal bovine serum and 1% penicillin streptomycin at 37°C overnight. Cells were then incubated at 37°C for 72 hours with the following treatments in media: 10 nM DBZ, 4 μg/mL Notch-1 monoclonal antibody, 4 μg/mL IgM antibody, a combination of DBZ and Notch-1 at the same concentrations, or a combination of DBZ and IgM at the same concentrations. After treatment, an MTT bioreagant was used to replace the solutions in the wells. After 3 hours, DMSO was added and left to react for 10 minutes. The absorbance was then analyzed on a Biotek Synergy H1M plate reader and values were normalized to untreated groups.

EdU Proliferation Assay. To determine the level of proliferation in MDA-MB-231 cells, cells were plated at 100,000 cells/well in a 24-well plate and incubated in DMEM with 10% fetal bovine serum and 1% penicillin streptomycin at 37°C overnight. Cells were then incubated at 37°C for 72 hours with the following treatments in media: No treatment, 10 nM DBZ, 4 μg/mL Notch-1 monoclonal antibody, or 4 μg/mL IgM antibody. At the 66-hour time point, EdU was added to each well at a concentration of 10 μM in media with the exception of a negative EdU, untreated control group.

Cells were then trypsinized and collected into centrifuge tubes. Cells were pelleted by centrifugation and the supernatant was removed. Cells were then resuspended in in 3% BSA in PBS, pelleted by centrifugation, and the supernatant was removed. Then, cells were resuspended in 100 μL of 4% formaldehyde in PBS and incubated for 15 minutes at room temperature. The fixative was then removed by adding 900 μL of 3% BSA in PBS. Cells were pelleted by centrifugation and the supernatant was removed. Next, cells were resuspended in 100 μL of Triton-X and left to incubate for 20 minutes at room temperature.

The permeabilization buffer was then removed by adding 900 μL of 3% BSA in PBS, pelleting the cells through centrifugation and removing the supernatant. During this wash, two Click-iT® reaction cocktails were prepared, one with azide and one without azide (another untreated control group). 100 μL of reaction cocktail was then added to each tube and left to incubate for 30 minutes at room temperature, protected from light. After two more wash steps, the samples were run on the flow cytometer.

Western Blotting Assay. To determine the expression level of p53 in MDA-MB-231 cell line, cells were plated at 100,000 cells/well in a 24-well plate and incubated in DMEM with 10% fetal bovine serum and 1% penicillin streptomycin at 37°C overnight. Cells were then incubated at 37°C for 72 hours with the following treatments in media: No treatment, 10 nM DBZ, 4 μg/mL Notch-1 monoclonal antibody, or 4 μg/mL IgM antibody. Following the 72-hour time point, the treatments were removed and the cells were rinsed once with PBS. Then, 25 μL of lysis buffer with protease inhibitor was added to each well. Cells were lysed, transferred to centrifuge tubes, and put on ice for 30 minutes. The samples were centrifuged for 5 minutes at 12,000xg. The supernatants of samples were kept, and the pellets discarded. A DC Assay was used to measure protein content in the remaining lysate.

Samples of cell lysate were added to Laemmli Sample Buffer in equal parts and left to incubate in Thermomixer set to 99°C and 400 rpm for 20 minutes. 4-12% agarose gel was prepared in a container with MES SDS running buffer. The gel was then loaded with lysate samples and an 11-250 kDa protein ladder. The gel was run at a constant 110 V for 1 hour. The gel was then placed onto an activated membrane and inserted into a turbo blotting system. The

membrane was then incubated at 4°C overnight on a rocker, submerged in p53 primary antibody at a 1:1000 dilution in 5% milk in TBS-T. Next, the membrane was washed and submerged in a secondary antibody solution (Affinity Purified Antibody Peroxidase Labeled IgG) at a 1:5000 dilution in milk buffer for 1 hour. The membrane was then developed in ECL solution and imaged using a ChemiDoc-It Imager. Image analysis was done using the program ImageJ.

Synthesis of PLGA Nanoparticles. PLGA was dissolved in acetone (1 mg PLGA /1 mL of acetone) for 20 minutes. The dissolved PLGA, and any material to be encapsulated (such as DBZ), was added dropwise to water in a 1:3 ratio by volume. The mixture was left to stir for 2 hours without a cover to allow the acetone to evaporate. The water and PLGA sample was then transferred to a centrifuge filter tube and centrifuged at 4200 rpm, 4°C, for 30 minutes. The filtrate was removed and the purified nanoparticles were resuspended in DI H_2O.

Antibody-conjugated PLGA Nanoparticles. A solution of 1 mg EDC and 1 mg sulfo-NHS in 1 mL of H_2O was made up and added to 250 μL of PLGA NPs in H_2O. The solution was left on a rocker for 20 minutes to prime crosslinkers for the addition of primary amines on antibodies. 25 μL of antibody was then added to the solution and was left on a rocker for 1 hour at room temperature. The solution was then transferred to a centrifuge filter tube and centrifuged at 4200 rpm, 4°C, for 30 minutes. The purified conjugated nanoparticles were resuspended in DI H_2O.