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Effect of Combining Bcl-2 Inhibition and Radiation on Apoptotic Cell Death in Human Breast Cancer Cell Lines as Determined by Clonogenic Assay and Western Blots

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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May 2012

Honors Capstone Project in _____ Biology _____

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Abstract

Cancer is a group of diseases characterized by abnormal control of cell growth and cell survival. Current treatment for cancer employs a combination of modalities including surgery, radiation, chemotherapeutic drugs, and immunotherapy. Our lab focuses on personalized medicine and treatments that can specifically target molecular features of a tumor as presented by the particular patient being treated. Cancers often exhibit an over-expression or under-expression of certain proteins that play a large role in regulating the process of apoptosis, which is programmed cell death. This study focuses on proposing drug treatment for cancer that is personalized in that it targets a protein family that is often over-expressed. This study was conducted on four human breast cancer lines: MDA-468, MDA-231, BT-20 and T47D. The protein family studied was the BCL-2 family member proteins which are divided in pro- and anti- apoptotic proteins. Two of the four cell lines were characterized by an over-expression of anti-apoptotic proteins which offers an option for specific targeted treatment by invoking inhibition of the over-expressed proteins. A small molecule BCL-2 inhibitor, Obatoclax (GX15-070) was used in this study in combination with radiation therapy to observe the effects on growth of these cancerous cells and to determine whether apoptosis was induced. This study shows that Obatoclax and radiation in combination is more effective inducing apoptosis in neoplastic cells lines that over-express anti-apoptotic BCL-2 family members. This finding is significant because it demonstrates the potential value of personalized, specific targeted treatment.

Not all tumors have an over-expression of anti-apoptotic BCL-2 family members, therefore Obatoclax would not be an effective treatment for such cancers. Since this drug would be most effective in treating tumors with an over-expression of anti-apoptotic BCL-2 family members, this study proposes a method to discover which tumors may over-express anti-apoptotic BCL-2 family members and allows for quantification. This allows for the specific personalized treatment of certain cancers and may be a more effective approach to treatment than just the standard treatment options.

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Background

Apoptosis is a normal physiological process in which cells are “programmed” to die. Apoptosis may occur when a cell has been damaged beyond repair, it has outlived its usefulness, during embryonic development, when a cell has aged (senescent), and/or after potentially harmful mutations have damaged the cell, among other reasons. The morphological changes that occur in the nucleus during this process include chromatin condensation, nuclear fragmentation and DNA laddering. In the cytoplasm, apoptotic bodies are formed. The signature characteristic that indicates that apoptosis is occurring is the blebbing of the cell membrane (1). These morphological changes are all due to proteins called caspases. The process of apoptosis also affects the mitochondria of the cell. Changes include mitochondrial swelling through the formation of membrane pores, or they increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out. The intrinsic apoptotic pathway is characterized by increased permeability of the mitochondria and release of cytochrome c into the cytoplasm. Cytochrome c then forms a multi-protein complex known as the ‘apoptosome’ and initiates activation of the caspase cascade through caspase 9.

The process of apoptosis involves a family of proteins called the BCL-2 super family, which includes proteins that promote (pro-apoptotic) and inhibit (anti-apoptotic) apoptosis. The pro-apoptotic BCL-2 family members are further divided by their sequence homology with those that possess a sequence homology to the BH1, BH2, BH3, BH4 regions, multi-domain pro-apoptotic proteins, and those that possess homology only with the BH3 proteins (2). Bcl-2 Homology

(BH) domains are found in all proteins belonging to the Bcl-2 family. Anti-apoptotic Bcl-2 family proteins (including Bcl-2, Bcl-xl and Bcl-w) share BH1 and BH2 domains and in some cases, a BH4 domain while most pro-apoptotic BCL-2 family proteins contain a BH3 domain. The BH3 domain is also found in anti-apoptotic proteins. The figure below represents a schematic of some of the domains in some of the proteins in the BCL-2 family member proteins (3).

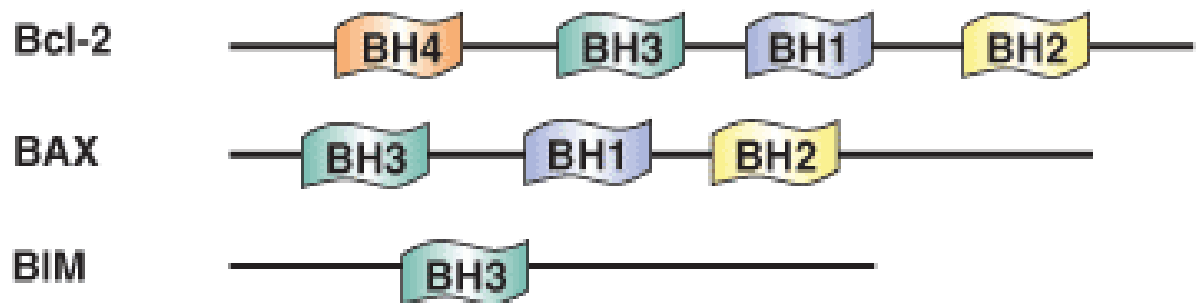


Figure 1: Figure shows the different BH domains to several of the BCL-2 family member proteins that play a role in regulating apoptosis (4).

Multi-domain pro-apoptotic proteins such as BAX and BAK work directly to induce apoptosis by receiving a death signal which then allows the mitochondrial membrane to become permeable for the release of cytochrome c. Cytochrome c then leads to the activation of caspases which play a large role in promoting apoptosis. Anti-apoptotic proteins from the BCL-2 super family work by inhibiting the release of cytochrome c from the mitochondrial membrane thereby blocking the activation of the BAK or BAX (pro-apoptotic) proteins (3). Proteins in the pro-apoptotic BCL-2 multi-domain family include BAX, BAK, BOK, and BOO among others while the pro-apoptotic BCL-2 BH3 family include BIK, BAD, BIM, NOXA and PUMA among others. Proteins in the anti-apoptotic

family include BCL-2, BCL-XL, BCLW, MCL1 among other proteins. Table 1 below represents a chart with the different anti-apoptotic and pro-apoptotic proteins found in the BCL-2 super family of proteins. BCL-2 is often overexpressed in many cancers including small cell lung carcinomas (SCLC), prostate, ovarian, bladder and breast cancer among many other cancers (3).

Table 1: BCL-2 Superfamily

Pro-Apoptotic		Anti-Apoptotic
BH3- only proteins	Multi-domain (BH)	
BIK	BAX	BCL-2
BAD	BAK	BCL-XL
BIM	BOK	BCL-W
HRK	BOO	MCL-1
BCLG	BCLG	BCL-B
HR	BCLB	+ viral homologs
NOXA	BCL-RAMBO	
PUMA		
+ others		

Breast Cancer is one of the most common forms of cancer among women. One in eight women will develop some form of breast cancer in her lifetime. In 2011, it was estimated that 230,480 new cases of invasive breast cancer were

expected to be diagnosed and an additional 57,650 new cases of non-invasive breast cancer in the United States (5). It was expected that 39,520 women would die from breast cancer with only lung cancer surpassing the number of deaths due to cancer for women. Since the 1990's there has been a decreasing trend in the number of deaths attributed to breast cancer due to several reasons including early detection, awareness, and advances in treatment options.

Significance of research

The process of apoptosis is deregulated in many cancers which can cause tumors to form. Since the BCL-2 family of proteins plays such a major role in apoptosis, these proteins are important to study because it offers a new option of treatment for certain cancers that over-express anti-apoptotic proteins. Those diagnosed with cancers known to have BCL-2 over-expression like certain breast cancers and lung cancers can have more treatment options available. Cancer treatments today may include surgery, chemotherapeutic drugs, immunotherapy, and/or radiation. Treatments that are effective in one type of cancer such as breast cancer can be applied to the treatment of other cancers, such as liver cancer or lung cancer if the mechanisms supporting these cancers are in fact similar. The pathological behavior of these cancers can be very different from patient to patient and using the same generalized non-specific treatment in all individuals may not be very effective since individuals react in different ways. Current research in the study of the BCL-2 family of proteins offers scientific researchers

a way to utilize this over-expression to offer personalized treatment that may be effective in more than one kind of cancer if those cancers derive from similar underlying molecular anomalies.

Methods and Materials

I. Cell Line and Reagents- Cell lines used were four human breast cancer cell lines which include MDA-MB-231 (MDA-231), MDA-MB-468 (MDA-468), BT-20 and T47-D. The MDA-231 cell line was obtained from a patient in 1973 at M. D. Anderson Cancer Center. This cell line is epithelial-like in morphology and exhibits spindle shaped morphology (6). MDA-468 was isolated in 1977 by R. Cailleau, et al., from a pleural effusion of a 51-year-old Black female patient with metastatic adenocarcinoma of the breast (7). BT-20 was established in 1958 by E.Y. Lasfargues and L. Ozzello. This cell line is epithelial-like in morphology (8). T47-D line was isolated from a 54 year old female patient with an infiltrating ductal carcinoma of the breast in 1979. These cells are epithelial-like in morphology and form a single layer in culture (9). The BT-20 line was maintained in Dulbecco's Modified Eagle's medium (DMEM) along with Ham's **F-12** Nutrient Mixture in a 1:1 ratio (Hyclone Laboratories, Logan, UT) with 10% fetal bovine serum (FBS), 2% penicillin/streptomycin, 10mmol/L HEPES, 1% sodium pyruvate, 2% sodium bicarbonate and 1% Non- Essential Amino Acids. T47-D, MDA-MB-468 (MDA-468), and MDA-MB-231 (MDA-231) were maintained in DMEM, which contained 10% fetal bovine serum (FBS), 2% penicillin/streptomycin, 10mM HEPES, 1% sodium pyruvate, 2% sodium bicarbonate and 20mM Glucose.

II. Drug Treatments and Irradiation- The main drug used is Obatoclax (GX15-070)

which is a small molecule BCL-2 inhibitor developed by Gemin X (Montreal, QC). Figure 2 shows the structure of Obatoclax.

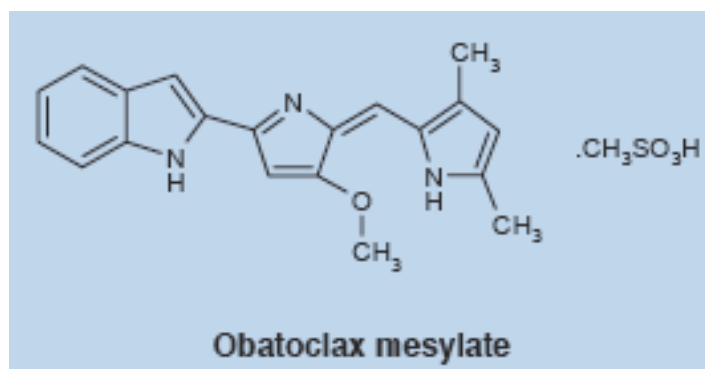


Figure 2: Chemical structure of Obatoclax mesylate (10).

Obatoclax was dissolved in DMSO at a stock concentration of 1mM and working dilutions were prepared in DMEM medium. Untreated control cells had the equivalent amount of DMSO added as the agent was diluted. Mock-irradiated cells (0 Gy) in plates were manipulated in the same way as those that received 2 Gy dose of x-irradiation. The X-ray source was a RS 2000 X-ray Biological Irradiator. Four hours after irradiation, cells were treated with 1uM of obatoclax (GX15-070) in a 37⁰C 5% CO₂ incubator. Assays were performed at 48 hours post-irradiation.

The following section describes the specific assays and protocols used to determine the effect of Bcl-2 Inhibition and radiation combination on apoptotic cell death in breast cancer lines. The protocols listed include the (III) colony assay, (IV) western blot (V) Growth-Inhibition Assay (Cytotoxicity assay) (VI). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (VII) PCR array.

(III.) The colony assay: protocol includes the following steps:

1. Place approximately 2×10^5 cells/well in a 6-well plate. Treat appropriately the next day.
2. Once the experimental treatment of cells is complete, plate limiting dilutions of cells (200-400 cells per well) in triplicate. One cell line per day.
3. Cells treated with radiation or agent may need to be plated with more cells however, keep the plating numbers as close to each other as possible.
4. Count at least 100 cells/side on hemacytometer using both sides to take an average.
5. Make 1:10 serial dilutions to get required number of cells in at least 100-200 microliters.
6. Plate cells in 5ml medium/well.
7. Leave plates for 8-12 or more days depending on the cell line.
8. On last day, rinse cells with PBS two times to get rid of the medium.
9. Add 0.5ml of 0.25% Crystal Violet in methanol (1.25 g CV in 500mL methanol-with 0.5mL of 37% formaldehyde).
10. Leave Crystal Violet on for 15 minutes.
11. Gently rinse plates with distilled water several times to get rid of crystal violet residue.
12. Count colonies (with at least 40 cells per colony) ideally about 40-50 colonies per treatment.
13. Normalize the number of treated colonies and number of cells plated to the control colonies and number of cells plated.

$$\text{Plating efficiency (PE)} = \frac{\text{Colonies counted} \times 100}{\text{Cells plated}}$$

The colony measures the ability for a cell to divide and form a colony. Senescent cells cannot form colonies. It is the gold standard for the evaluation of cell sensitivity to radiation and various drugs.

(IV). WESTERN BLOT PROTOCOL USING AMERSHAM ECF KIT
 (Amersham Cat #RPN 5780): ** Revised 7-08-2011 **

Before Transfer: Cut PVDF blotting membrane to 2.5 x 3 inches.

- a) Pre-wet PVDF membrane in 100% methanol for 20 seconds.
- b) Wash in distilled water 2X5 minutes.
- c) Equilibrate membranes and gels in transfer buffer 20-30 minutes.

To store blot after transfer:

- a) Rinse membrane in TBS.
- b) Dry (protein side up).
- c) Store between two sheets of whatman 3MM paper at 40C for up to three months (once dry, membrane will need prewetting in 100% methanol for 10 seconds before use).

Antibody hybridization immediately following the transfer:

- a) Place PVDF membrane in TBS-tween-20 (TBS-t) for 5 min.
0.1%tween/TBS=0.4ml/400ml
- b) Block in TBS/t with 5% blocking agent overnight. Milk block tends to work the best. Shaker @ R.T. (0.5g non-fat dry milk in 10ml TBS/blot).
- c) Rinse 2 times w/ TBS-t to get excess blocking solution off.
- d) For polyclonal and monoclonal primary antibody: 5% membrane (milk) block in TBS/t for 1.5 hours @ RT on shaker.
- e) Rinse 2 times w/ TBS-t. Wash 4x 20 min w/ TBS/t-shaker @ RT.
- f) Add secondary antibody (anti-mouse-alk phos. 1/10,000 in 2.5% milk block in TBS/t) for 1.5 hour. Shaker @ RT.
- g) Follow step e (above) except final in TBS only (no tween).
- h) Add ECF reagent to membrane for 2 min. at room temp. (200ul working stock/3.60 ml TBS (no tween)) in the dark.
- i) Drain excess ECF reagent and dry between filter paper.

Reprobing membrane:

1. Strip ECF reagent from membrane in 70% methanol for 45 minutes. Shaker@ RT.
2. Rinse in TBS for 5 minutes.
3. Block in 5% block buffer overnight on shaker in cold room @ 4 degrees.
4. Hybridize with antibody to 'housekeeping protein' e.g. anti-beta-actin (1/10000=1ul/10ml for MA1-93199) TBS- tween w/ 5% milk, 1 ½ hours on shaker @ RT.
5. Rinse 2 times w/ TBS-t. Wash 1x 5min and 3x 20 min w/ TBS/t-shaker @ RT.
6. Add secondary antibody (anti-mouse-alk phos. 1/10,000 in 2.5% milk block in TBS/t) for 1.5 hour. Shaker @ RT.
7. Follow step e (above) except final in TBS only (no tween).
8. Add ECF reagent to membrane for 2 min. at room temp.

(240ul working stock/3.60 ml TBS (no tween)) in the dark.

9. Drain excess ECF reagent and dry between filter paper.

10. Image with Phospho-Imager set @ 700V

Notes*

See Antibodies notebook for recommended dilutions.

Some protein lysates are available to use as positive controls

Western Blots allow us to visualize the whether specific proteins are present. It also allows us to be able to predict which cell lines would respond better to the BCL-2 inhibitor. We tested four cell lines to see the varying amounts of four anti-apoptotic BCL-2 proteins: A1, BCL-2, MCL-1, BCL-XL, BCL-W.

(V). The Cytotoxicity assay/Growth Inhibition Assay

1. Determine the amount of viable cells using the Countess Automated Cell Counter using 10 microliters of Trypan Blue and 10 microliters of the sample of cells.

2. Cells were then plated in 96- well plates with 2×10^4 - 5×10^4 in each well.

3. Cells are assayed after 48 hours after exposure to the different treatments.

4. WST-1 is added to each well to determine the amount of live cells still in each of the wells and it is evaluated by measuring the optical density at 440nm using 650nm as the reference on a Tecan microplate reader using Magellan software (Tecan) WST-1 accumulates in viable cells and absorbance readings of 440nm/650nm reference have a linear correlation with the number of viable cells.

The Cytotoxicity assay was utilized to determine the most effective treatment doses so that all cells were not killed because this would be toxic to the host but kill enough cells that shows that the drug will be effective.

(VI). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay-Fluorescence Microscopy

1. Cell lines are seeded in a 6-well plate at a concentration of 2×10^5 - 3×10^5 cells per well in 1ml of media.
2. The 6-well plates were placed in a 37°C 5% CO₂ incubator for 24 hours.
3. After 24 hours, the cells were irradiated at a 2 Gy dose of radiation with the RS 2000 X-ray Biological Irradiator. Mock irradiated samples (0 Gy) were handled in the same manner as the irradiated plates (2GY) to keep all conditions constant except for the treatment.
4. Four hours after irradiation, cells were treated with 1μmol/L obatoclax for an additional 44 hours in a 37 °C 5% CO₂ incubator.
5. Cells were collected 48 hours post irradiation.
6. The media from the 6 well plates was collected and placed in polystyrene round bottom tubes.
7. The cells that adhered to the plate had 250 ml of 0.25% trypsin with EDTA added to each well. The media that was previously collected was added to wash the corresponding well.
8. The cell suspension is centrifuged at 1000 rpm for 10 minutes.
9. The supernatant is decanted with the cell pellet left behind.
10. 1 ml of 4% formaldehyde in Dulbecco's was added and vortexed at a low speed.
11. The solution containing the cells and 4% formaldehyde was held at room temperature for a period of 10 minutes.
12. The suspension was centrifuged at 1200 rpm for 10 minutes.
13. The supernatant was decanted, with 1 ml of 80% ethanol in distilled water added to the pellet followed by placement in a -20 °C freezer until preparation for the TUNEL procedure.
14. After a minimum of 24 hours in the -20 °C freezer, the experiment was carried out according to the manufacturer's directions. The samples were placed in a 37 °C 5% CO₂ incubator for 3.5 hours in the dark, with gentle resuspension at 30-minute intervals.
15. After 3.5 hours, 300 ml of Tris Buffered Saline was added to each tube. The tubes were measured using fluorescein isothiocyanate (FITC) as a fluorescent marker for labeling various cellular modes of death.
16. Acquisition was with a BD LSRII flow cytometer with a 488 nm argon ion source and FACSDiVa and analysis with FlowJo software. Immediately following flow cytometry the cells remaining in the tubes were cytospun onto glass slides and DAPI-containing vectashield was placed on the cytospun cells, coverslipped and visualized with an Olympus BX 60 fluorescent microscope.
17. Four to five fields per slide with at least 100 cells total per slide were photographed at 400X magnification and examined using MagnaFire software. The TIFF files were adjusted using Adobe Photoshop.
18. All adjustments to contrast and color balance were made to all cells simultaneously and identically.

TUNEL is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. It allows for the visualization of apoptotic and non-apoptotic cells.

(VII). RNA extraction/ PCR array

Step 1- Collect cells

A- Count Cells- do not use more than 1×10^7

B- For adherent cells- DO NOT TRYPSINIZE. Remove medium and replace with ice cold PBS. Scrape with rubber policeman. **Keep on ice while doing this. Centrifuge for 5 minutes at 3000 rpm.

For non-adherent cells- Do not add PBS, just spin down for 5 minutes at 6000 rpm.

C- Completely aspirate/decant supernatant

Step 2- Disrupt cells by adding Buffer RLT

A. Loosen pelleted cells by thoroughly flicking the tube

B. Add appropriate amount of Buffer RLT

# of Cells	Volume of RLT (μ l)
$<5 \times 10^6$	350
$5 \times 10^6 - 1 \times 10^7$	

Step 3- Homogenize the Lysate

A- Pass lysate at least 5 times through a blunt 20 gauge needle fitted to an RNase-free syringe

Step 4- Add 1 volume (350 or 600ul) of 70% ethanol to the homogenized lysate, mix well by pipetting (Do not centrifuge)

Step 5- Transfer up to 700 ul of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2ml collection tube (supplied). Close the lid gently and centrifuge for 15 seconds at $\geq 10,000$ rpm. Discard the flow through and reuse the collection tube in step 6

Step 6- **RNase-Free DNase Set Protocol- (instead of first wash)

Prepare the DNase I stock solution by dissolving the lyophilized DNase I (1500 Kunits) with 550 ul of RNase-free water provided. Mix gently by inverting and store at either -20°C for up to 9 months or $2-8^\circ\text{C}$ for up to 6 weeks

A- Add 350 ul Buffer RW1 to the RNeasy spin column. Close lid gently, and centrifuge for 15 seconds for $\geq 10,000$ to wash the spin column membrane. Discard flow-through, reuse collection tube in step D

B- Add 10 ul DNase I stock solution to 70 ul Buffer RDD. Mix gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube

C- Add the DNase I incubation mix (80 ul) directly to the RNeasy spin column membrane, and place on the benchtop for 15 minutes

D- Add 350 ul Buffer RW1 to the RNeasy spin column. Close lid gently and centrifuge for 15 seconds at $\geq 10,000$ rpm. Discard flow-through and continue with the first buffer RPE wash step in the relevant protocol

Step 7- Add 500 μ l Buffer RPE to the RNeasy spin column. Close lid gently, and centrifuge for 15 seconds at $\geq 10,000$ rpm to wash the spin column membrane. Discard the flow through, reuse the collection tube in step 8.

Step 8- Add 500 μ l Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 minutes at $\geq 10,000$ to wash the spin column membrane.

**After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through

Step 9- **Optional-Place RNeasy spin column in a new 2ml collection tube, and discard the old collection tube with the flow-through. Close lid gently, and centrifuge at full speed for 1 minute.

Step 10- Place RNeasy spin column in a new 1.5 ml collection tube. Add 30-50 μ l RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 minute at $\geq 10,000$ to elute RNA.

Step 11- If the expected RNA yield is > 30 μ g, repeat step 10 using another 30-50 μ l RNase-free water, or using the eluate from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10.

NOTES:

Should recover 3-4 μ g total RNA, least amount would be 100 ng/ μ l.

β -mercaptaethanol needs to be added to RLT buffer, 10 μ l of β -mercaptaethanol per 1 ml of RLT.

PCR array- RT2 First Strand Kit Procedure

- 1- Briefly (10-15 seconds) spin down all reagents
- 2- Prepare the DNA Elimination Mixture
 - a. For each RNA sample, combine the following in a sterile PCR tube:

Total RNA	25.0 ng to 5.0	μ g
GE (5X gDNA Elimination Buffer)	2.0	μ l
H2O to final volume of	10.0	μ l

- b. Mix the contents gently with pipettor followed by brief centrifugation
- c. Incubate at 42°C for 5 min
- d. Chill on ice immediately for at least one minute

- 3- Prepare the RT Cocktail

RT Cocktail	1 reaction	2 reactions	4 reactions
BC3 (5X RT Buffer 3)	4 μ l	8 μ l	16 μ l

P2 (Primer and External Control Mix)	1 μ l	2 μ l	4 μ l
RE3 (RT Enzyme Mix 3)	2 μ l	4 μ l	8 μ l
H2O	3 μ l	6 μ l	12 μ l
Final Volume	10 μ l	20 μ l	40 μ l

- 4- First Strand cDNA Synthesis Reaction:
- a. Add 10 μ l of RT Cocktail to each 10 μ l Genomic DNA Elimination Mixture
 - b. Mix well but gently with a pipettor
 - c. Incubate at 42°C for exactly 15 minutes then immediately stop the reaction by heating to 95°C for 5 minutes
 - d. Add 91 μ l of H2O to each 20 μ l of cDNA synthesis reaction. Mix well
 - e. Hold the finished First Strand cDNA Synthesis Reaction on ice until the next step or store overnight at -20°C

Results

Clonogenic assays, Western blots, Cytotoxicity assays and TUNEL assays were used to determine whether the relative amounts of anti-apoptotic BCL-2 family member proteins in the different breast cancer lines were consistent with the sensitivities of those cell lines to the BCL-2 inhibitor Obatoclax.

The four cell lines selected to test the effect of Obatoclax have all been shown to over-express anti-apoptotic BCL-2 proteins. Lines T47D and BT20 express anti-apoptotic BCL-2 proteins to a lower extent than do MDA-231 and MDA-468 lines. Thus, by treating these four selected lines with the BCL-2 inhibitor in combination with radiation we are able to quantify the effects of the combination treatment on the inhibition of anti-apoptotic proteins with the resultant re-introduction of apoptosis.

Obatoclax at 1 μ M was shown to produce significant inhibition in the cytotoxicity assay. Irradiation of the cells did not have a significant effect as determined with the cytotoxicity assay. Figure 3 shows the results of the growth inhibition assay using 1 μ M Obatoclax and 2GY radiation.

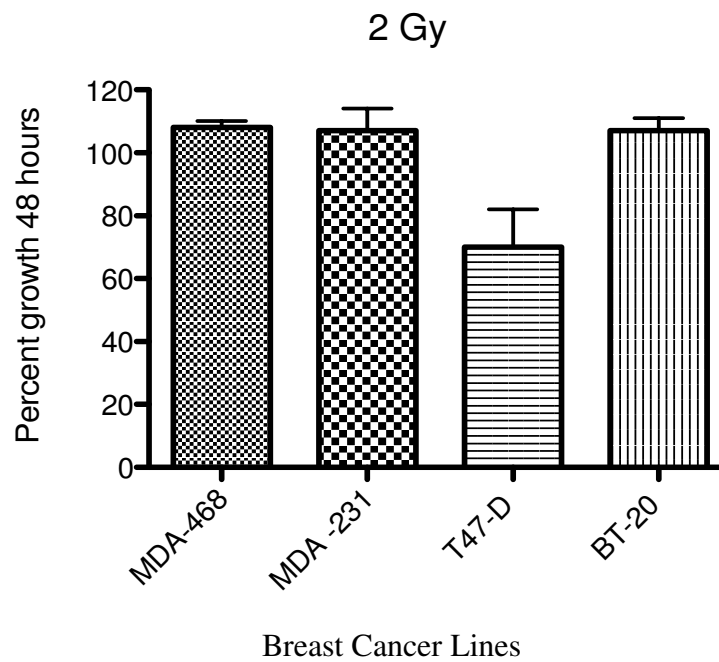
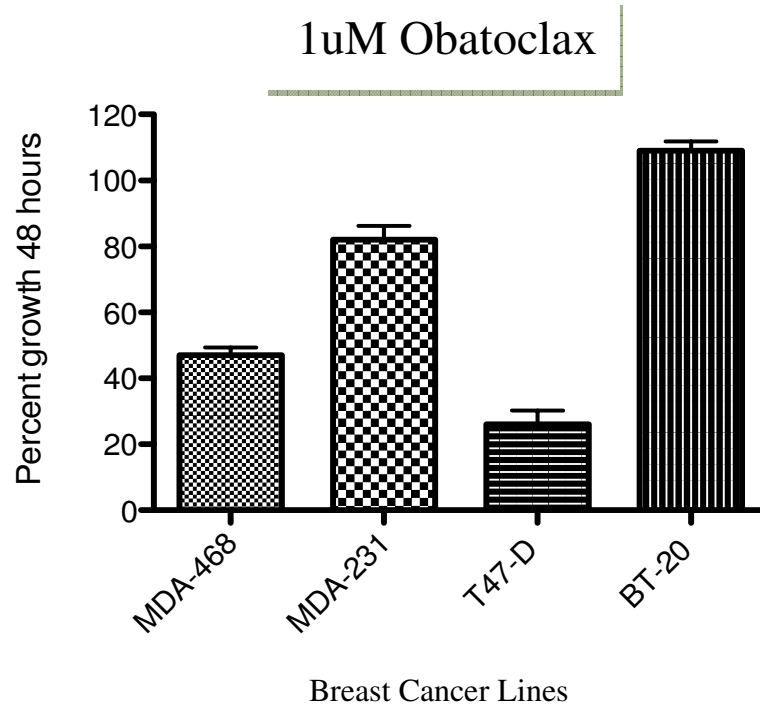
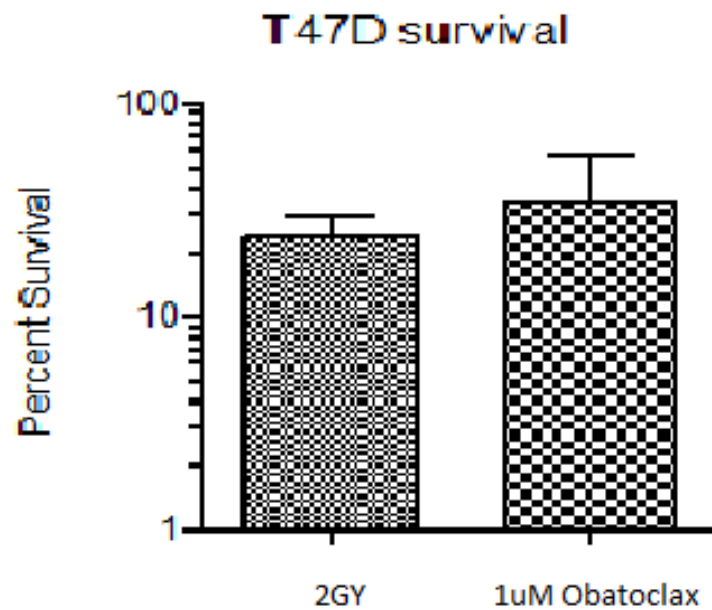


Figure 3: Growth Inhibition Assay. Each of the four cell lines were treated with 1 uM Obatoclax or DMSO solvent alone. Cell numbers were estimated by a colorimetric assay using 3 hour conversion of the tetrazolium salt, WST-1 to formazan, and evaluated by measuring the optical density at 440nm using 650nm as the reference on a Tecan microplate reader using Magellan software (Tecan, Austria, GmbH) (11).

When treated with 1 uM obatoclax, MDA-468, MDA-231 and T47-D seemed to have responded the most to the drug treatment. Radiation with 2Gy did not seem to have a significant effect on the cell lines.

Figure 4 below shows the effects of 1 uM Obatoclax and 2 Gy radiation for the T47D and the MDA- 231 breast cell lines and the surviving fraction determined by clonogenic assay.



T47D

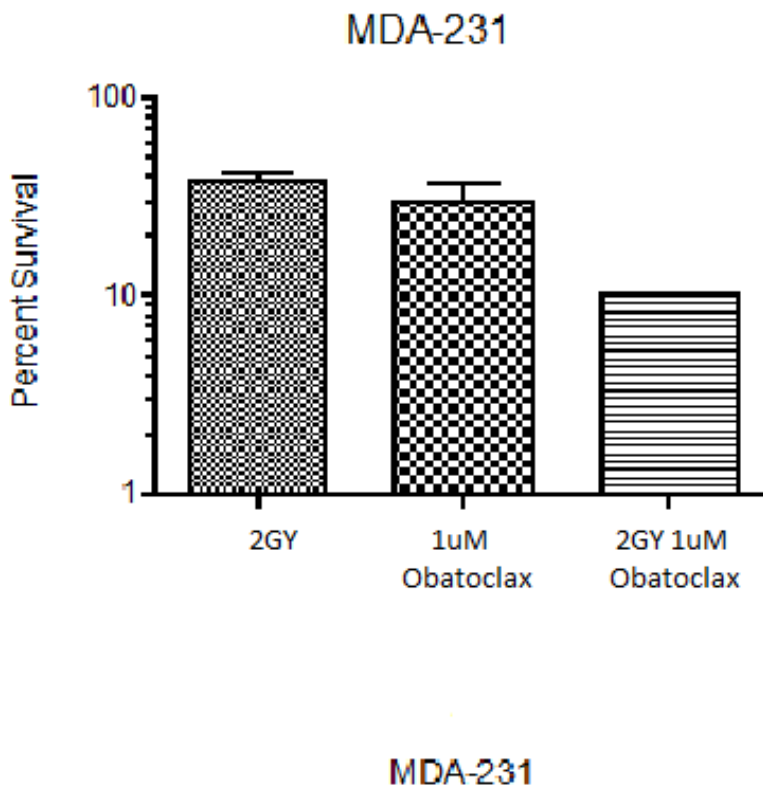


Figure 4: Toxicity of Obatoclox and Ionizing radiation as determined by colony assay for surviving cells. Cells were treated with either DMSO, 1 uM Obatoclox in DMSO, or DMSO plus 2 Gy ionizing radiation, plated at low densities in 6-well plates, and surviving colonies stained and counted 10 days later (11).

Reliable and reproducible data for the human breast cancer lines of MDA-468 and BT-20 could not be obtained due to poor plating efficiencies below 1% even at low cell seeding densities. Even though data for the human breast cancer cell lines of MDA-231 and T47D was obtained, it should be noted that these cell lines along with MDA-468 and BT-20 did not grow well when seeded at low cell densities. The breast cancer cell line of MDA-231 had a plating efficiency of twenty-five percent while T47-D has a plating efficiency of ten percent. Survival measurements for MDA 231 (25% plating efficiency) and T47-D (10% plating

efficiency) in the presence of obatoclax are likely to be confounded by the interaction between the stress response and the pro-apoptotic effects of obatoclax. Thus it is very likely that we have underestimated the surviving fraction from obatoclax alone that would occur at higher cell seeding densities (11). From the graphs, it is shown that 1uM Obatoclax had a similar toxicity to that of 2Gy. A combination of Obatoclax and radiation treatment could not be obtained for the T47D cell line due difficulty in interpreting results. The combination treatment of Obatoclax and radiation of 2GY was shown to be more effective than the single agents to treatment.

TUNEL-Fluorescence Microscopy

The determination of whether the relative amount of BCL-2 family member proteins would be related to induction of apoptosis was also tested. The amount of apoptosis was quantified using TUNEL stained cells visualized by fluorescent microscopy after treatment with 1 uM obatoclax and / or 2 Gy radiation. Figure 5 below shows the TUNEL stained cells.

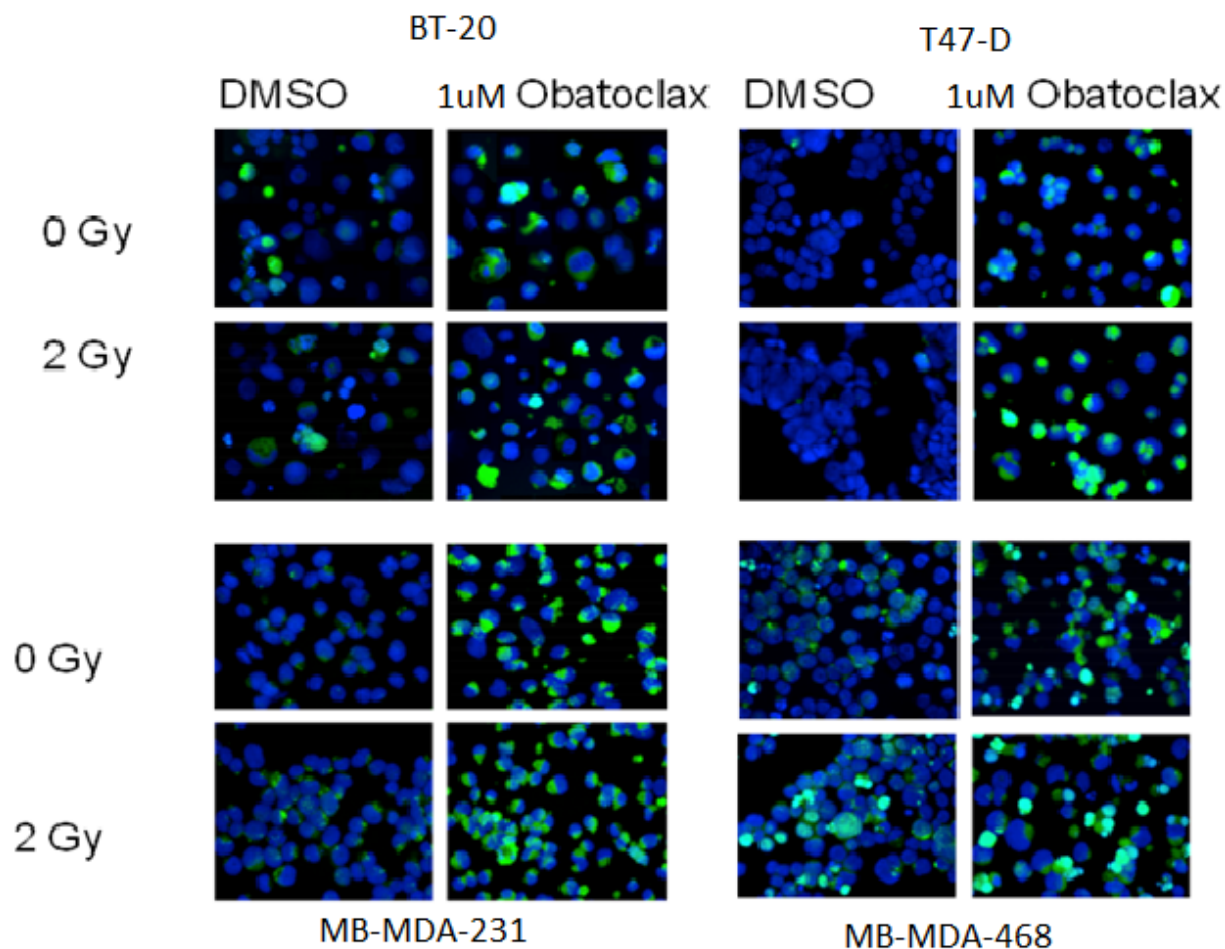


Figure 5) Fluorescent microscopy of TUNEL stained cells (11)

These images allow analysis of the untreated controls which are shown at 0GY(mock-radiation) for each of the four cell lines. In these images, the cell nuclei are stained with DAPI, a blue dye. The green fluorescent stained cells that are undergoing apoptosis. Using TUNEL fluorescence microscopy, it is seen that the combination treatment of 1uM Obatoclox with 2GY radiation had more of an effect on each of the cell lines more so than the treatment of Obatoclox or 2GY radiation alone. Specifically, the combination treatment induced apoptosis in the cell lines of MDA-231 and MDA-468 to a greater extent than the cell lines of BT-

20 and T47-D. This data was supported by data from TUNEL by Flow Cytometry (data not shown).

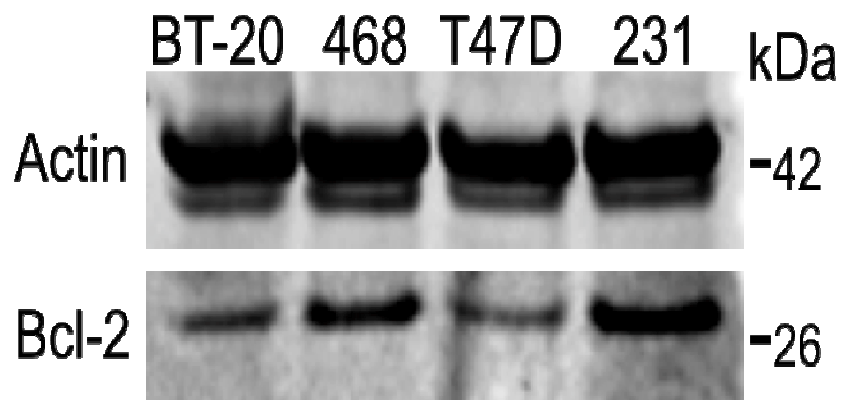
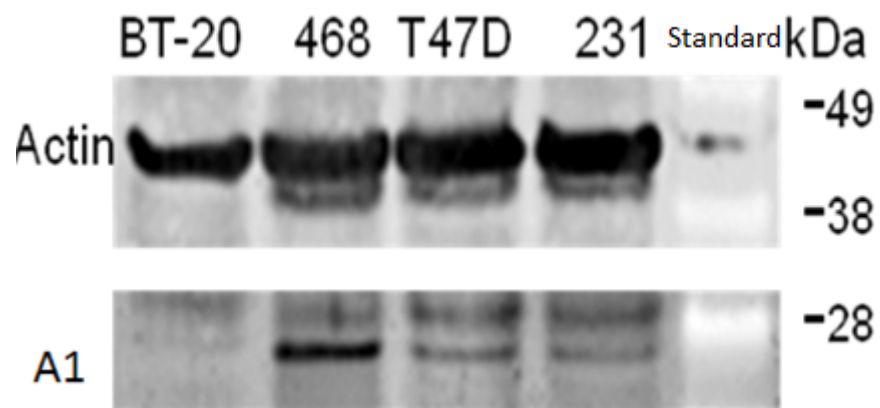
RT-PCR

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) can be used to quantify mRNA from clinical samples. SA biosciences RT-PCR apoptosis microarray was used to determine whether RT-PCR microarrays could be used to determine the relative sensitivity to obatoclax-induced apoptosis. This array uses primers to detect all the Bcl-2 family members – both pro and anti apoptotic - as well as most of the XIAP family of genes that act downstream of the Bcl-2 family and might provide important information. RT-PCR quantifies doubling cycles (Cp) to reach a specific detectable point. We compared the normalized Cp's (measured as \log_2) for each of the apoptotic genes from the cell lines with similar normalized values for breast human mammary epithelial cells (HMECs) as a point of comparison (data not shown).

Western Blot

Since RT-PCR microarrays quantify mRNA levels, it is assumed that this test would also be related to protein levels. Western blots were used to confirm that the overexpressed mRNAs resulted in higher levels of their protein products. Western blots were used to quantify the relative amount of commonly over-expressed anti-apoptotic BCL-2 family members. Commonly expressed anti-apoptotic BCL-2 family members include A1, BCL-2, BCL-XL, MCL-1 and

BCL-W. The relative amount of these five proteins was determined for each of the cell lines. Labeled antibodies were used to quantify the amounts of the anti-apoptotic Bcl-2 family members on the Western blots, which were then normalized by determining the amount of beta-actin present in each sample. We compared the protein levels to BT-20 since it had the lowest level of mRNA expression. Below shows the Western blot images obtained.



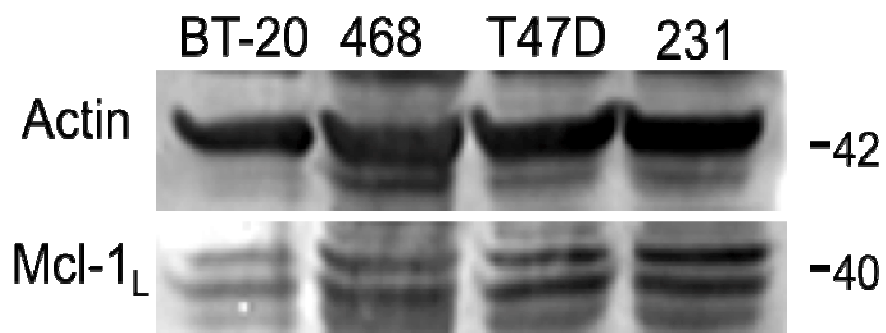
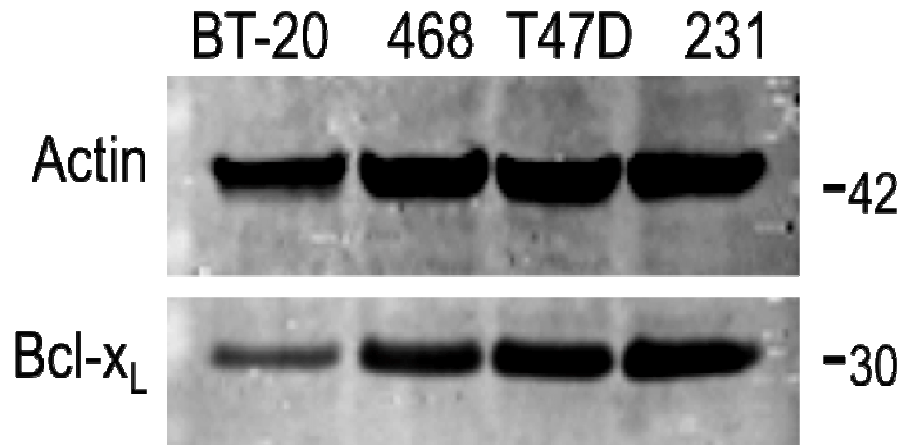
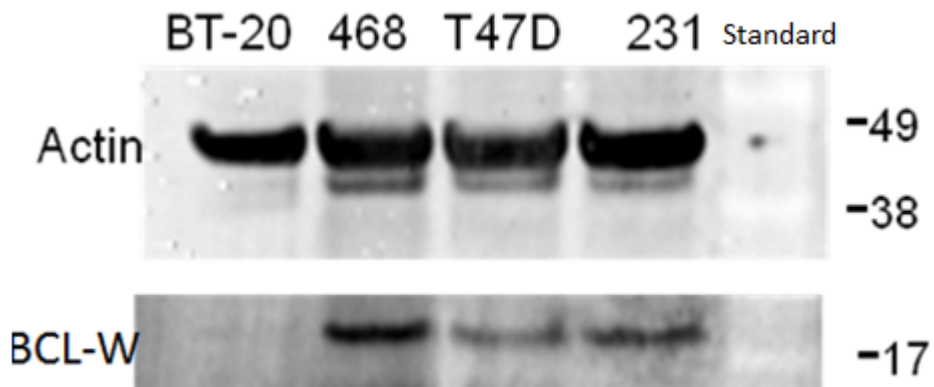


Figure 6) Western analysis of Bcl-2 family member proteins A1, Bcl-2, Mcl-1, Bcl-w, and Bcl-x_L. Varying levels of anti-apoptotic Bcl-2 family members are found within and among the four breast cancer cell lines BT-20, MDA-MB-231, MDA-MB-468 and T47-D. Total protein from four different breast cancer cell lines was analyzed by Western blotting, with antibodies to each of five anti-apoptotic Bcl-2 family proteins. β -actin was used to control for loading differences (11).

The results from the Western blots were very similar to the RT-PCR microarray data(not shown). MDA-468 had an elevated level of two of the anti-apoptotic BCL-2 family proteins including A1 and BCL-2 while MDA-231 had an elevated level of two anti-apoptotic BCL-2 family proteins including BCL-2 and BCL-XL. T47-D had high levels of Bcl-2 but relatively low levels of the other proteins, and BT-20 had the lowest levels of all the five proteins. The major difference between the microarray data and the Western blot data was that levels of BCL-XL were higher in MDA468, MDA 231, and T47D compared to BT-20. However it is possible that mRNA levels under-expressed in BT-20 relative to normal HMECs. Table 2 shows the Western blot data for each of the five proteins.

Anti-apoptotic Proteins

Cell Lines	A1	MCL-1	BCL-2	BCL-xl	BCL-w
MDA-231	-----	-----	Elevated higher	Elevated higher	-----
MDA-468	Elevated higher	-----	Elevated higher	-----	-----
BT-20	Relatively low level	Relatively low level	Relatively low level	Relatively low level	Relatively low level
T47D	Relatively low level	Relatively low level	Relatively high level	Relatively low level	Relatively low level

Table 2: Western Blots data organized in a chart showing that MDA-231 and MDA-468 had higher levels of anti-apoptotic proteins

Note: Much of this data has been submitted and is pending patent by the Hudson laboratory and is currently confidential.

Discussion

Several techniques and assays were used to determine the effect of a BCL-2 inhibitor in combination with radiation therapy on human breast cancer cell lines. Before the start of this project, it has been found that several cancers have over-expressed proteins belonging to the anti-apoptotic BCL-2 family member proteins. The results from this study can be used to increase the efficacy of current treatments. The ultimate goal of the study was to determine the effect of the combination treatments and to see if it were possible to predict which patients will respond to which treatments. Using microarrays (PCR-RT), it was shown that the breast cancer cell lines with the highest levels of BCL-2 family member expression respond to obatoclax and radiation with greater levels of apoptosis than those with lower levels of BCL-2 family member levels. These results can be useful in a variety of ways including the testing of individual patient tumors for specific BCL-2 family member levels to aid in selection of specific treatments. Individualized patient treatment would target specific tumors instead of relying on generalized treatments including surgery, radiation chemotherapy and immunotherapy.

The drug used for this study was Obatoclax, a BCL-2 inhibitor. Drugs such as Obatoclax would mostly likely be combined with other therapies including radiation and surgery to treat local tumors. This study showed that Obatoclax combined with other therapies such as radiation induces a higher level of apoptosis than what would be seen with single agent treatment of Obatoclax or

radiation alone. The combination results are roughly in proportion to the relative amount of Bcl-2 family member over-expression in the cell lines.

Over-expression of any combination of the BCL-2 family members is capable of inhibiting apoptosis and several other pathways can also contribute. Over-expression of a single BCL-2 protein 25-fold may be more easily detected than over-expression of several BCL-2 family proteins 25-fold spread over several different equally functioning family members. Although a 25-fold increase in a single BCL-2 family protein is easier to detect, this does not diminish the importance of the 25-fold total over-expression spread over several different BCL-2 family members. Being able to quantify the sum of multiple but relatively small over-expressions in individual proteins and then taking the sum of the total expression is the goal for this experiments and future experiments. The highly specific Bcl-2 inhibitors will only be effective in proportion to the relative contributions of the subset of those proteins affected by the inhibitor.

Observed in this study was that either multiple pro-apoptotic Bcl-2 family members are up-regulated along with over-expression of downstream XIAP (X-linked inhibitor of apoptosis protein) family members, or none of the Bcl-2 family members or the downstream XIAP family members are over-expressed. It was observed that the breast cancer cell line MDA468 did not only have the anti-apoptotic family members of BCL-2 including A1 over-expressed but also had the over-expression of pro-apoptotic BCL-2 family members of Bax and Bim roughly to the same extent as the over-expression of the anti-apoptotic BCL-2 members. Downstream anti-apoptotic XIAP family proteins are also over-expressed. In

contrast, BT20 and T47D have apparently managed to achieve the same anti-apoptotic goal primarily by lowering the pro-apoptotic Bax and Bim expression.

The model that seems best to explain our results is that pro-growth pathway activation leads to increased pro-apoptotic protein Bax and Bim expression, which, in turn make the cells more sensitive to pro-apoptotic signals as part of a feedback inhibition mechanism. Resistance to apoptosis arises secondary to selection for resistance to stresses such as hypoxia, which would otherwise induce apoptosis (11). The four cell lines used in this study, were chosen either because of their high levels of anti-apoptotic BCL-2 and XIAP family members or the fact that they seem to have a disabled over-expression of the pro-apoptotic proteins. Bcl-2 inhibitors such as obatoclax, induce apoptosis in proportion to the amount of unopposed Bax members following treatment, which in turn is proportional to the total pre-existing Bax members of the family (but not the sequestered BH3 only members) plus the total inhibiting Bcl-2 family members (11).

A recent study from Al-Harbi et al. presented a simple RT-PCR-based system to quantify sensitivity to the inhibitor ABT737 which is another BCL-2 inhibitor. ABT737 inhibits Bcl-2, Bcl-XL, and Bcl-W, but not Mcl-1 or A1 (11). The ratio of these proteins are important because the cells are sensitive to treatment only when the MCL-1 and A1 to BCL-2 is low which occurs only when Bcl-2 levels are high and Mcl-1 and /or A1 are low. If both groups are low or both groups are high, the cells are resistant to the treatment. The advantage in this study is that it can be broadly applied to distinguish whether the BCL-2 family

proteins are over expressed and specifically determine which proteins are over-expressed. Data is not shown for experiments using the drug ABT737 and the four cell lines used in the study but it was found that the four cell lines used were not sensitive to the drug ABT737. Presumably this is due to the high levels of MCL-1 and A1 transcript levels found in these cell lines and it was found that ABT737 does not inhibit these specific proteins. BCL-2 proteins are over-expressed in two of the four cell lines used, MDA-231 and MDA-468 and not so much in the other two cell lines used T47D and BT-20. That the Bcl-2 family members are over-expressed is evident both from the increase in the individual anti-apoptotic Bcl-2 family members, and from the increases in the Bax family and the downstream XIAP family (11).

This study shows the importance of identifying tumors which over-express BCL-2 family members because it plays an important role in breast cancer pathobiology and treatment options. RT-PCR arrays capable of quantifying mRNA transcripts from all of the Bcl-2 family members and related pathways may provide a means to tailor specific treatments to individual tumors (11).

Summary

Cancer is a group of diseases that is characterized by abnormal control of cell growth and survival. Cancer can and may arise in different tissues and organs in the body including the brain, blood, breast, and bone. Some cancer cells have the ability to metastasize which means to invade and spread to other tissues in the body and form new cancers. In the Hudson lab, we focus on breast cancer which is one of the most common forms of cancer among women. In fact, one in eight women will develop some form of breast cancer in her lifetime. In 2011, it was estimated that 230,480 new cases of invasive breast cancer as well as an additional 57, 650 new cases of non-invasive breast cancer would be diagnosed in the United States (2). An expected 39, 520 women were expected to die from breast cancer with only lung cancer surpassing the number of deaths due to cancer for women. Since the 1990's there has been a decreasing trend in the number of deaths attributed to breast cancer due to several reasons including early detection, awareness, and advances in treatment options.

Many labs are working to try to find better and more effective treatments for cancer which include the personalization of treatments. Currently, available treatments include surgery, immunotherapy, radiation, and/or chemotherapy and are pretty much generalized because these treatment options are used to treat basically all cancers no matter the location, size and type. Personalization of treatment will allow certain cancers to be handled in a way that would target specific cancer cells and be more effective treatment.

Apoptosis is a normal process in which certain cells in the body are “programmed” to die. This can be due to several reasons including but not limited to when a cell has been damaged beyond repair, it has outlived its usefulness, during embryonic development, when a cell has aged (several replications), and/or after potentially harmful mutations have damaged the cell.

In most cancers, the apoptotic pathway is disrupted or destroyed so cells that are suppose to undergo apoptosis don't and they continue to multiply and grow unchecked. The BCL-2 family of proteins plays a role in regulating the apoptotic pathway and this offers an entry point for conducting research. The BCL-2 family member of proteins is divided into pro-apoptotic proteins which work to promote cell death and anti-apoptotic proteins which inhibit cell death. The balance of these proteins is important for cell homeostasis (balanced internal environment). In previous studies, it has been shown that several cancers over-express proteins in the BCL-2 family. In the lab, we work with a small molecule BCL-2 inhibitor called Obatoclax. For the study, four breast cancer cell lines were used: MDA-231, MDA-468, BT-20 and T47-D. In this study, these four cell lines were treated with the drug inhibitor in combination with radiation therapy.

The effects of this treatment was observed and analyzed through a series of tests. Western blots were used to test the levels of anti-apoptotic proteins expressed in the different cell lines. It would also allow the lab to predict which cell lines would respond best to the treatment because of the different level of anti-apoptotic protein levels. The colony assay was an important test because it allowed us to see if the drug treatments in combination with radiation were

affecting the breast cancer cell lines. The growth inhibition assay allowed us to understand and evaluate the proliferation of tumor cells (breast cancer cells) and calculate the potency of the treatment. The TUNEL assay allowed us to visualize and detect DNA fragmentation, due to the apoptotic process, using fluorescence microscopy. RT-PCR was used to quantify messenger RNA (mRNA) to determine the relative sensitivity to obatoclax and its role in inducing apoptosis.

These assays have given us some insight into how to personalize treatments for patients with cancers that over-express the BCL-2 family member proteins. This study has shown that the BCL-2 inhibitor drug, Obatoclax had more of an impact on the breast cancer cell lines of MDA-231 and MDA-468 when treated in combination with radiation therapy. RT-PCR data (not shown) showed that these two cell lines over-expressed the anti-apoptotic proteins more so than the other two cell-lines examined. Since MDA-231 and MDA-468 over-expressed these proteins at a higher level than the other cell lines, it makes sense that these two cell lines would respond to the treatment better. Radiation therapy was also tested and it has a similar effect as treatment with Obatoclax. Combination therapy of drug treatment and radiation therapy had more of an effect than drug treatment or radiation therapy alone.

These results show the importance of personalized treatment for cancer therapy treatment. Standard therapy includes surgery for localized tumors, chemotherapy and radiation. These standard treatments are options for most cancers despite the differences between cell types, location, aggressiveness and other differentiating characteristics. Through analysis such as the one conducted

in this study, clinicians and researchers are better able to understand the differences between the cancers. Using research methods such as RT-PCR microarrays, could allow some cancers to be treated differently and more effectively than others. Not all cancers would benefit from a certain treatment therapy as shown in this study because this drug targets cancers that over-express the BCL-2 family of proteins.

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