Targeting chemotherapeutic resistance in breast cancer

By

Jonathan Avery

An Undergraduate Thesis Submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Baccalaureate of Science in BioResource Research,
Biotechnology Option

06/01/2016

This work contains proprietary information – Do not publish

APPROVED:

Siva Kolluri, Environmental and Molecular Toxicology	Date
Hyo Sang Jang, Environmental and Molecular Toxicology	Date
Martin Pearce, Environmental and Molecular Toxicology	Date
Katharine G. Field, BRR Director	Date

I understand that my project will become part of the permanent collection of the Oregon State University Library, and will become part of the Scholars Archive collection for BioResource Research. My signature below authorizes release of my project and thesis to any reader upon request.

Your Name	**Date**

Summary

The Bcl-2 family of proteins regulate cell survival and are targets for cancer therapy. This protein family has also been implicated in chemotherapeutic resistance, the cause of approximately 90% of treatment failure in advanced cancer. We analyzed the expression and the role of the Bcl-2 proteins in chemotherapy resistant breast cancer cells. Further, we investigated the role of a new class of molecules developed in our laboratory that target Bcl-2. These compounds effectively induced death in chemotherapy resistant breast cancer cells and reduced their ability to form colonies. Therefore, our studies have identified a group of compounds that have the potential to treat chemotherapy resistant breast cancer.

Significance

Chemotherapy is a standard treatment approach for breast cancer. However, some breast tumors that initially respond well to treatment stop responding, leading to the development of chemotherapeutic resistant tumors. These resistant tumors are hard to treat and are responsible for breast cancer mortality. We investigated the expression of pro-survival Bcl-2 proteins in chemotherapy resistant breast cancer cells, and the utility of a new class of compounds targeting Bcl-2. The Bcl-2 targeting compounds have been found to be more effective in treating a chemotherapeutic resistant cancer cells. These findings provide evidence of a novel way to target chemotherapeutic resistant cancer and improve the long term treatment outcomes for breast cancer patients.

Highlights

- Bcl-2 family of proteins are dysregulated in chemotherapy resistant breast cancers.
- A group of compounds developed to target Bcl-2 are able to suppress growth of chemotherapeutic resistant cancer cells.
- Resistance to one chemotherapy drug leads to cross resistance to another chemotherapy drug.

1. Introduction

Breast Cancer is a heterogeneous disease that encompasses a variety of distinct morphological and histological features, as well as varying clinical behaviors. This diversity has led to multiple attempts to classify the disease into different subtypes. One of the most accepted classifications is the Triple Negative Breast Cancer (TNBC) subtype (Badve et al. 2011). It has been uniformly defined as tumors that lack expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2. Triple negative breast cancer accounts for approximately 15% to 20% of all breast cancers, and approximately 15% of TNBC cases will metastases to the brain (O'Reilly et al. 2015). It is typically high grade and has a very high rate of proliferation. (Cleere 2010). TNBC has a poor prognosis overall due to its highly aggressive nature (Cleere 2010). TNBC diagnosis disproportionally affects women who are under the age of 40, non-Hispanic black, or Hispanic (Bauer et al. 2007).

Approximately 80% of TNBCs are also classified as basal- like breast cancer (Foulkes, Smith, and Reis-Filho 2010). The two terms, TNBC and basal-like breast cancer, have been used interchangeably in the past, but they are not the same. Basal-like breast cancers are characterized by low levels or absence of the estrogen receptor, a lack of HER2 overexpression, as well as expression of genes usually found in normal basal or myoepithelial cells (Foulkes, Smith, and Reis-Filho 2010).

Initial treatment of triple negative breast cancers commonly involves anthracyclines (e.g. doxorubicin) and taxanes (e.g. paclitaxel) (Foulkes, Smith, and Reis-Filho 2010), which are general cytotoxic agents. Common hormone receptor targeting therapies are ineffective because drug targets (ER, PR and HER2) are absent. One taxane, paclitaxel, is commonly used and a core component of TNBC therapy. Paclitaxel is a chemotherapeutic that targets

rapidly dividing cells. This results in apoptosis induction in primarily cancer cells, but it also kills non-carcinogenic fast dividing cells, such as blood cells. Paclitaxel acts by inhibiting microtubule formation, which is necessary for cells to successfully complete mitosis. Paclitaxel inhibits the ability of cells to divide, and eventually leads to cell death through a calcium dependent manner(Pan and Gollahon 2013). Treatment with paclitaxel, directly or indirectly, releases apoptotic factors that stimulate mitochondrial membrane permeabilization, which results in apoptosis. The underlying mechanism to this phenomenon is currently unclear, but it has been shown that both paclitaxel and Bcl-2 make use of calcium release from the endoplasmic reticulum, and they are linked in some way with Bcl-2 expression at the ER membrane negotiating resistance to paclitaxel (Pan and Gollahon 2013).

Chemotherapy treatments, such as paclitaxel, are generally effective in the beginning of a cancer treatment regimen. However, resistance to this treatment (either primary or acquired) is a major barrier to successful long-term treatment. Primary resistance is innate. The chemotherapeutic is not effective because of an innate characteristic of the cancer, such as the lack of the HER2 receptor with HER2 targeting therapies. Acquired resistance is gained over time. A chemotherapeutic may initially cause tumor size and density to decrease, however the cancer may stop responding to therapy and start growing again. Paclitaxel is commonly used in TNBC because it is initially effective and leads to a reduction in tumor size below detectable limits. Despite this initial success, clusters of cells that did not fully respond to paclitaxel due to acquired resistance may regrow to form a therapy resistant tumor. This is referred to patient relapse and at this stage the patient has a very poor prognosis and very few treatment options. The relapse rate for TNBC patients in the first 3-5 years is significantly higher than for women presenting with hormone positive breast cancer (O'Reilly et al. 2015). This resistance is not limited to paclitaxel and is common barrier to the successful treatment of cancer patients treated with the majority of cancer therapeutics.

Chemoresistance can be attributed to multiple mechanisms. The target may be may be mutated, or the drug is getting degraded at an increased pace, among other ways. One possible mechanism is a defective apoptotic pathway. Of particular interest is the B-cell lymphoma 2 (Bcl-2) intrinsic death pathway. The Bcl-2 family of proteins are defined by the presence of conserved alpha-helical Bcl-2 homology domains (BH domains) and consists of both pro- and anti-apoptotic family members. The anti-apoptotic (pro-survival) proteins consist of several major players, some of which are Bcl-2, Bcl-xL, and Mcl-1. Anti-apoptotic proteins generally have four conserved BH domains (BH4, BH3, BH2, BH1), and a transmembrane domain for membrane localization. It is a crucial checkpoint in the intrinsic apoptotic pathway. Cell death through the intrinsic pathway is initiated by the prior mentioned pro-apoptotic proteins (BAX and BAK), while the anti-apoptotic proteins inhibit their action, acting as a regulator. The other pro-apoptotic BH3 domain only proteins (e.g. BIM and PUMA) can bind to all anti-apoptotic members, while others (e.g. BAD and NOXA) bind only to certain anti-apoptotic proteins. Apoptotic stimulus causes the activation/upregulation of BH3 only proteins, which then sequester the anti-apoptotic Bcl-2 family members. Sequestration of the anti-apoptotic members results in the availability of pro-apoptotic BAX and BAK to initiate the permeabilization of the outer mitochondrial membrane, which leads to activation of the caspase cascade and ultimately cell death. The relative amounts of anti- and pro- apoptotic proteins at the mitochondrial membrane determines the cells fate(Shi et al. 2010).

The role of Bcl-2 in resistance to chemotherapeutics has been demonstrated (Fesik 2005). It is commonly upregulated in cancers, and the overexpression of Bcl-2 inhibits BAX oligomerization and mitochondrial outer membrane permeabilization does not occur, thus inhibiting chemotherapeutic induced apoptosis (Teijido and Dejean 2010). This upregulation of anti-apoptotic Bcl-2 family members has been selectively targeted in the past using BH3 mimetics. BH3 mimetics bind to and inhibit anti-apoptotic Bcl-2 family members in a manner similar to BH3

domain only pro-apoptotic proteins (BIM/PUMA). This frees up BAX and/or BAK in order to initiate the apoptotic cascade. One of the compounds is known as ABT-737, and is able to kill cells only when BAX or BAK is present (Cragg et al. 2009). The dependency on these proteins indicate the cell death is initiated only through the mitochondrial apoptotic pathway (Chen et al. 2007, 1).

ABT-737 has been shown to be an effective chemotherapeutic in targeting paclitaxel resistance in melanoma cell lines (Watanabe et al. 2013). In the same study it was found that the level of Bcl-2 was well correlated with the paclitaxel resistance. However, there is conflicting evidence within other cell lines. In ovarian carcinoma, Bcl-2 was found to be associated with paclitaxel binding**,** and downregulation of Bcl-2 was a novel mechanism of paclitaxel resistance. (Ferlini et al. 2003, 2).

As previously stated, ABT compounds work by inhibiting Bcl-2, and this has been found effective in cell lines overexpressing Bcl-2. An emerging issue with ABT compounds in cancer treatment is resistance to the ABT compounds themselves (Mazumder et al. 2012). If Bcl-2 is upregulated further, ABT compounds will not be able to inhibit all Bcl-2 present. This resistance, coupled with the cytotoxicity that ABT compounds possess, has left the opening for other Bcl-2 targeting strategies to be viable.

As shown above, targeting of Bcl-2 is an established concept. Previously, the nuclear receptor Nur77 was found to bind Bcl-2 and induce cell death through functional conversion of Bcl-2(Lin et al. 2004). A 9-mer fragment of Nur77 (NuBCP) was shown to be capable of inducing cell death in the same manner (Kolluri et al. 2008). This identified a novel way to target Bcl-2 in cancer and exploit a mechanism cancer cells typically use to evade cell death (in resistance), in order to induce cell death. NuBCP was found to bind to the loop domain (the domain between BH4 and BH3) of Bcl-2 and expose the previously hidden BH3 domain in Bcl-2 that then blocks the activity of anti-apoptotic Bcl-xL (Kolluri et al. 2008). This Nur77 peptide could potentially be

synergistic to paclitaxel and alleviate its resistance, however it is a peptide and is subject to proteasomal degradation as well as immunogenicity issues, leading to difficulty in *in vivo* usage.

The Kolluri Lab has recently developed potential anticancer therapeutics that work in the same manner as the 9-mer Nur77 fragment. They are known to bind to the loop domain of Bcl-2 and functionally convert it from a protector of the cell to a killer of the cell. As the amount of Bcl-2 is increased, ABT compounds become increasingly less effective, however when Bcl-2 is upregulated further Bcl-2 functional converters have an increased efficacy.

We believe that paclitaxel resistance is dependent on Bcl-2, and targeting this dependency with BFCs could potentially lead to a selective killing of chemoresistant cell lines. The goal of this project is to test the efficacy of Bcl-2 functional converters in targeting paclitaxel resistance in triple negative breast cancer cell lines.

2. Experimental Procedures

2.1 Drugs. Paclitaxel was obtained from Sigma Aldrich with a stock concentration of 10 mM dissolved in DMSO. Bcl-2 Functional Converters (BFC 1108, BFC1111, BFC1103, BFC1101) were obtained from Chembridge with a stock concentration of 20 mM, but their structures are currently proprietary. The final DMSO concentration never exceeded 0.5% in either treated or control samples. DMSO control concentrations matched the highest concentration of DMSO used on a compared treatment.

2.2 Cell Culture. The MDA-MB-468 triple negative breast cancer cell line were purchased from ATCC and unfrozen from liquid nitrogen vapor phase stock, cultured in 90% Dulbecco's Modification of Eagle's Medium with 4.5 g/L glucose, L-glutamine and sodium pyruvate and 10% Fetal Bovine Serum (FBS) with Penicillin Streptomycin. Cells were propagated in a 10 cm culture plate and were trypsinized when cellular density reached 80%. When the cultures were over 60% density, and two days had passed since the last treatment, they were treated with paclitaxel. The dose started at 10 nM, and was increased by 10 nM each treatment until the 100 nM treatment was reached, over a period of 4 months. Identical MDA-MB-468 cells were cultured alongside these cells, but without the paclitaxel treatment, and designated as the control line. Cells were cultured in a Nauire IR Autoflow CO_2 Water-Jacketed Incubator at 37 °C with a 5% CO_2 concentration. Cells were tested at 10% FBS conditions, 1% FBS conditions, and low glucose DMEM with 10% FBS conditions.

2.3 Western Blots. Cell lysates were taken from aspirated samples, after being centrifuged for 5 minutes at 1100 RPM. The lysates were diluted in 2X Laemmli Buffer and loaded into 12% Polyacrylamide gels. The samples were transferred onto PDVF membranes from Thermo Scientific using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell for 30 minutes at 25 Volts. The

membrane was then blocked in 5% nonfat dry milk and Tris-buffered saline/Tween 20 (TBST). The following primary antibodies were used: mouse monocolonal anti Bcl-2 sourced from Santa Cruz Biotechnology (sc-509) with a 1:200 dilution, polyclonal rabbit anti-Mcl-1 sourced from Serotec (AHP472) with a 1:400 dilution, mouse monocolonal Bcl-X sourced from Invitrogen (AHO0222) with a 1:200 dilution, and mouse monoclonal GAPDH sourced from Santa Cruz Biotechnology (sc-365062) with a 1:500 dilution. All dilutions were in 5% nonfat dry milk and 1X Tris-Buffered saline/Tween 20. The 5 milliliters of the primary antibody solutions were heat sealed with the PDVF membrane for 24 hours on a rotator at 4°C. Secondary antibodies were horseradish peroxidase labeled and sourced from Southern Biotech(mouse: 1010-05, rabbit: 4050-05), diluted to 1:2000 with 5% nonfat milk and TBST, and incubated in a heat sealed membrane on a rotator at room temperature for one hour before imaging.

2.4 Cell Viability Assay. Cells were counted using a Bright Line Hemocytometer using the average from four counts. They were plated in 96-well flat bottom plates from greiner bio-one (655086). 2000 cells were plated per well using 200 microliters of media per well (10 cells per microliter). After a 24 hour incubation period, cells were treated using the desired treatment diluted into 5 microliters of media, and then treated in triplicates. The plate viability was read after 72 hours of treatment using a TR717 Microplate Luminometer from applied Biosystems. Standard protocol for the CellTiter-Glo Luminescent Cell Viability Assay from Promega was used. The assay detects the amount of ATP, and the amount of ATP closely correlates to the amount of viable cells.

2.5 Colony Formation Assay. Cells were plated at a density of 500 cells per well in 6 well plates from greiner bio-one (657160) using 2 milliliters of media. After 24 hours of incubation, cells were directly treated with desired drug concentrations After a 9 day incubation period, the media was suctioned out, and the wells were stained for 20 minutes using methylene blue. Plates were counted by hand, with treatments in triplicates.

2.6 Statistical Analysis. Each treatment was divided by the DMSO treatment average in order to get the relative percent. The triplicates were then analyzed using a two tailed t-test with equal variance. All p-values listed are the comparison between the control line and the resistant line for each treatment.

3. Results

3.1 Anti-Apoptotic proteins are upregulated in response to paclitaxel. A 48 hour treatment with different paclitaxel doses caused two key anti-apoptotic Bcl-2 protein levels to increase. Bcl-xL showed a dramatic increase over the DMSO treated vehicle control (Figure 1a), and MCL-1 showed an increase in a dose dependent manner, up to the highest treated dose of 100 nM (Figure 1b).

Figure 1. Initial upregulation of Bcl-2 proteins in MB468 cells. (A) Western blots performed on the lysates from a 48 hour paclitaxel treatment on MDA-MB-468 cells show a strong increase in Bcl-xL levels, with the highest upregulation response occurring at 0.1 nM. GAPDH is used as a loading control.

(B) Western blots using lysates from a 48 hour paclitaxel treatment on the MB468 cells show MCL-1 upregulation. While the MCL-1 band strength remains even the decrease in loading control (GAPDH) over increasing paclitaxel treatment concentrations indicates an upregulation of MCL-1.

3.2 Resistant MB468 cells have a 2-5 fold increase in paclitaxel resistance compared to sensitive parental line. To determine the resistance of the cells treated with paclitaxel we employed two methods. First we looked at the resistance to paclitaxel through the cellular viability assay. We saw a 2 to 5 fold increase in cellular viability in the resistant line (Figure 2a). Second, paclitaxel's' ability to inhibit colony formation in the control line was far more pronounced than in resistant line. The paclitaxel resistant line colony counts were similar to the vehicle treated counts, while no growth occurred in the control line (Figure 2b).

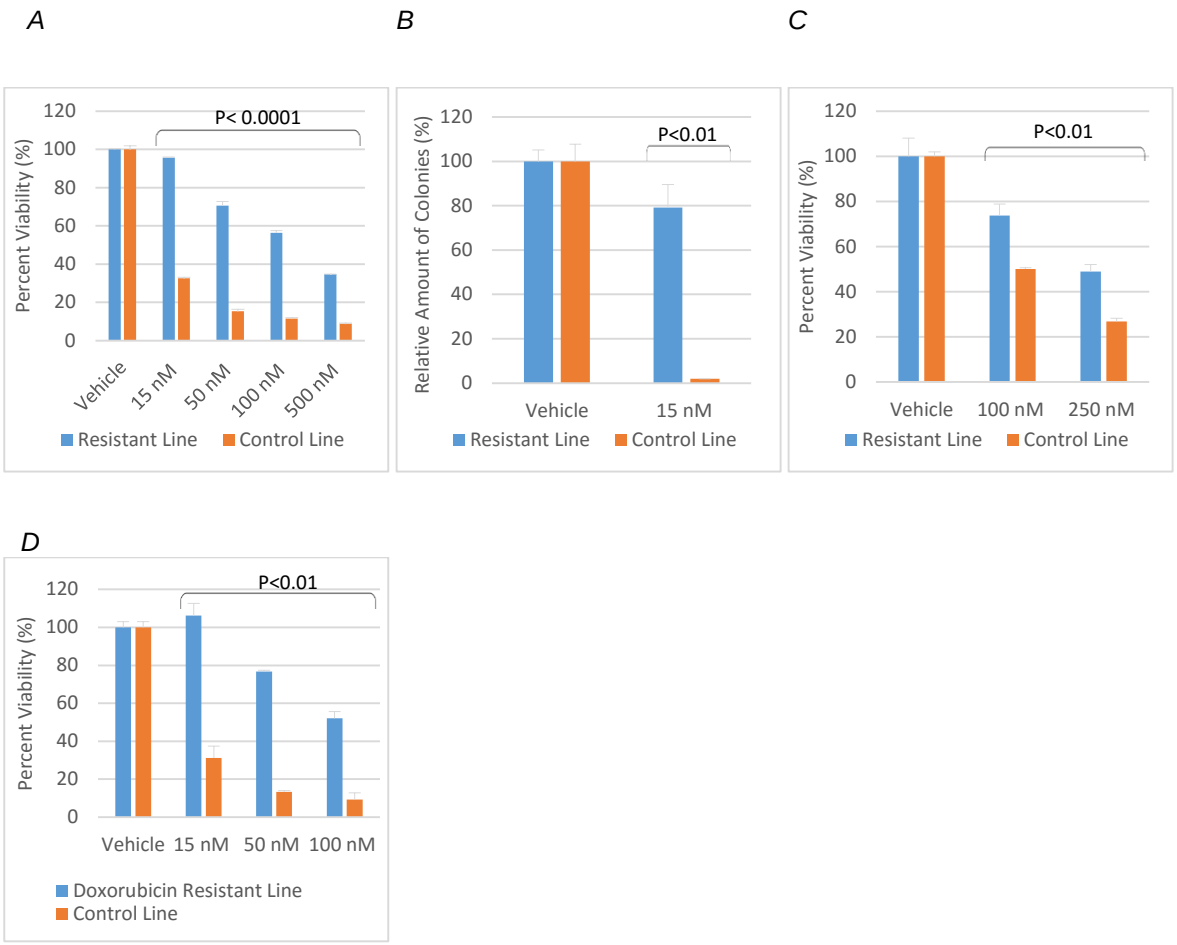

Figure 2. Paclitaxel Resistance

(A) The control line cellular viability is significantly reduced in comparison to the resistant cell line when treated with increasing doses of paclitaxel. There is a 2.5 to 5 fold difference in cellular viability. Results charted in percentage relative to the DMSO treated vehicle.

(B) The resistant line has a large increase in colony formation in comparison to the control line when treated with paclitaxel. 15 nM paclitaxel was enough to completely inhibit colony formation in the parental line, while the resistant line was able to form colonies comparable to the DMSO treated vehicle.

(C) Both lines were treated with multiple doses of doxorubicin, another chemotherapeutic. Neither had previously seen doxorubicin, but the resistant line showed a significant increase in resistance to doxorubicin in a glo-viability assay.

(D) In a doxorubicin resistant MB468 cell line there was a large increase in resistance to paclitaxel. The effect appeared in a dose dependent manner, with increasing doses of doxorubicin found on the x-axis. The relative viability was plotted against the DMSO vehicle which was set to 100% viability.

3.3 Paclitaxel resistance leads to cross resistance to doxorubicin. Both cell lines saw doxorubicin for the first time in a glo-viability assay. The parental cell line had a significant decrease in viability compared to the resistant line after the doxorubicin treatment (Figure 2c). We also found cross resistance to paclitaxel in a doxorubicin resistant cell line (Figure 2d).

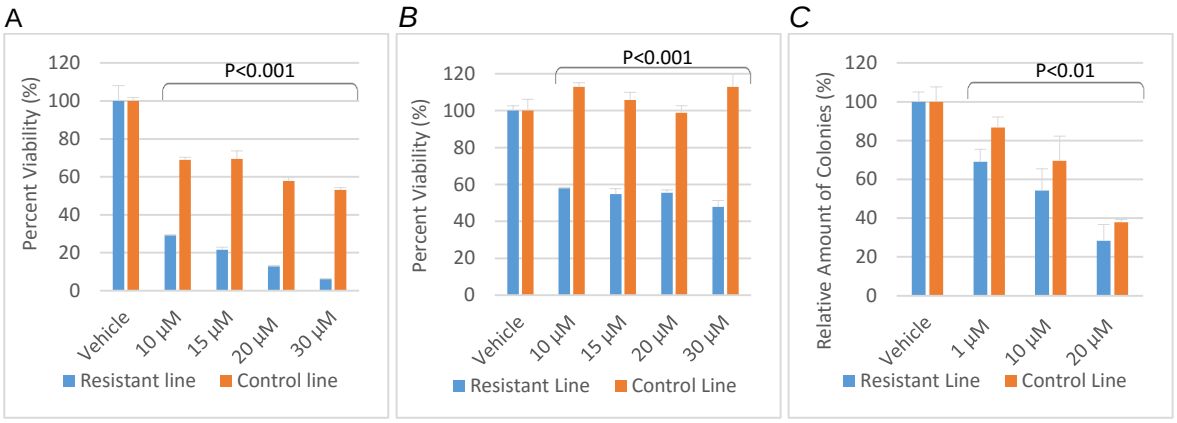

Figure 3. BFC1108 is effective in resistant cell lines. (A) In 1% FBS medium, BFC1108 treatment decreased the cellular viability of the resistant line by approximately 2 to 7 fold in comparison to the control line, relative to the DMSO vehicle. The effect was dose dependent, and all doses listed are of BFC1108.

(B) In a low glucose medium, BFC1108 treatment decreased cellular viability of the resistant cell line by approximately 2 fold in comparison to the control line relative to the DMSO treated vehicle. The effect is not dose dependant, and all doses listed are of BFC1108.

(C) A colony formation assay in 10% FBS medium did not show a significant reduction in colony formation when treated with BFC1108, however there is a consistent trend to the data.

3.4 BFC1108 efficiently targets paclitaxel resistant TNBC cells. When given doses of BFC1108 ranging from 10 µM to 30 µM in 1% FBS conditions the resistant line had up to a seven fold decrease in viability, and the effect occurred in a dose dependent manner (Figure 3a). Under low glucose conditions there was a 2 fold decrease in cellular viability of the

resistant cell line (Figure 3b). When looking at colony formation data there was not a significant decrease in colonies, however there was a consistent trend (Figure 3c).

3.5 BFC1103 reduces cellular viability as well as inhibits colony formation. When the resistant cell line was treated with doses of BFC1103 in low glucose medium there was a significant reduction in cellular viability (Figure 4a). In conjunction with this data, the colony formation assay showed a significant decrease in the resistant cell line's potential to form new colonies (Figure 4b).

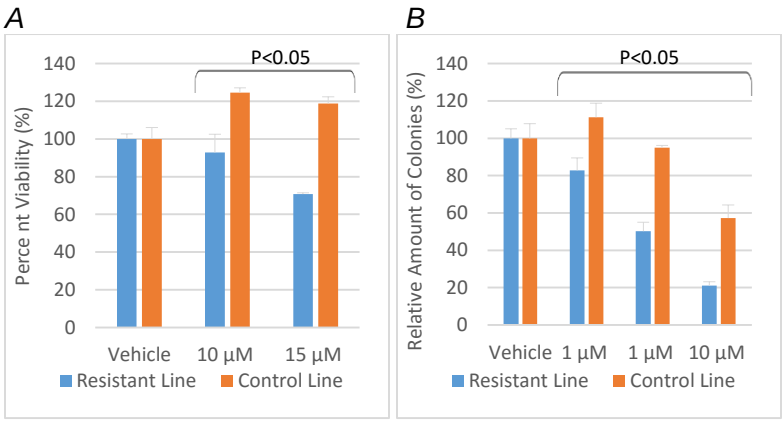

Figure 4. BFC1103 is effective in both colony formation inhibition as well as in decreasing cell viability. (A) In low glucose medium, BFC1103 treatment decreased callular viability of the resistant line by approximatly 30-45% in comparison to the control line relative to the DMSO treated vehicle. The effect was dose dependant, and all doses listed are of BFC1108.

(B) When colony formation was assessed, BFC1103 was found to significantly reduce the number of colonies that the resistant line was able to form in a dose dependent manner. They were graphed relative to the amount of colonies that the DMSO treated vehicle formed.

3.6 BFC1101 is effective in low glucose medium, but not in 1% FBS medium at targeting paclitaxel resistant TNBC. When the resistant line was treated with BFC1101 doses ranging from 1 µM to 30 µM, in 1% FBS medium, there was a significant reduction of cellular viability in the control line when compared to the resistant line (Figure 5a). This effect was reversed under low glucose conditions. BFC1101 lead to a significant reduction of viability of the resistant cell line under low glucose conditions (Figure 5b.)

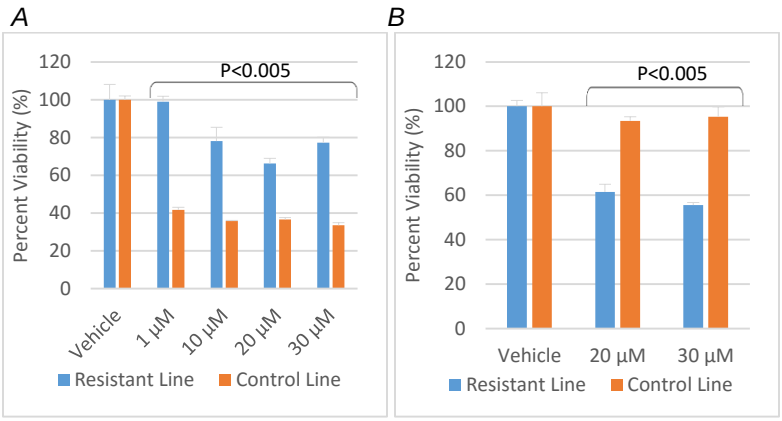

Figure 5. BFC1101 has varied effects under different conditions (A) When the resistant line was treated with multiple doses of BFC1101 in 1% FBS medium it exhibited a significantly higher viability than the control line. This effect was not dose dependent. The data was normalized to the DMSO treated vehicle.

(B) When the resistant line was treated with BFC1101 in low glucose there was a significant decrease in resistant cell viability. The results were normalized to a DMSO treated vehicle that was set to 100% viability.

3.7 BFC1111 reduces cellular viability in low glucose conditions, as well as inhibits colony formation. When treated under low glucose conditions with doses of 10 µM and 15 µM, BFC1111 was able to significantly reduce the cellular viability, as measured by a glo-viability assay (Figure 6a). When treated in 10% FBS, BFC1111 was able to significantly reduce the amount of colonies that formed (Figure 6b).

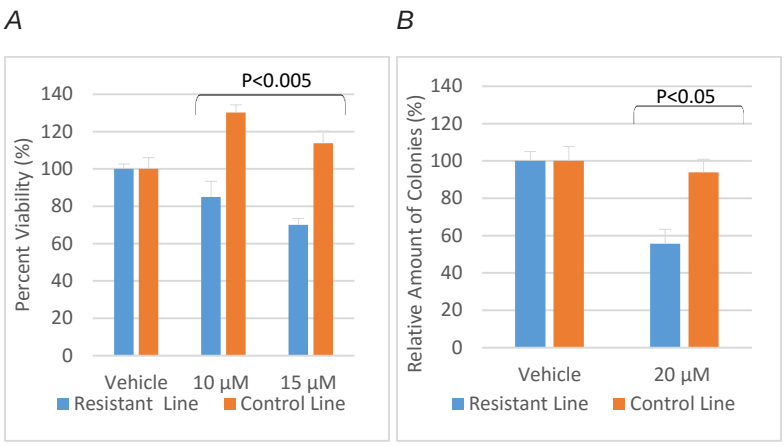

Figure 6. BFC1111 is effective in both colony formation inhibition as well as in decreasing cell viability. (A) Under low glucose conditions doses of both 10 µM and 15 µM BFC1111 were found to have a significant reduction in viability within the resistant cell line in comparison to the control line. The results were normalized using the DMSO treated vehicle, which was set to 100% viability.

(B) In 10% FBS medium BFC1111 was effective at reducing the number of colonies formed in the resistant cell line at a dose of 20 µM. The results were normalized using the DMSO treated vehicle, which was set to 100% viability.

3.8 The paclitaxel resistant line has a decrease in all Bcl-2 anti-apoptotic protein members. A western blot of cell lysates showed that the key targets of the BFCs are downregulated in the resistant cell line (Figure 7a). The other main anti-apoptotic proteins (Bcl-xL and MCL-1) were also found to be downregulated (Figure 7b).

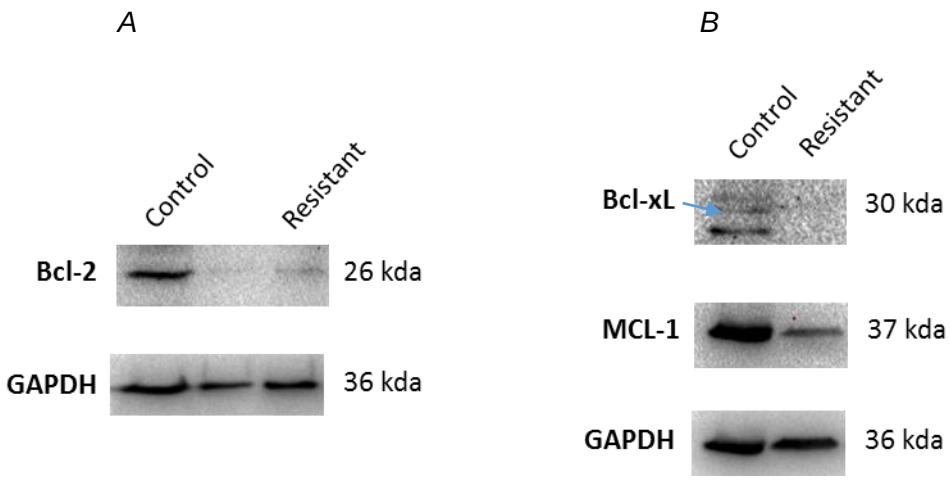

Figure 7. Key anti-apoptotic Bcl-2 family members are downregulated in the paclitaxel resistant TNBC cell line (A) Bcl-2 was found to be downregulated in the resistant cell line. The center band is the resistant cell line after being under constant paclitaxel pressure following cyclic treatment. The constant paclitaxel treatment also lead to a downregulation of Bcl-2.

(B) Bcl-xL and MCL-1 are downregulated. The arrow designates the line that Bcl-xL is. The antibody also recognized Bcl-xS, which is present at 23 kda.

4. Discussion

Bcl-2 functional converters are able to successfully target a paclitaxel resistant cell line. Each converter held different activities, yet they were all effective. This strongly suggests that the resistant cancer is dependent on Bcl-2, and it is able to be targeted.

4.1 Anti-apoptotic proteins show an initial upregulation in response to paclitaxel. The upregulation in response to the low doses of paclitaxel suggests that Bcl-xL and Mcl-1 are involved with the paclitaxel response, and that this response can be potentially targeted in resistance.

4.2 Paclitaxel resistance leads to an increase in resistance to doxorubicin. In the resistant cell line there was an increase in resistance to doxorubicin. Initially we believed that the cross-resistance was due to the slower growth rate of the resistant line (approximately 80% of the control line). However, cross resistance was also seen to paclitaxel in a doxorubicin resistant cell line. The doxorubicin resistant cell line has a similar growth rate compared to that of the control line. This strong increase in resistance has implications reaching into the clinic. Doxorubicin and paclitaxel are the two main chemotherapeutics used to treat TNBC. An alternative way to combat resistance is to use a therapy that the cancer is not resistant to. When resistance to one therapy increases the resistance to another, using the other method as an alternative is not viable because of cross-resistance thus increasing the need for a therapy that can target resistance.

4.3 Each BFC compound is effective in different medium. Tumors have variable microenvironments. These environments tend to be deficient in certain nutrients, like glucose (Weber and Kuo 2012). This seems counterintuitive at first for a highly proliferating cell, however cancer cells have adapted to these harsh environments. Because of this it is

reasonable to test environments with limiting nutrients in order to simulate these conditions. All the test compounds were effective in one medium or another. This suggests that they act in slightly different manners, but each has its merits in resistance treatment. The individual BFC merits should be taken into consideration in future testing.

4.4 BFC1101 has variable effects dependent on the culture medium. In 1% FBS medium there was a decrease in viability in the control line relative to the resistant line. This result was not found in any other compound, or with BFC1101 in different mediums. BFC1101 is unique in that it is a FDA approved compound that treats a different disease. It was found to have a binding affinity to Bcl-2, and was able to act in a Bcl-2 dependent manner in addition to its current role in the clinic. However, while we do not know the reason for the differential effects, we speculate that it is related to known alternative targets of BFC1101.

4.5 Bcl-2 is downregulated in the resistant cell line. Bcl-2, the target for the BFCs, was found to be downregulated, as well as all of the other anti-apoptotic Bcl-2 family members. Previous literature showed upregulation of Bcl-2 as the targetable aspect of resistance. However the BFC compounds were able to successfully target resistance in cell line that has downregulation of all the anti-apoptotic Bcl-2 proteins. This case means that we can look beyond the upregulation of Bcl-2 in order to treat resistance, thus expanding the prior target scenario that was limited to only Bcl-2 upregulation.

4.6 The anti-apoptotic to pro-apoptotic ratio may be the mechanism behind the Bcl-2 dependency. In the future we will be looking at the pro-apoptotic protein expression levels. If they are downregulated to an extent greater than that of the anti-apoptotic proteins the relative ratio may still be skewed to favor the anti-apoptotic proteins, despite them being downregulated. This ratio could be an indicator to be used in the clinic relating to the efficacy of Bcl-2 functional converter treatment beyond the prior indicator of Bcl-2 overexpression.

4.7 Paclitaxel and Bcl-2 functional converters have potential for synergistic treatment. As seen in the initial response to paclitaxel (Figure 1) there was an increase in anti-apoptotic proteins. Since Bcl-2 functional converters generally target this upregulation, paclitaxel treatment could lead to a synergistic effect within the clinic. Paclitaxel could upregulated Bcl-2 and provide a new target for the Bcl-2 functional converter leading to an effect greater than that of either paclitaxel or Bcl-2 functional converters alone.

Our results show the efficacy of using BFCs as a treatment for resistant cancer. This promising data can help to both further the application of BFC compounds, as well as further the progress of BFCs towards the eventual goal of clinical use. These applications need to be investigated in further *in vitro* models. Alongside this, efficacy and safety profiling *in vivo* needs to occur in order to push the development of Bcl-2 functional converters forward into later stage development, such as clinical trials. These Bcl-2 functional converters have the potential to provide an exciting new treatment option for patients who currently have limited treatment options and poor prognosis.

References

Badve, Sunil, David J. Dabbs, Stuart J. Schnitt, Frederick L. Baehner, Thomas Decker, Vincenzo Eusebi, Stephen B. Fox, et al. 2011. “Basal-like and Triple-Negative Breast Cancers: A Critical Review with an Emphasis on the Implications for Pathologists and Oncologists.” *Modern Pathology* 24 (2): 157–67. doi:10.1038/modpathol.2010.200.

Bauer, Katrina R., Monica Brown, Rosemary D. Cress, Carol A. Parise, and Vincent Caggiano. 2007. “Descriptive Analysis of Estrogen Receptor (ER)-Negative, Progesterone Receptor (PR)-Negative, and HER2-Negative Invasive Breast Cancer, the so-Called Triple-Negative Phenotype.” *Cancer* 109 (9): 1721–28. doi:10.1002/cncr.22618.

Cleere, Darrel. 2010. “Triple-Negatvie Breast Cancer: A Clinical Update.” *Community Oncology* 7: 203–11.

Cragg, Mark S., Claire Harris, Andreas Strasser, and Clare L. Scott. 2009. “Unleashing the Power of Inhibitors of Oncogenic Kinases through BH3 Mimetics.” *Nature Reviews Cancer* 9 (5): 321–26. doi:10.1038/nrc2615.

Ferlini, Cristiano, Giuseppina Raspaglio, Simona Mozzetti, Mariagrazia Distefano, Flavia Filippetti, Enrica Martinelli, Gabriella Ferrandina, Daniela Gallo, Franco Oreste Ranelletti, and Giovanni Scambia. 2003. “Bcl-2 Down-Regulation Is a Novel Mechanism of Paclitaxel Resistance.” *Molecular Pharmacology* 64 (1): 51–58. doi:10.1124/mol.64.1.51.

Fesik, Stephen W. 2005. “Promoting Apoptosis as a Strategy for Cancer Drug Discovery.” *Nature Reviews Cancer* 5 (11): 876–85. doi:10.1038/nrc1736.

Foulkes, William D., Ian E. Smith, and Jorge S. Reis-Filho. 2010. “Triple-Negative Breast Cancer.” *New England Journal of Medicine* 363 (20): 1938–48. doi:10.1056/NEJMra1001389.

Kolluri, Siva Kumar, Xiuwen Zhu, Xin Zhou, Bingzhen Lin, Ya Chen, Kai Sun, Xuefei Tian, et al. 2008. “A Short Nur77-Derived Peptide Converts Bcl-2 from a Protector to a Killer.” *Cancer Cell* 14 (4): 285–98. doi:10.1016/j.ccr.2008.09.002.

Lin, Bingzhen, Siva Kumar Kolluri, Feng Lin, Wen Liu, Young-Hoon Han, Xihua Cao, Marcia I Dawson, John C Reed, and Xiao-kun Zhang. 2004. “Conversion of Bcl-2 from Protector to Killer by Interaction with Nuclear Orphan Receptor Nur77/TR3.” *Cell* 116 (4): 527–40. doi:10.1016/S0092-8674(04)00162-X.

Mazumder, Suparna, Gaurav S. Choudhary, Sayer Al-harbi, and Alexandru Almasan. 2012. “Mcl-1 Phosphorylation Defines ABT-737 Resistance That Can Be Overcome by

Increased NOXA Expression in Leukemic B Cells." *Cancer Research* 72 (12): 3069–79. doi:10.1158/0008-5472.CAN-11-4106.

O'Reilly, Elma A., Luke Gubbins, Shiva Sharma, Riona Tully, Matthew Ho Zhing Guang, Karolina Weiner-Gorzel, John McCaffrey, et al. 2015. "The Fate of Chemoresistance in Triple Negative Breast Cancer (TNBC)." *BBA Clinical* 3 (June): 257–75. doi:10.1016/j.bbacli.2015.03.003.

Pan, Zhi, and Lauren Gollahon. 2013. "Paclitaxel Attenuates Bcl-2 Resistance to Apoptosis in Breast Cancer Cells through an Endoplasmic Reticulum-Mediated Calcium Release in a Dosage Dependent Manner." *Biochemical and Biophysical Research Communications* 432 (3): 431–37. doi:10.1016/j.bbrc.2013.01.130.

Shi, Lei, Jian Chen, Jian Yang, Tianhong Pan, Shuguang Zhang, and Zhimin Wang. 2010. "MiR-21 Protected Human Glioblastoma U87MG Cells from Chemotherapeutic Drug Temozolomide Induced Apoptosis by Decreasing Bax/Bcl-2 Ratio and Caspase-3 Activity." *Brain Research* 1352 (September): 255–64. doi:10.1016/j.brainres.2010.07.009.

Teijido, O., and L. Dejean. 2010. "Upregulation of Bcl2 Inhibits Apoptosis-Driven BAX Insertion but Favors BAX Relocalization in Mitochondria." *FEBS Letters* 584 (15): 3305–10. doi:10.1016/j.febslet.2010.07.002.

Watanabe, Ayano, Shinji Yasuhira, Tsuyoshi Inoue, Shuya Kasai, Masahiko Shibazaki, Kazuhiro Takahashi, Toshihide Akasaka, Tomoyuki Masuda, and Chihaya Maesawa. 2013. "BCL2 and BCLxL Are Key Determinants of Resistance to Antitubulin Chemotherapeutics in Melanoma Cells." *Experimental Dermatology* 22 (8): 518–23. doi:10.1111/exd.12185.

Weber, Cynthia E., and Paul C. Kuo. 2012. "The Tumor Microenvironment." *Surgical Oncology* 21 (3): 172–77. doi:10.1016/j.suronc.2011.09.001.