THE EFFECT OF VINORELBINE ON MCF-7 AND MDA-MB-231 HUMAN BREAST CANCER CELL LINES

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ABSTRACT

Vinorelbine, otherwise known as Navelbine or KW-2307, is a semi-synthesized chemotherapeutic drug part of the class of compounds known as *Vinca* alkaloids. Like other *Vinca* alkaloids, vinorelbine interacts with microtubules, specifically non-polymerized monomeric tubulin proteins. This interaction prevents the polymerization and subsequent assembly of the microtubules, thereby arresting cells in the G2/metaphase stage. Vinorelbine is commonly used in treating breast cancers and non-small cell lung cancers (NSCLC). The aim of this research is to investigate the mode of cell death caused by vinorelbine in MDA-MB-231 versus MCF-7 human breast cancer cell lines, which are metastatic and non-metastatic respectively, and to evaluate whether metastasis plays a significant role in the drug interaction. Three XTT growth inhibition assays were performed on each cell line in the presence and absence of vinorelbine after 24 or 48 hours, cell death was visualized by DAPI staining each cell line in the presence and absence of vinorelbine after 24 or 48 hours, and Western blotting was used to analyze the mode of cell death in the presence and absence of vinorelbine after 24 or 48 hours by determining the presence of Beclin, LC3, and Caspase-3 proteins.

INTRODUCTION

Cancer is characterized by cells that are in a state of uncontrolled growth with a low rate of death. Carcinogenesis, the process by which cells become cancerous, is composed of two major steps, though each step is made up of a series of cellular events. The first major step is the appearance of oncogene and tumor suppresser mutations in a cell or group of cells (Rovini and others 2011). The presence of these genetic mutations may lead to the second major step of carcinogenesis, disorderly and uninhibited cell proliferation (Rovini and others 2011). The formation of tumors is ultimately caused by this unrestrained cell division and can lead to the growth and spread of the cancer within the body (Rovini and others 2011). Breast cancer is the most frequently occurring of the cancers that are considered “female” cancers, though in rare cases it has been shown to occur in males (Zhang and others 2012).

Surgery, radiation therapy, and chemotherapy are all common, effective treatments of breast cancer, though chemotherapy is the most common of the three (Boulikas and Tsogas 2008). Chemotherapy, which works by inducing cell death in the cancer cells, is a rapidly advancing treatment option (Zhang and others 2012). Constant developments in the area of chemotherapy have helped to revolutionize cancer treatment and change the way in which a cancer diagnosis is viewed (Chien and Moasser 2008).

THE CHEMOTHERAPEUTIC DRUG VINORELBINE

More than 700 chemotherapeutic drugs have been approved by the FDA for clinical use since the 1970’s (Boulikas and Tsogas 2008). Chemotherapeutic drugs are organized into six major groups which include platinum coordination complexes, antimetabolites, antitumor drugs,

alkylating agents, antimicrotubule agents, and those that do not fall into the preceding categories, but target specific biological pathways (Boulikas and Tsogas 2008).

Vinca alkaloids are a subset of antimicrotubule agents that originate in the family of periwinkle plants and have been derived from one of *Catharanthus roseus*, *Vinca rosea*, *Lochnera rosea*, or *Ammocallis rosea* (Boulikas and Tsogas 2008). All *Vinca* alkaloids are cell cycle specific, causing cell death by arresting mitosis though binding tubulin monomers and stopping microtubules from polymerizing (Bourgarel-Rey and others 2009; Boulikas and Tsogas 2008; Depenbrock and others 1995).

Vinorelbine, otherwise known as Navelbine or KW-2307, is a semi-synthesized, chemotherapeutic drug and is part of the class of compounds known as *vinca* alkaloids (Chiu and others 2012; Boulikas and Tsogas 2008; Hayakawa and others 2008; Fukuoka and others 2001; Liu and others 2001). Vinorelbine may be used alone or in conjunction with other chemotherapeutic drugs and it is regularly prescribed in the treatment of non-small cell lung cancer (NSCLC) and breast cancer (Chiu and others 2012; Boulikas and Tsogas 2008; Hayakawa and others 2008). Like other *Vinca* alkaloids, the action of vinorelbine is targeted to microtubules; in specific, vinorelbine interacts with the non-polymerized monomeric tubulin proteins, and prevents polymerization and subsequently the assembly of the microtubules, thereby arresting cells in the G2/metaphase stage (Chiu and others 2012; Hayakawa and others 2008; Fukuoka and others 2001).

Compared to other chemotherapeutic drugs of the same class, vinorelbine has relatively mild side effects, though some unpleasant effects persist (Hayakawa and others 2008). The most common side effects associated with vinorelbine are mild nausea and vomiting, lower intestinal

tract upsets, increased tendency to bruise or bleed, mild peripheral neuropathy, acute chest pain during administration, mild vesicant properties, general fatigue, and myelosuppression in the form of leucopenia causing a reduced resistance to infections, which tends to be the dose limiting effect (Boulikas and Tsogas 2008). In a clinical setting, vinorelbine is quickly distributed over the peripheral tissues because of its lipophilic properties, so the highest concentration of vinorelbine (at injection of 20-30mg/m^2 of body area) is found to be approximately 1mM, which reduces to 100nM in one hour, and to 10nM in 24 hours (Chiu and others 2012).

Vinca alkaloids are known to induce a range of apoptotic signal transduction pathways, some of which are known to be specifically activated by certain drugs (Bourgarel-Rey and others 2009; Wang and others 1999). The effect of vinorelbine is mediated through the Bcl-2 family of genes and the caspase protein family, which are involved in regulating and inducing apoptosis (Chiu and others 2012; Saussede-Aim and others 2009; Hayakawa and others 2008). The apoptotic effect of vinorelbine has also been associated with a response from the intrinsic mitochondrial apoptotic pathway, though this has been less well characterized (Bourgarel-Rey and others 2009).

MCF-7 AND MDA-MB-231 CELL LINES

The MCF-7 cell line is a non-metastatic cell line that was isolated from a tumor in the breast of a 69 year old Caucasian woman in 1970 (Soule and others 1973). In 1973 the cell line was established at the Michigan Cancer Foundation by Soule and his colleagues (Soule and others 1973). MCF-7 cells are positive for estrogen and progesterone receptors (Brown and others 1984; Masiakowski and others 1982; Soule and others 1973). MCF-7 cells contain the *p53* gene, and encode the wild-type *p53* tumor suppressor, but do not easily undergo *p53*-mediated

apoptosis (Bourgarel-Rey and others 2009; Fan and others 1995). Further, MCF-7 cells are caspase-3 deficient and may not undergo DNA fragmentation; however the caspase-3 deficiency does not inhibit MCF-7 cells from undergoing cell death (Kagawa and others 2001). Therefore, apoptosis must be mediated through another pathway; it has been proposed that in the presence of vinorelbine caspase-8 mediates apoptosis in caspase-3 deficient cells (Hayakawa and others 2008).

MDA-MB-231 cells have a high rate of proliferation, are able to form new tumors in nude mice, lack estrogen receptors, and are from a line of human breast cancer cells (Hardwick and others 2001). The MDA-MB-231 cells are metastatic, as indicated by the novel tumors they produce in nude mice. The metastasis of cancerous cells is the most common cause of death in cancer patients (Siri, Chen and Chen 2006).

HOW DOES VINORELBINE AFFECT THESE CELLS?

Though they may share similar mechanisms, chemotherapeutic drugs have different interactions with each cell type. In MCF-7 cells vinorelbine increases the cellular level of β3-Tubulin mRNA, but does not appear to interact directly with the other isotypes of Tubulin mRNA (Saussede-Aim and others 2009). Vinorelbine affects the β3-Tubulin mRNA through the AP-1 binding site, a DNA promoter region that can be bound by homo or heterodimeric associations of Jun and Fos proteins (Saussede-Aim and others 2009).

MCF-7 cells also show an increase in *p38* and *p58* activity in the presence of vinorelbine (Liem and others 2003; Liu and others 1999). *p38, p58*, ERK (extracellular signal kinase), and JNK (c-Jun terminal kinases) responses are coordinated in breast cells, and lead to apoptosis (Liem and others 2003). ERK2 in MCF-7 cells has a large and rapid response to the presence of

vinorelbine, and increases in concentration dramatically (Liu and others 2001). *p53* is considered an important gene in a cancer cell's response to chemotherapy, and can determine if the cell will undergo apoptosis (Liem and others 2003).

Vinorelbine causes a decrease in Bcl-2 protein in MCF-7 cells. Bcl-2 protein helps preserve the mitochondrial membrane and stops mitochondria from releasing apoptogenic molecules that activate caspases, thus inhibiting apoptosis (Bourgarel-Rey and others 2009). The mechanism causing the decrease of Bcl-2 by vinorelbine involves *p53,* which binds to a consensus site 1Kb upstream from the Bcl-2 P1 promoter to down regulate it's expression (Bourgarel-Rey and others 2009). Thus down-regulation of Bcl-2 may be key to inducing apoptosis in MCF-7 cells with vinorelbine (Bourgarel-Rey and others 2009).

The effect of vinorelbine on MDA-MB-231 cells has not been as well studied as its effect on MCF-7 cells. Most of the studies reviewed that tested the effects of vinorelbine on MDA-MB-231 cells were testing the synergistic effect of other drugs with vinorelbine, and thus the resulting effects cannot be contributed solely to vinorelbine. As a single agent, vinorelbine has been shown to indirectly alter receptor binding of EGF in MDA-MB-231 cells by cytoplasmic mechanisms (Depenbrock and others 1995).

WHAT IS APOPTOSIS?

Apoptosis, or programmed cell death, is a way in which a cell may die without negatively impacting other cells around it and is an important method of the destruction of cancer cells by chemotherapy (Yang and others 2012). Depending on the manner in which cell death was triggered, a cell may undergo apoptosis by two pathways, intrinsic or extrinsic, (Lin and others 2012). An initiation phase, a decision phase, and an execution phase make up the mechanism of

apoptosis (Lin and others 2012). The initiation phase starts when the cell is signaled to prepare to die by extrinsic factors (Ashkenazi and Dixit 1998). In the decision phase, the cell may still be allowed to live (Green and Reed 1998), but if the cell has committed to die, it begins the execution phase, in which cell death and clearance is controlled by downstream caspases (Susin and others 1997). The mechanism of the execution phase may happen in one of two ways: by an apoptotic signal transducing pathway involving the fas-associated death domain protein (FADD) and the death-inducing signaling-complex (DISC), which catalyzes the conversion of procaspase-8 into caspase-8, or by a mitochondria-mediated process (Hayakawa and others 2008). In mammals, a balance between suppressor and promoter gene products is what regulates apoptosis (Liu and others 2001).

Apoptosis plays an important role in higher level, multi-cellular organisms, especially in tissue development in embryos, and homeostasis of tissues in the body (Yang and others 2012; Hayakawa and others 2008; Liu and others 2001). Apoptosis is characterized by the breakdown of the cell by catabolic enzymes, shrinking of the cell, membrane blebbing, and the fragmentation of the cell’s DNA, which can be used as a way of determining if a cell culture is undergoing apoptosis *in vitro* (Yang and others 2012).

WHAT IS AUTOPHAGY?

Literally meaning self-eating, autophagy is an essential process in which old or damaged cell constituents are degraded and often recycled (Fu and others 2012; Yang and others 2012). Double-membraned autophagosomes encapsulate targets in the cytoplasm, and then fuse with lysosomes allowing lysosomal enzymes to break down the contents (Yang and others 2012).

Autophagy is a process to important cellular survival, but excessive autophagy can lead to autophagic cell death (Fu and others 2012; Yang and others 2012).

Autophagy is independent from apoptosis, though there is evidence that the two cycles can interact (Fu and others 2012). The initiation of autophagy enhances apoptosis in some cases, but destruction of damaged mitochondria by autophagy can hinder the progress of apoptosis (Fu and others 2012; Yang and others 2012).

RESEARCH DESIGN AND METHODOLOGY

This research was made up of 5 phases, which are as follows: culturing and maintaining cell lines (MDA-MB-231, MCF7), determining growth inhibition by an XTT assay, observing cell death by DAPI staining, and determining the method of cell death by Western Blot analysis. Finally, the data was analyzed using statistical methods.

CULTURING AND MAINTAINING CELL LINES

The MDA-MB-231 and MCF-7 cell lines were maintained in RPMI (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich). The cell lines were grown at 37°C in an incubator at 5% CO_2 in flat sided cell culture flasks (Fisher Scientific). The cell lines were maintained throughout the course of the research by changing the media and splitting the cells as needed.

XTT ASSAY

Two 96-well plates were seeded with a starting concentration of $1X10^4$ cells/well for MDA-MB-231 cells and $2X10^4$ cells/well for MCF-7 cells. Ten replicates for each cell line were set up, five replicates of treatment and five of non-treatment. The cells were allowed to adhere for 24 hours in 100 µl of media at which point the treatment was applied. The treatment cells had 100 µl 0.5nM vinorelbine solution replace the old media, and the non-treatment cells will had 100

µl of fresh media replace the old media. The treatment was left for 24 and 48 hours, after which a cell proliferation assay was performed. To determine the amount of viable cells per well, Biological Industries' XTT based Cell Proliferation kit was used following the manufacturer's protocols as has been described previously (Jost and others 1992; Roehm and others 1991; Hansen and others 1989; Weislow and others 1989; Scudiero and others 1988; Tada and others 1986).

DAPI STAINING

MDA-MB-231 and MCF-7 cells were adhered to glass cover slips in all wells of a six-well plate. The cells were applied for 24 hours to the cover slips at a starting concentration of $1X10^4$ cells/well (MDA-MB-231) and $2X10^4$ cells/well (MCF7). After 24 hours a treatment of vinorelbine (0.5nM concentration) was applied to three of the wells and the other three wells were mock treated. The treatment was left for 24 or 48 hours, after which the media was removed and the cells were fixed. The cells were then washed with PBS (Phosphate Buffered Saline) and stained with DAPI (Invitrogen), after which they were viewed under a fluorescent microscope. Photographs were taken and transferred to a laptop, where they were magnified and a minimum of 200 cells per treatment were counted and determined to be apoptotic, autophagic, or normal.

WESTERN BLOT

Two 12-well plates for each cell line were seeded with a starting concentration of $1X10^4$ cells/well (MDA-MB-231 cells) or $2X10^4$ cells/well (MCF-7 cells). After allowing the cells to adhere for 24 hours six wells on each plate had a vinorelbine solution (0.5nM concentration) replace the old media, and six had fresh media replace the old media. The treatment was left

for 24 or 48 hours. After the treatment time had elapsed, the media was removed and the cells washed with 100 µl 2X SSB (100mM Tris-Cl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200mM dithiothreitol) and collected. The SDS-PAGE gels were poured with a 5% acrylamide stacking gel and a 12% acrylamide separating gel. The presence of Beclin-1, LC3 (LC3-I or LC3-II), and caspase-3 (procaspase-3 or caspase-3) was determined using indirect antibody detection. Mouse anti-caspase-3 (Cell Signaling Technology) diluted 1:1000 with a 5% milk-TBST solution, Rabbit anti-Beclin-1 (Cell Signaling Technology) diluted 1:1000 with a milk-TBST solution, and Rabbit anti-LC3B (Sigma-Aldrich) diluted 1:1000 with a 5% milk-TBST solution were used as primary antibodies. Anti-Rabbit IgG-peroxidase (Sigma-Aldrich) diluted 1:500 with a 5% milk-TBST solution and anti-mouse IgG-peroxidase (Sigma-Aldrich) diluted 1:500 with a 5% milk- TBST solution were used as the secondary antibodies.

DATA ANALYSIS

After all the results were gathered, statistical analyses were performed using Excel. The results were analyzed using the Student's t-Test.

RESULTS

XTT ASSAY

The results of the XTT assays were compiled and are as follows:

After 24 hours of treatment with vinorelbine (0.5 nM concentration) the MCF-7 cells had an average absorbance of 0.217 at 450 nm (10 replicates), and the non-treatment cells had an average absorbance of 0.213 at 450 nm (10 replicates). After 48 hours of treatment with 0.5 nM vinorelbine the MCF-7 cells had an average absorbance of 0.162 at 450 nm (10 replicates), and the non-treatment cells had an average absorbance of 0.172 at 450 nm (10 replicates).

After 24 hours of treatment with 0.5 nM vinorelbine the MDA-MB-231 cells had an average A_{450} of 0.228 (10 replicates), and the non-treatment cells had an average A_{450} of 0.356 (10 replicates). After 48 hours of treatment with 0.5 nM vinorelbine the MCF-7 cells had an average A_{450} of 0.223 at 450 nm (10 replicates), and the non-treatment cells had an average A_{450} of 0.655 (10 replicates).

A student t-test is run on the results and it is found that the vinorelbine treatment does not cause a significant difference in growth of the MCF-7 cells after 24 or 48 hours; however, the treatment causes a significant decrease in the growth of MDA-MB-231 cells after 24 and 48 hours. The data is summarized in the following figure.

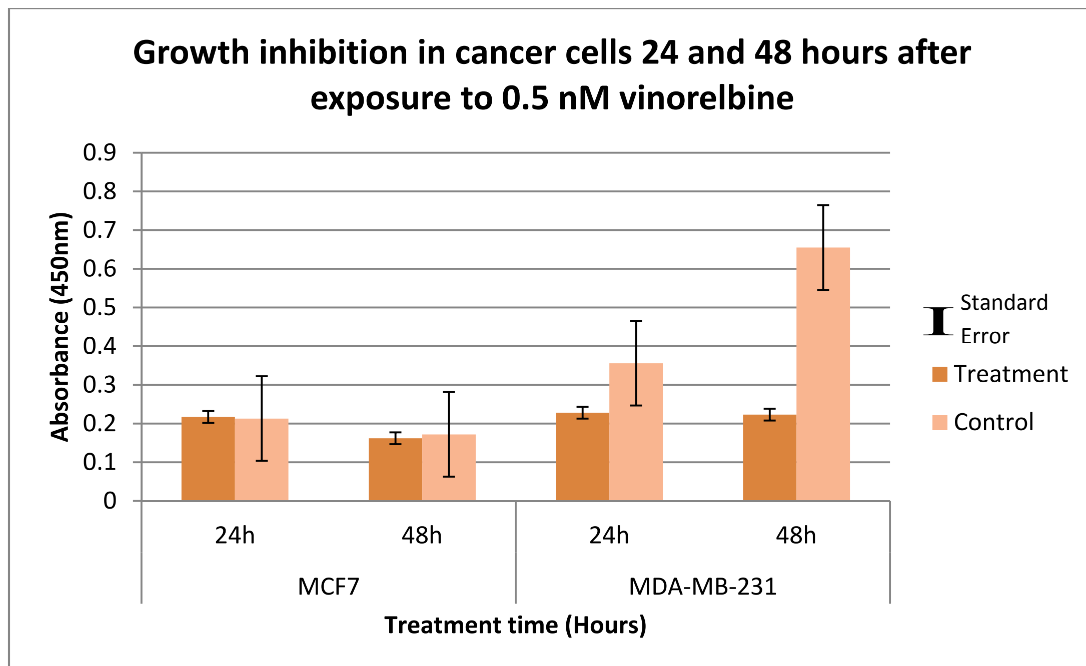

Figure 1. Results of growth inhibition after 24 or 48 hours treatment with 0.5 nm vinorelbine on MCF-7 and MDA-MB-231 cells determined by A_{450} after XTT assay.

DAPI STAINING

After DAPI staining, the percentage of apoptotic cells was determined by counting cells with characteristic membrane blebbing as representative of apoptosis relative to the total number of cells. After 24 hours of vinorelbine treatment 29% of MCF-7 cells and 0% of the non-treatment MCF-7 cells were determined to be apoptotic. After 48 hours of vinorelbine treatment 31% of MCF-7 cells and 0% of the non-treatment MCF-7 cells were determined to be apoptotic. After 24 hours, 41% of vinorelbine treated MDA-MB-231 cells were determined to be apoptotic, compared to 0% of the non-treatment cells. After 48 hours, 54% of the vinorelbine treated MDA-MB-231 cells and 0% of the non-treatment cells were determined to be apoptotic. This data is summarized in the following figure.

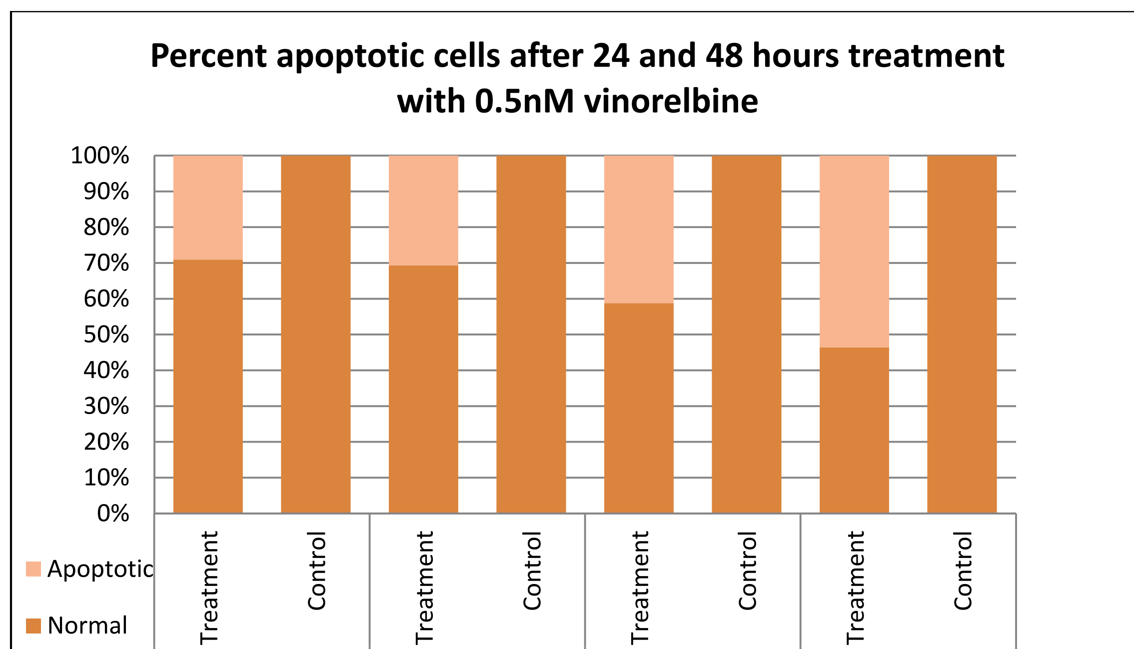

Figure 2. A comparison of the percentage of apoptotic cells after 24 or 48 hours as determined by DAPI stain treatment, followed by counting the number of cells showing nucleic membrane blebbing as representative of apoptosis relative to the total number of cells.

WESTERN BLOT

Western blotting showed that Beclin-1 and LC3-I, but not LC3-II proteins were present in both cell lines after 24 hours and 48 hours in treatment and non-treatment conditions. A Western Blot for caspase-3 (proenzyme and enzyme) was attempted three times, but no results could be identified. The following photos of the blots show the presence of the Beclin-1 and LC3-I proteins.

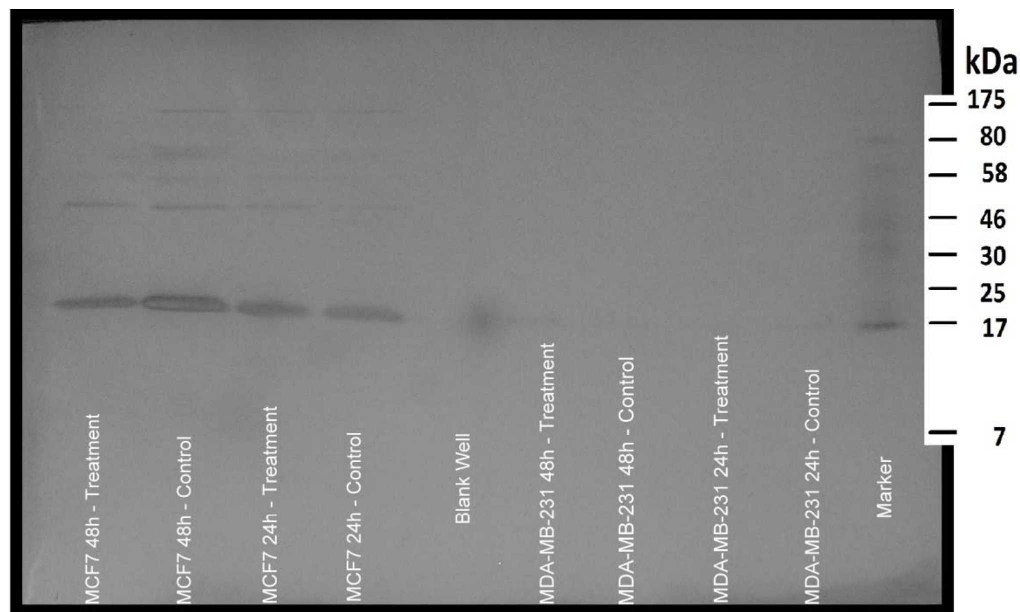

Figure 3. Western blot of a SDS-PAGE gel (5% stacking, 12% separating) with 10μl samples of MCF-7 and MDA-MB-231 cell lysates after 24 or 48 hours. The blot was intended to find LC3B and was completed with Rabbit anti-LCB (Sigma-Aldrich) primary and anti-Rabbit IgG-peroxidase (Sigma-Aldrich) secondary. The LC3-II protein occurs at the 17 kDa mark. If the LC3-II protein were present it would appear at the 14 kDa mark.

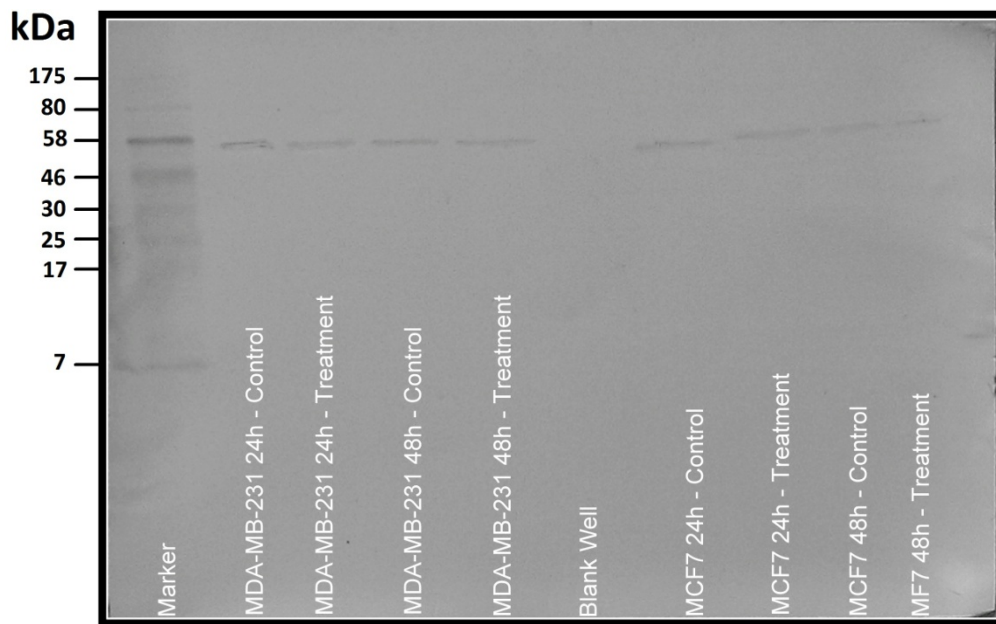

Figure 4. Western blot of a SDS-PAGE gel (5% stacking, 12% separating) with 10µl samples of MCF-7 and MDA-MB-231 cell lysates after 24 or 48 hours. The blot was intended to find Beclin-1 and was completed with Rabbit anti-Beclin-1 (Cell Signaling Technology) primary and anti-Rabbit IgG-peroxidase (Sigma-Aldrich) secondary. The Beclin-1 protein occurs at the 60 kDa mark.

Note: the western blot for caspase-3 was performed three times and each time, no results were seen, and thus no photos of the membrane were included here.

DISCUSSION

XTT ASSAY

The results of the XTT assays indicate that the growth of the MCF7 cells was not affected very strongly by the vinorelbine treatment. After 24 and 48 hours there was no significant difference between the treatment cells and the no-treatment cells. The growth of the MDA-MB-231 cells was significantly decreased in the vinorelbine treatment groups in as little as 24 hours after treatment. These results indicate that the vinorelbine affected the MDA-MB-231

cells to a higher degree than the MCF7 cells. This is interesting because the MCF7 cells are not metastatic and as such are not as aggressive; however, because vinorelbine targets cells as they are replicating, the slower growth rate of the MCF7 cells could have contributed to the vinorelbine working less efficiently.

The MDA-MB-231 cells are metastatic and as such have a faster growth rate than a non-metastatic cancer, which could have allowed the vinorelbine to target the growing cells more effectively. The literature seems to concur that due to its microtubule stabilizing action, vinorelbine targets highly proliferative cells (Klotz and others 2012; Jordan and Wilson 2004). The larger affect of vinorelbine on faster growing cells seems to be a logical and accepted conclusion.

DAPI-STAIN AND CELL COUNT

DAPI staining and observing both cell lines under a microscope revealed that a considerable number of the treatment cells of each cell line were undergoing what appeared to be apoptosis. Further, there were far fewer cells that had been treated with vinorelbine on the slides compared to those that had not been treated. This was observed for both cell lines. Though the XTT assays would indicate that there is no significant difference in the growth of the treatment and non-treatment MCF7 cells, when the cells were DAPI stained and observed under a fluorescent microscope it was clear that the non-treatment cells looked healthier and were more abundant than the treatment cells.

No apoptotic cells were seen in any of the non-treatment cells regardless of cell line. The MDA-MB-231 cells treated with vinorelbine had a higher percentage of cells that appeared to

be undergoing apoptosis than the MCF7 cells treated with vinorelbine, which agrees with the trends seen in the XTT assays.

The literature agrees that treatment with vinorelbine induces apoptosis in both MDA-MB-213 cells and MCF7 cells. Zeybek and others in 2011 saw apoptotic cells in MCF7 cells after treating with vinorelbine, while Weigei and others in 2009 see apoptosis in both cell lines. It appears that treatment with anti-microtubule agents, such as vinorelbine, is widely accepted to induce apoptosis, though the mechanism by which this is achieved depends on the drug used (Wang and others 1999).

WESTERN BLOT

The presence of Beclin-1 indicates that there was adequate protein in the samples, as it is a ubiquitous protein and so was expected to be in each sample of cell lysate regardless of treatment.

The results of the western blot for LC3B indicates that neither cell line is undergoing autophagy, since LC3-I (17 KDa) is visible as a band, but not LC3-II (14 KDa). LC3-I is the cytosolic pro-form of LC3-II, and it is not until it is conjugated with phosphatidylethanolamine to become LC3-II that the peptide is indicative of the number of autophagosomes (Mizushima and Yoshimori 2007). Therefore, we can conclude that neither the MCF-7 cells, nor the MDA-MB-231 cells are undergoing autophagy.

Because no band was visualized in the western blot for caspase-3, the test is inconclusive. Though no bands associated with caspase-3 were expected in the MCF7 cell lysates, because the MCF7 cells lack the caspase-3 apoptotic pathway, either procaspase-3 or caspase-3 should

have been seen in the MDA-MB-231 cell lysates depending on if the caspase-3 apoptotic pathway was activated.

Though the activation of the caspase-3 pathway could not be proven conclusively here, Hayakawa and others in 2008 found that caspase-3,-8, and -9 are involved in vinorelbine mediated apoptosis of Jurkat cells. Further, Klotz and others in 2012 found that vinorelbine induces caspase-3 in APC negative cells, common in colorectal cancers, indicating the caspase cascade is implicated in apoptosis induced by vinorelbine treatment.

Going forward, it would be interesting to run this experiment with higher concentrations of vinorelbine, as this may induce significant growth inhibition in the MCF7 cells, and produce more exaggerated results. The literature suggests that concentrations ranging from 10nM to 100nM have been used previously (Klotz and others 2012; Bourgel-Rey and others 2009; Hayakawa and others 2008). Further, figuring out what went wrong in the caspase-3 western blot and rectifying it so that concrete results could be obtained would be a crucial next step. Finally, though MCF-7 cells lack the caspase-3 pathway, they were seen to be undergoing apoptosis in the DAPI stain that was completed. It would be worthwhile to investigate these results further, and perhaps investigate the claim that vinorelbine induced apoptosis is mediated through the caspase-8 pathway put forward by Hayakawa and others in 2008.

CONCLUSION

Two important notions emerge from these findings. First, the results of the XTT assay suggest that the MDA-MB-231 cells are more sensitive to vinorelbine treatment than the MCF-7 cells. Because of the nature of vinorelbine and its interference in G2/Metaphase, it is logical that the more aggressive cells with a higher rate of proliferation would be most affected. These

findings would suggest that vinorelbine would be most useful in treating cancers that have metastasized and are growing quickly; indeed, vinorelbine is commonly used clinically as in cases where the patient’s cancer is non-responsive to other treatments (Boulikas and Tsogas 2008).

Second, it is obvious, when considering a combination of the visualization of DAPI stained cells and the LC3B western blot, that neither cell line is undergoing autophagy. Only a combination of apoptotic and healthy cells were seen in the results of the DAPI stain, and even more convincingly, neither of the cell line produced LC3-II in any of the treatments as evidenced by the western blot. While I cannot say with certainty that the MDA-MB-231 cells underwent apoptosis, due to the failed caspase-3 western blot, I can say that they did not undergo autophagy.

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