Spring 2012

Defeating Cytoplasmic Sequestration of p53 in Human Breast Cancer Cells; Is Mortalin Involved?

Sarah Yunes
University of New Hampshire - Main Campus

Defeating Cytoplasmic Sequestration of p53 in Human SKBR3 Breast Cancer Cells; Is Mortalin Involved?

Sarah Yunes (BMCB)

Faculty Mentor: Dr. Charles Walker (MCBS)

Abstract:

Some human breast cancer cell lines are characterized by lack of p53 function in cell cycle control and apoptosis, sometimes due to cytoplasmic sequestration of p53 in the cytoplasm. The molecular mechanism causing this phenotype is unknown, but two mechanisms have been suggested. One involves a defective protein normally promoting nuclear import of p53. Another mechanism that might be involved is cytoplasmic tethering of p53 by mitochondrial heat shock protein, mortalin. When mortalin is over-expressed or otherwise mutated as to hyperactivate the binding site, it binds up all the p53 so that is unable to enter the nucleus of the cell. In this project, treatments of SKBR3 breast cancer cells with MKT-077, a competitive binder of mortalin, were used to confirm the second mechanism of cytoplasmic sequestration of p53. The migration of p53 and subsequent apoptosis was confirmed using immunocytochemistry, Romanowsky staining, and a TUNEL assay. The majority of the migration of p53 occurred between 8 hours and 24 hours of treatment with MKT-077, with some evidence of apoptosis occurring after 24 hours.

Introduction:

The tumor suppressor protein p53 is considered to be one of the most pivotal components of cancer progression, as well as being an indicator of resistance to traditional treatment methods[1]. A mutated form is present in 60% of human cancers[2]. Its widespread involvement in malignancy is most likely due to its important role in cell

cycle regulation, DNA repair, and apoptosis or programmed cellular death. When DNA damage is detected, the intracellular concentration of p53 increases and the protein is localize in the nucleus in non-malignant cells where the protein acts as transcription factor for a variety of proteins that can stop the cell cycle until DNA is repaired or so that apoptosis can be initiated if the damage is too extensive[2, 3]. When p53 is somehow prevented from performing its job adequately, the cell is prevented from fixing DNA and from undergoing apoptosis[1, 3].

It is not necessary for p53 to be mutated or absent from the cell for it to be unable to perform its transcriptional functions. As it interacts with many other proteins, a change in their expression level or in their sequence of amino acids could incapacitate wild type p53. One key protein is mortalin (the mitochondrial Hsp70), a member of the heat shock family of proteins (Hsps) that have been implicated in stress response within the cell can bind to p53[4]. As p53 is responsible for halting the cell cycle following its translocation to the nucleus in the cell cycle, having it active within the nucleus constantly is not advantageous. Therefore, there are times that p53 needs to be sequestered in the cytoplasm. In humans, mortalin routinely binds to p53, as the carboxy terminal end of p53 has a binding site for the amino terminal region of mortalin[1]. This relationship has been further supported by observations in clams (*Mya arenaria*) of the return of p53 to the nucleus after cytoplasmic sequestration when the intracellular mortalin concentration is decreased by blocking the active binding site or by causing DNA damage that results in an increase in p53 concentration that vastly surpasses the amount of available mortalin[5]. This relationship could exist in human cancers, as the binding site for p53 in clam mortalin is 97% identical to that of humans[5]. Also, an interaction between mortalin and

p53 has been shown to occur in the mitochondria in relation to a nucleus-independent apoptotic pathway[6]. If mortalin is modified in such a way that it no longer releases p53 when it is required in the nucleus, this abrogate p53 control mechanisms and could cause uncontrolled proliferation and an inability of the cell to undergo apoptosis[1-5]. There is evidence of this occurrence in human neuroblastoma, colon carcinoma, osteocarcinoma and in breast cancer MCF7 cells where an increase in the concentration of the mortalin-binding portion of p53 caused wild-type p53 to be released from the cytoplasm and stop the cell cycle[1]. Over sixty percent of patient-derived cancerous tissue tested in one study showed an overexpression of mortalin, which when transplanted to nude mice resulted in tumors[4].

As such, exposing cancerous cells to a competitive binder for mortalin could be an effective therapy. MKT-077, a water-soluble rhodacyanine dye has been shown to inhibit growth of cancerous tissue[7]. This inhibition is a result of MKT-077 interacting with the p53-binding domain of mortalin, disrupting mortalin's interaction with p53. As such, MKT-077 could free p53 from mortalin and allow it to initiate apoptosis either through its nuclear function or its mitochondrial function. This research aims to determine whether p53 is sequestered in the cytoplasm of SKBR3 breast adenocarcinoma cells, whether treatment of the cells with MKT-077 over time will initiate nuclear translocation of p53, and whether or not the translocation will result in cells undergoing apoptosis.

Statement of Hypothesis:

If SKBR3 breast adenocarcinoma cells are treated with MKT-077, it will result in translocation of cytoplasmically sequestered p53 to the nucleus followed by apoptosis of the breast cancer cells.

Methods:

Cell Culture: Human breast adenocarcinoma (SKBR3) cells were obtained from American Type Culture Collection (ATCC). The cells were cultured in McCoy's 5A medium (ATCC) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1% penicillin/streptomycin solution (Sigma). Cells were kept at 37°C and 5% carbon dioxide.

Treatment with MKT-077: MKT-077 (Sigma) was added directly to cell culture flasks to a final concentration of 7 μM when the cells were 60-80% confluent. Flasks were covered in aluminum foil to avoid potential photoinactivation of MKT-077. Cells were treated for two, four, six, eight, and twenty-four hours and subsequently prepared as cytospins along with untreated control cells.

Immunohistochemistry to determine the distribution of p53 protein: Detection of p53 protein employed the DO-1 antibody (Santa Cruz Biotechnology) in phosphate buffered saline (PBS) diluted to 1 μg/mL. Slides were developed with the Vectastain ABC Peroxidase Mouse IgG kit (Vector Laboratories). Negative control slides received identical treatment minus primary antibody. Preparations will be observed on a Zeiss Axioplan II microscope at 400X-630X to detect distribution of p53 into the cells.

Romanowsky Stain: Romanowsky stain (Kwik Diff staining system; Thermo) was used to observe the morphology of both control and treated cells. Preparations were observed on a Zeiss Axioplan II microscope at 200-630X.

TUNEL Assay for Apoptosis: Breast cancer (SKBR3) cells untreated and treated with MKT-077 were assayed using the fluorescent terminal dUTP nick-end labeling (TUNEL) assay (DeadEnd™ Fluorometric TUNEL System, Promega) to identify DNA damage as an indicator of apoptosis. Negative controls received deionized water instead of the rTdT enzyme. A drop of ProLong® Antifade Gold Reagent (Life Technologies) was added to each slide and covered with a coverslip as a counterstain. After incubation at room temperature in the dark for 24 hours, the coverslips were sealed to the slides with clear nail polish. Preparations were observed on a Zeiss Axioplan II at 400X-630X to detect fluorescein and DAPI fluorescence.

RNA Extraction: RNA was extracted from SKBR3 breast carcinoma cells for analysis with qPCR. The media was removed and 1 mL of TRIzol (Ambion, Life Technologies) for each 10 cm^2 of surface area of the container that held the growing cells was added and agitated until the cells lysed. After a five minute incubation period, the cell lysates were separated into even volumes. For every milliliter of TRIzol used, 0.2 mL of chloroform was divided evenly amongst the samples. They were vortexed for fifteen seconds, incubated on ice for fifteen minutes, and centrifuged at 12,000 RCF for 15 minutes at 4°C. Each aqueous layer was combined with an amount of 100% isopropanol equal to 0.5 mL for every 1 mL of TRIzol used that was split evenly among the samples. The samples

were kept at -20°C for 2 hours. After thawing the samples, the samples were centrifuged at 12,000 RCF at 4°C for 15 minutes or until pellets developed. The pellets were washed with 1 mL of 75% ethanol for every milliliter of TRIzol used split evenly among the samples by vortexing and then centrifuged at 7500 RCF for five minutes at 4°C. The ethanol was removed and the pellets were allowed to air dry. After drying, each pellet was resuspended 75 μL in RNase-free water. A 1:100 dilution of each sample was analyzed using an Ultrospec 3100. The extracted RNA was stored at -80°C.

DNase Treatment: One microgram of RNA was combined with 1 μL of 10X DNase I reaction buffer from a DNase I kit (Invitrogen) and 9 μL of nuclease-free water. One microliter of DNase I, Amplification Grade was added and the mixture was incubated for a maximum of fifteen minutes. After, a microliter of 25 mM EDTA was added and the mixture was incubated at 65°C for ten minutes. The samples were stored at -80°C.

cDNA Synthesis: cDNA was synthesized using SuperScript First Strand Synthesis System for RT-PCR (Invitrogen). Eight microliters of DNase treated RNA was combined with 1 μL of 50 μM oligo(dT) and 1 μL of 10 mM dNTP mix and incubated at 65°C for five minutes. The mixture was placed on ice until needed. Separately, with n equaling the number of reactions plus one, 2n microliters of 10X RT buffer was combined with 4n microliters of 25 mM magnesium chloride (MgCl$_2$), 2n microliters of 0.1 DTT, 1n microliters RNaseOUT and 1n microliters of SuperScript III RT and then mixed. Ten μL of this mixture was added to the mix containing the DNase treated RNA samples, which were mixed and centrifuged briefly. The samples were incubated at 50°C for one hour and then incubated for 85°C. The samples were then kept on ice for a minute followed by a brief centrifugation and a twenty minutes incubation period at 37°C. The cDNA

samples were stored at -20°C.

Real-time quantitative polymerase chain reaction: A 1:10 dilution of a combination of all DNase treated RNA samples was made. A 1:5 dilution containing equal amounts of all cDNA samples, called solution one, was made with RNase-free water (Fischer), vortexed and centrifuged. Fifteen microliters of solution one were mixed with 15 µL of RNase-free water to create solution two, which was vortexed and centrifuged briefly. Fifteen microliters of solution two were vortexed with fifteen µL of RNase-free water and centrifuged to make solution three. Fifteen microliters of solution three and 15 µL of RNase-free water were vortexed and centrifuged for a short time to make solution four. To make solution five, 6 µL of solution two was combined with 24 µL of RNase-free water, vortexed, and briefly centrifuged. Two microliters of solution 2 and 18 µL of RNase-free water were vortexed and centrifuged to make solution six.

Primers for p53, mortalin, and GAPDH (Qiagen) were used, with GAPDH acting as a reference, resulting in three separate master mixes. In the following cases, n represents number of wells in a 96 well plate to be used plus one. To make the master mix solution for the mortalin primer so that the concentration in the well was 200 nM, 5n microliters of RT^2 SYBR Green ROX qPCR Mastermix (Qiagen), 4.4n microliters of RNase-free water, and 0.2n microliters of the 10 µM mortalin primer were combined, vortexed, and centrifuged briefly. For the p53 mastermix solution, so that the concentration in the well was 400 nM, 5n microliters of RT^2 SYBR Green ROX qPCR Mastermix was combined with 0.4n microliters of 10 µM p53 primer and 4.2n microliters of RNase-free water. This mastermix was vortexed and centrifuged for a short amount of time. Finally, 5n microliters of RT^2 SYBR Green ROX qPCR Mastermix, 0.4n

microliters of 10 μM of GAPDH primer, and 4.2n microliters of RNase-free water were combined, vortexed and centrifuged to create a GAPDH mastermix solution that creates a 400 nM concentration in the wells.

To arrange a real-time quantitative polymerase chain reaction (RT-qPCR) for p53, 9.6 μL of the p53 mastermix solution was combined with 0.4 μL of each cDNA sample in duplicate. A no reverse transcriptase control was created in duplicate by combining 9.6 μL of the p53 mastermix solution with 0.4 μL DNase treated RNA. A PCR negative control was created in duplicate by combining 9.6 μL of p53 mastermix solution and 0.4 μL of double deionized water. To create a triplicate of standard curves, 9.6 μL of p53 mastermix was combined separately with solutions one through six made previously. This same process is replicated using the mortalin mastermix and the GAPDH mastermix solution. The well plate was centrifuged at 1000 RCF for three minutes. The well plate was analyzed using the ABI 7500 Fast Real-Time PCR System and the associated software. The data was then graphed and analyzed for efficiency and consistency.

Nuclear and Cytoplasmic Protein Extraction: One milliliter of SKBR3 cell suspension was centrifuged at 2000 RCF for ten minutes at 4°C, after which the supernatant was removed. The pellet was combined with 200 μL of ice-cold CER I from the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo) and vortexed. The sample was kept on ice for ten minutes, combined with 11 μL of CER II, vortexed and kept on ice for one minute. Then the sample was centrifuged for five minutes at 18,000 RCF at 4°C. The supernatant containing the cytoplasmic proteins was separated out and stored at -80°C. The pellet was resuspended in 100 μL ice-cold NER and the mixture was

vortexed. The sample was kept on ice for 40 minutes, with the sample being vortexed every ten minutes. After, the sample was centrifuged at 18,000 RCF for ten minutes at 4°C. The supernatant, which contained the nuclear proteins, was separated and stored at -80°C.

RNA Interference: A six-well plate was seeded with 3.7×10^5 cells per well. The cells were kept in 2300 µL of McCoy's 5A media at 37°C and 5 % carbon dioxide until the transfection complex was made. The transfection complex contained 150 ng of HSPA9 Silencer Select Pre-designed siRNA (Ambion, Life Technologies) dissolved in 100 µL of media mixed with 12 µL of HiPerfect Transfection Reagent (Qiagen) per well used so that when added to the cells the siRNA will be at a 5 nM concentration. The transfection complex was vortexed and incubated at room temperature for five to ten minutes. This mixture is then added to every well dropwise and the plate was swirled to evenly distribute the complexes. The cells were kept at 37°C and 5% carbon dioxide for 24 hours, after which they were used for RNA extractions and cytospins.

Results:

Cytospins analyzed using the Romanowsky stain aid in the depiction of cellular morphology by staining the nucleus dark blue or purple with the cytoplasm being stained light blue. SKBR3 cells appeared to have large nuclear regions and unique cytoplasm characteristics. For comparison, **Figure 1** shows an image of a breast cancer cell taken with an electron microscope that Dr. Charles Walker provided. Slight membrane irregularities are present because SKBR3 breast cancer cells are transformed versions of the secretory mammary cells responsible for milk production. The Romanowsky stain supported these observations **(Figure 2)**. Changes in cell morphology became more apparent as time treated with MKT-077 increased. Very little change occurred between untreated cells and the cells that were treated for two hours, with more obvious changes occurring after eight hours of treatment **(Figure 2)**, and becoming more drastic by twenty-four hours of treatment **(Figure 3)**. Changes included a decrease in overall cell size to nuclear size ratio and an increased number of membrane irregularities.
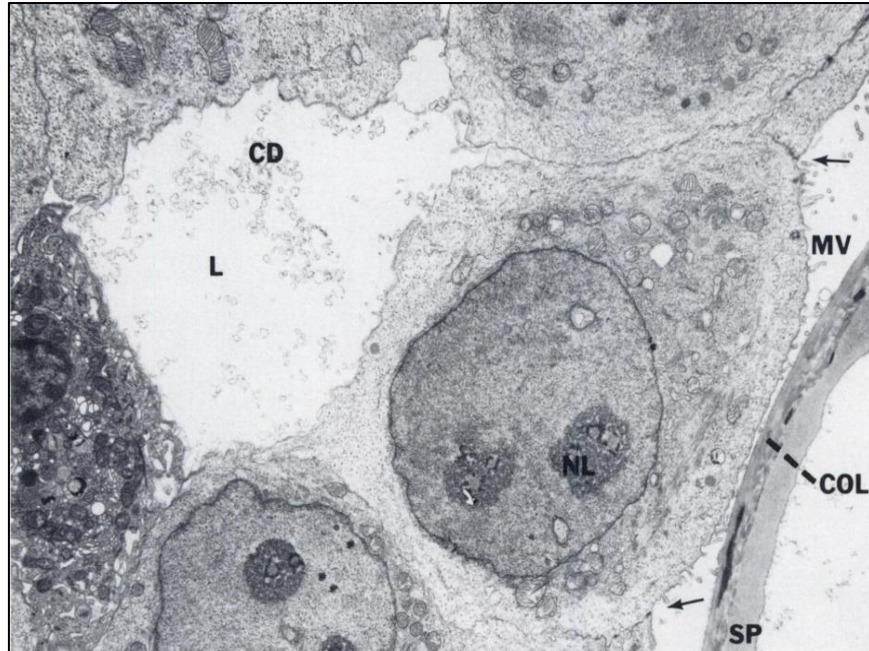
**Figure 1:** Electron microscope image of an SKBR3 breast cancer cell
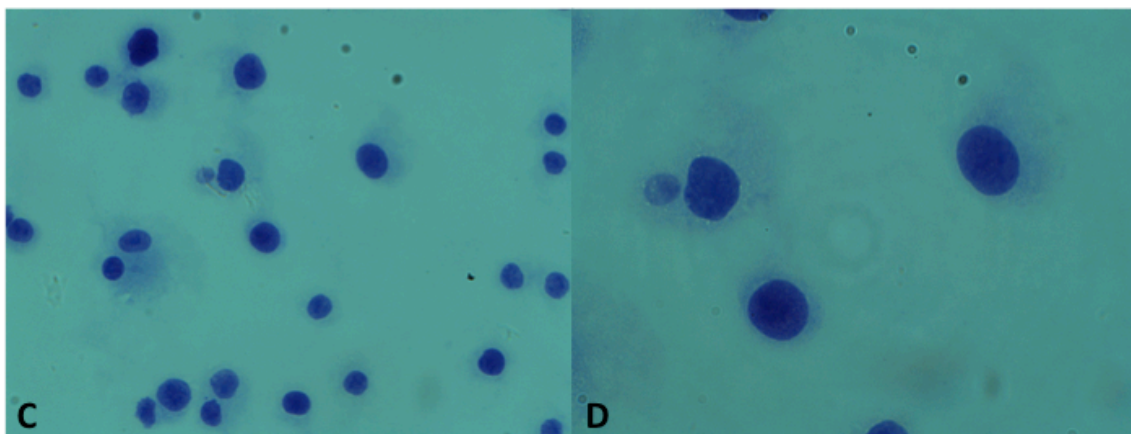
Figure 2: Images of Romanovsky stained SKBR3 cells. Figure 2A shows SKBR3 cells treated with MKT-077 for 2 hours at 200X and Figure 2B shows them at 400X. Romanovsky stained SKBR3 cells after an 8 hour MKT-077 treatments are also shown at 200X (Figure 2C) and 400X (Figure 2D).
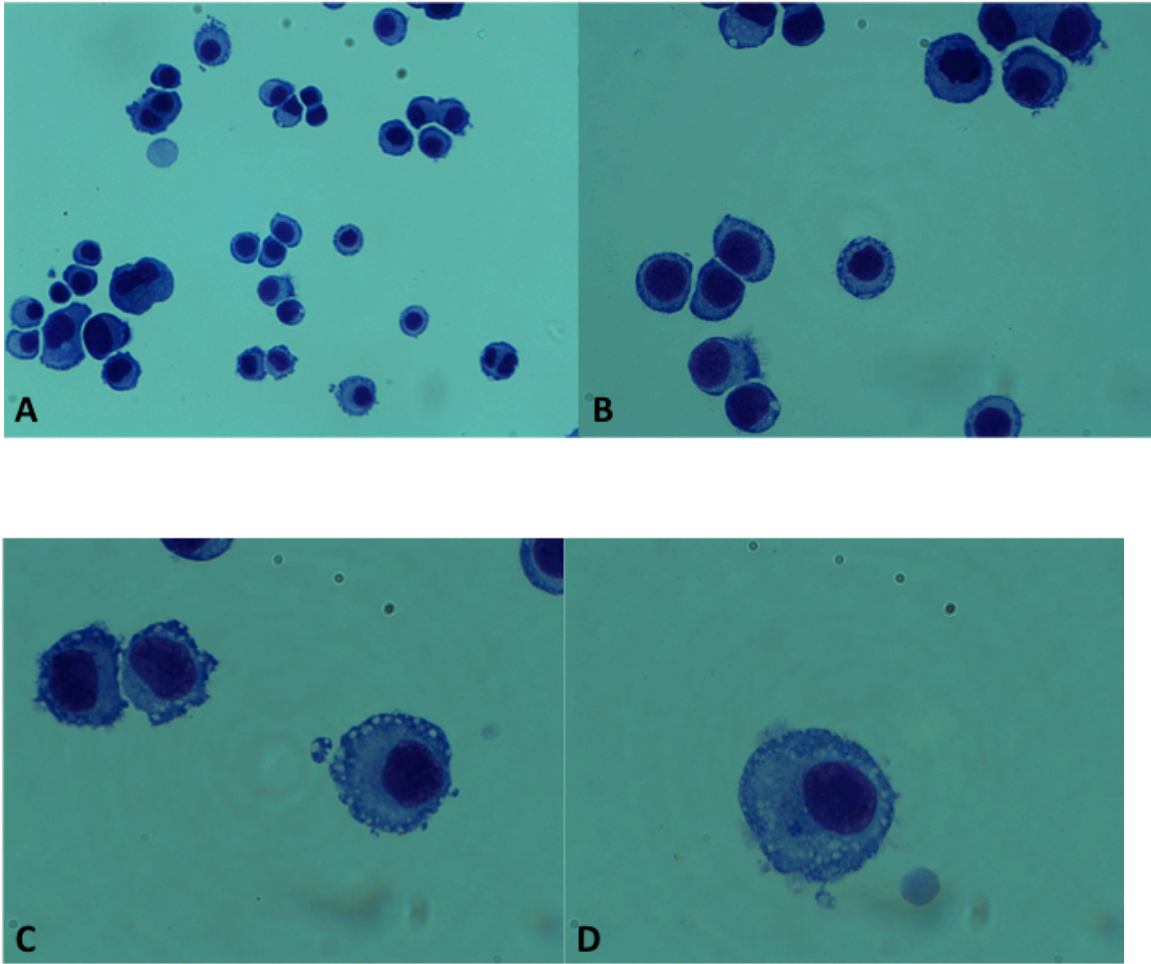
Figure 3: SKBR3 cells treated for 24 hours with MKT-077 were observed at 200X (Figure 3A) and 400X (Figure 3B) after a Romanowsky stain. These same cells are also shown at 630X (Figure 3C) with one image focusing on a single cell (Figure 3D).

Using a primary antibody that is supposed to be specific to p53, immunohistochemistry allows for the staining of a specific protein, indicating its location within a cell. The process used creates a dark brown color where the protein is localized with a yellow color marking the rest of the cell. All negative controls that did not receive primary antibody did not stain. The stain indicated p53 was localized around the periphery of the cell in the cytoplasm near to the

membrane, with little change after two hours of MKT-077 treatment **(Figure 4)**. After eight hours of treatment, distinct nuclear staining was present with diffuse staining throughout the cytoplasm, indicating p53 movement within the cell **(Figure 4)**. Membrane irregularities increased in number and severity by eight hours of treatment as well, corresponding to the membrane irregularities seen in the Romanowsky stain in **Figure 2**. By the time twenty-four hours of treatment with MKT-077 had passed, the nuclear region of the cells was very darkly stained, though some staining was present around the plasma membrane of the cells as before **(Figure 4)**. Also corresponding to the Romanowsky cells treated for 24 hours with MKT-077, membrane irregularities representing "blebbing" had increased drastically and the cell size compared to the nucleus had decreased **(Figure 4)**.
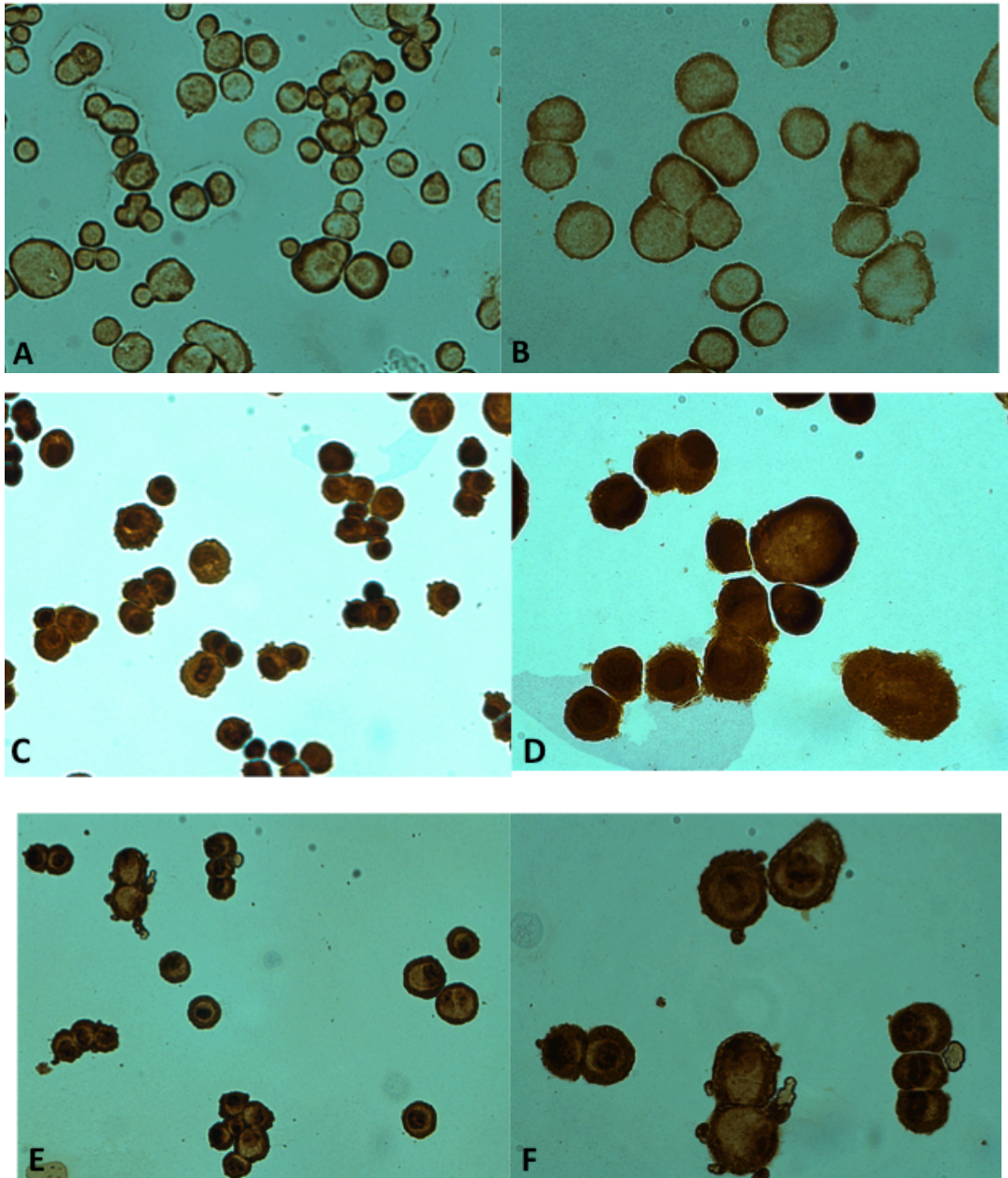
Figure 4: Immunohistochemistry stain of SKBR3 cells after 2 hours of MKT-077 treatment at 200X (Figure 4A) and 400X (Figure 4B), after 8 hours of MKT-077 treatment at 200X (Figure 4C) and 400X (Figure 4D), and after 24 hours of MKT-077 treatment at 200X (Figure 4E) and 400X (Figure 4F)

A TUNEL assay marks DNA damage in a cell, which is usually indicative of apoptosis. DNA damage is marked with a green fluorescence. A counterstain containing DAPI was used to mark the nucleus with blue-purple fluorescence. All fluorescence had to be taken in black and white, but the fluorescence light used will be indicated. The untreated cells showed DAPI fluorescence but no FITC fluorescence was present, indicating no or very low levels of DNA damage **(Figure 5)**. All samples that did not receive the rTdT enzyme acted as a negative control and did not exhibit FITC fluorescence (data not shown). FITC fluorescence was also absent from the SKBR3 cells that were treated with MKT-077 for two hours. Some FITC fluorescence appeared in the cells that had been treated for four hours. However, it is not in the nucleus where the genomic DNA is present but completely outside of it **(Figure 5)**. The cytoplasm was speckled with light FITC fluorescence. The strength of the FITC fluorescence increased in intensity and remained excluded from the nucleus after six hours of exposure to MKT-077 **(Figure 5)**. This was confirmed by comparing the FITC and DAPI fluorescence from the same group of cells. After eight and twenty-four hours of MKT-077 treatment, FITC fluorescence increased in quantity and intensity, though it remained exclusively in the cytoplasm **(Figure 6)**.
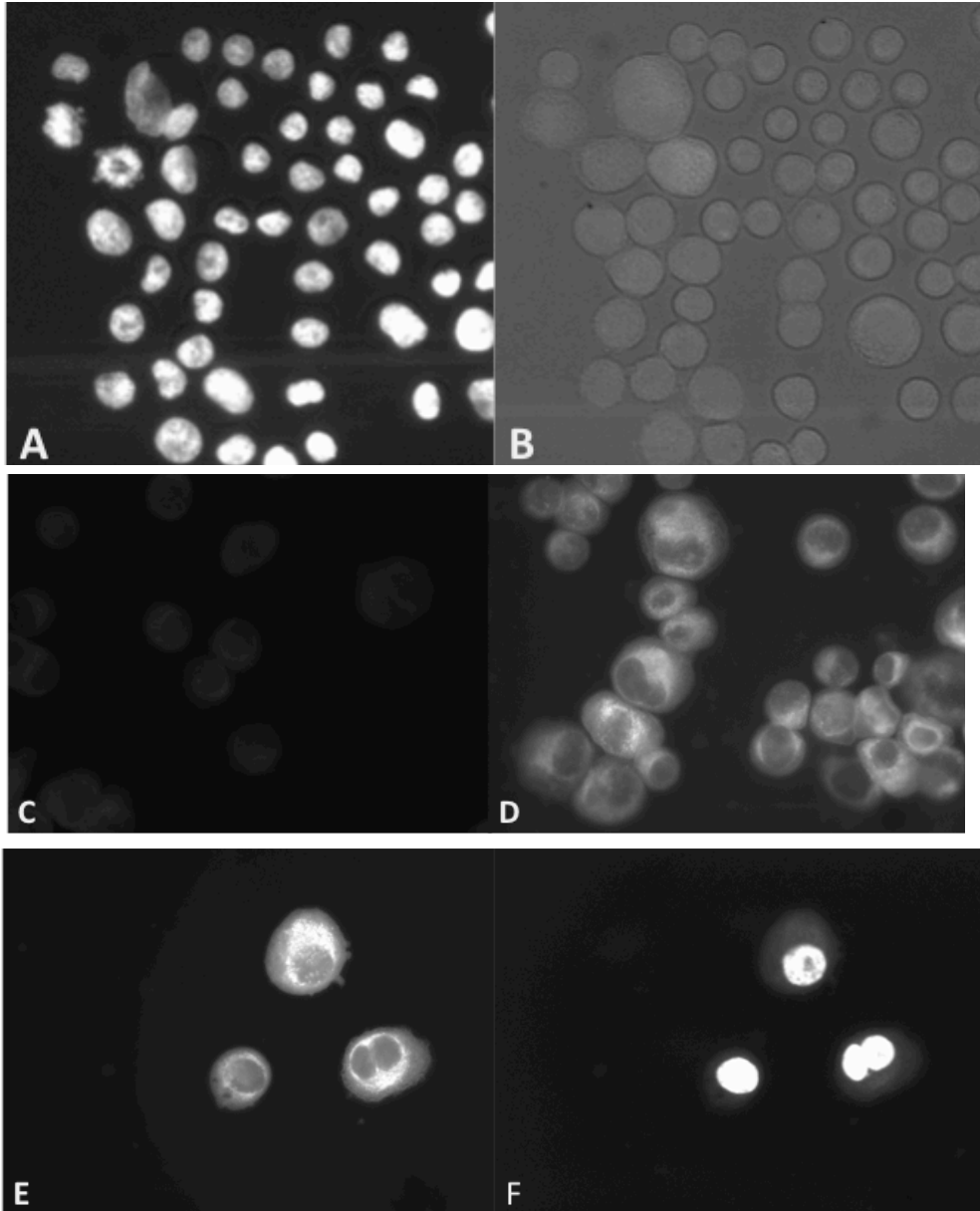
Figure 5: SKBR3 cells after fluorometric TUNEL assay and DAPI counterstain. Untreated SKBR3 cells are shown under DAPI fluorescence (Figure 5A) and FITC fluorescence, both at 200X. FITC fluorescence of SKBR3 cells at 400X after MKT-077 treatment for 2 hours (Figure 5C) and for 4 hours (Figure 5D). The same cells treated with MKT-077 for 6 hours are shown at 400X under both FITC fluorescence (Figure 5E) and DAPI fluorescence (Figure 5F).
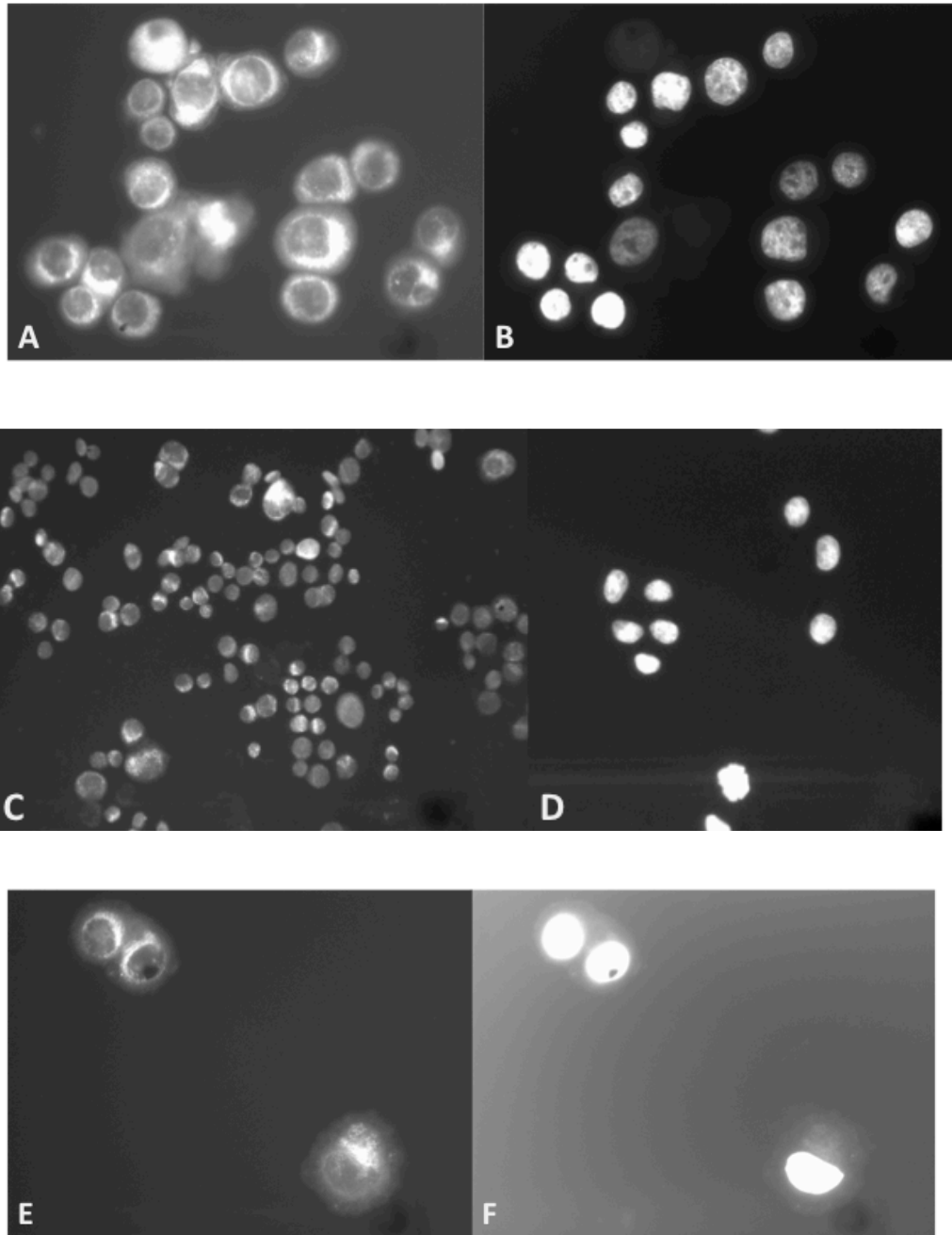
Figure 6: Fluorometric TUNEL assay on SKBR3 cells treated for MKT-077 for 8 hours at 400X under FITC fluorescence (Figure 6A) and DAPI fluorescence (Figure 6B). Cells treated for 24 hours observed under FITC fluorescence (Figure 6C) and DAPI fluorescence (Figure 6D) at 200X. Two images were also viewed of the same cells at 400X (Figure 6E and Figure 6F respectively).

The RNA extraction of the SKBR3 cells resulted in a RNA concentration of 366 µg/mL. The results of the real-time quantitative polymerase chain reaction (RT-qPCR) of the cDNA produced from the extracted RNA are still pending.

Discussion:

The Romanowsky stain indicated cell morphology changes as treatment time increased. The cells initially had large nuclei and cytoplasmic area. Their membranes are very intact with some slight membrane bubbling as the cell is undergoing the secretion process **(Figure 2)**. After eight hours of treatment, the cells have begun to shrink in size and the nuclei are less distinct, though this may be an irregularity in staining. Light blue dye was seen dissipating out from the cells, indicating potential cell degradation. After twenty-four hours, the nuclei are distinct and there are even some cells seen in the middle of cytokinesis **(Figure 3)**. The cytoplasm size to nuclear size ratio appeared to have decreased. Membrane bubbling has increased in size and in frequency. This progression of cell morphology suggests apoptotic processes were initiated by the MKT-077 treatment. This suggests that the disruption of mortalin binding allowed the cells to undergo apoptosis.

Immunohistochemistry was supposed to indicate the location of p53 in the cell. Before treatment and at low treatment times, the staining exhibited darker staining lining the interior of the plasma membrane, supposedly indicating that p53 is trapped in the cytoplasm in untreated SKBR3 cells **(Figure 4)**. As treatment time lengthens, some of the staining has moved to the nucleus, but some staining remains in the cytoplasm. Also, membrane irregularities increase in number and severity, indicating a loss of membrane integrity. According to this assay, MKT-077 treatment causes some translocation of p53 to the nucleus, which could initiate apoptosis as

indicated by the "blebbing" of the mitochondrial membrane. However, as will be addressed further later, there is some question as to whether this staining represents p53's location.

The fluorometric TUNEL assay measures DNA damage in the form of DNA breaks by tagging the breaks with a fluorescent dye fluorescin that emits green light under FITC. DAPI is used to compare the location of the FITC fluorescence to the nucleus. Fluorescence increases as the length of treatment time increases and no marked DNA damage appears before four hours of treatment **(Figure 5)**. The TUNEL assay does not indicate DNA damage in the nucleus, but rather in the cytoplasm. As the only DNA in the cytoplasm is in the mitochondria, these results suggest that in the times and concentrations used in this study, exposure to MKT-077 causes mitochondrial DNA damage. This suggests that the morphological signs of apoptosis in these cells could be caused by apoptosis activity initiated at the mitochondria.

After additional research, these changes may not be due to p53 translocation however. A later immunohistochemistry assay using an antibody from a different company indicated presence of p53 in the nucleus as well as the cytoplasm in untreated SKBR3 cells and no change in p53 location between the untreated cells and the cells that had been treated for twenty-four hours (data not shown). Also, when a western blot was performed of protein extracts from untreated cells, the antibody used did not stain at 53 kDa as expected but at about 73 kDa, though the protein that did react with the antibody appeared to be mostly in the cytoplasmic

fraction (data not shown). Also, the new antibody did not interact with the p53 standard protein. Considering this, it is impossible at this point to confirm that p53 is what is translocated into the nucleus after exposure to MKT-077. It can be said, however, that something was translocated to the nucleus after exposure to MKT-077 and that DNA damage indicative of apoptosis occurred at the mitochondria.

To elucidate this mysterious occurrence, another two western blots could be performed, staining each membrane with an individual antibody to determine which stains p53 more accurately using cellular extracts and a p53 protein standard. Also, extracted RNA was used to generate cDNA, which is in the process of being used in real-time quantitative polymerase chain reaction (RT-qPCR) to determine the expression levels of mortalin, p53, and GAPDH for comparison. From there, siRNA could be performed using a complimentary sequence to mortalin RNA to silence mortalin expression. Then RT-qPCR and the various staining techniques could be used to indicate changes in mortalin and p53 expression and whether this results in apoptosis in theses cells.

References:

1. Kaul, S., S. Aida, T. Yaguchi, K. Kaur, and R. Wadhwa. Activation of wild type p53 function by its mortalin-binding, cytoplasmically localizing carboxyl terminus peptides. Journal of Biological Chemistry 2005; 280(47): 39373-39379.

2. Kelley, M., P. Winge, J. Heaney, R. Stephens, J. Farell, R. Van Beneden, C. Reinisch, M. Lesser, C. Walker. Expression of homologues for p53 and p73 in the softshell clam (*Mya arenaria*), a naturally-occurring model of human cancer. Oncogene 2001; 20: 748-758.

3. Böttger, S., E. Jerszyk, B. Low, and C. Walker. Genotoxic stress-induced expression of p53 and apoptosis in leukemic clam hemocytes with cytoplasmically sequestered p53. Cancer Research 2008; 68(3): 777-782.

4. Wadhwa, R., S. Takano, K. Kaur, C. Deocaris, O. Pereira-Smith, R. Reddel, and S. Kaul. Upregulation of mortalin/mthsp70/Grp75 contributes to human carcinogenesis. International Journal of Cancer 2006; 118: 2973-2980.

5. Walker, C., S. Böttger, and B. Low. Mortalin-based cytoplasmic sequestration of p53 in a nonmammalian cancer model. American Journal of Pathology 2006; 168(5): 1526-1530.

6. Grover, A., D. Priyandoko, R. Gao, A. Shandilya, N. Widodo, V. S. Bisaria, S. Kaul, R. Wadhwa, D. Sundar. Withanone binds to mortalin and abrogates mortalin-p53 complex: computational and experimental evidence. The International Journal of Biochemistry and Cell Biology

7. Koya, K., Y. Li, H. Wang, T. Ukai, N. Tatsuta, M. Kawakami, T. Shishido, and L. Bo Chen. MKT-077, a novel rhodacyanine dye in clinical trials, exhibits

anticarcinoma acticivity in preclinical studies based on selective mitochondrial

accumulation. Cancer Research 1996; 56:538-543.