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ROLE OF AUTOPHAGY IN THE RESPONSE OF HS578T BREAST TUMOR CELLS TO RADIATION

Shweta Chakradeo
Virginia Commonwealth University

ROLE OF AUTOPHAGY IN THE RESPONSE OF HS578T BREAST TUMOR CELLS TO

RADIATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of

Science at Virginia Commonwealth University

By

Shweta Moreshwar Chakradeo

Bachelor of Pharmacy, University of Mumbai

Director: David A Gewirtz, PhD

Professor, Department of Pharmacology & Toxicology

Virginia Commonwealth University

Richmond, Virginia

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LIST OF ABBREVIATIONS

IR Irradiation

ROS Reactive Oxygen Species

LC3 Light Chain 3

1, 25 D3

DAPI 6- Diamidino 2- Phenylindole

TUNEL Terminal Deoxynucleotidyl transferase dUTP nick end labeling

AO Acridine Orange

AVO Acidic Vesicular Organelle

CQ Chloroquine

3MA 3-Methyladenine

BAF Bafilomycin A

FACS Fluorescence activated cell sorting

ABSTRACT

ROLE OF AUTOPHAGY IN THE RESPONSE OF HS578T BREAST TUMOR CELLS

TO RADIATION

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Breast cancer is the most commonly observed cancer type in women and is the second leading cause of cancer death in women. Radiation can be used to debulk tumors prior to surgery as well as to treat patients after surgery and/or chemotherapy. Previous studies from our laboratory have shown that the anti –malarial drug chloroquine sensitizes breast cancer cell lines to radiation by suppression of autophagy which is a conservative catabolic process that can be cytoprotective. The scientific literature has demonstrated

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that many tumor cell systems undergo cytoprotective autophagy and that pharmacological or genetic inhibition of autophagy leads to other modes of cell death such as apoptosis. Acridine orange staining was used for determination of acidic vacuole formation, an indication of autophagy and DAPI/TUNEL staining was used to identify apoptotic cells. Our studies in Hs578t breast tumor cells show the lack of sensitization by chloroquine upon autophagy inhibition with minimal apoptosis when cells are treated with $5 \times 2Gy$ radiation. The extent of apoptosis was not increased upon autophagy inhibition by Chloroquine as determined by DAPI/TUNEL assays and quantified by Flow Cytometry using AnnexinV/PI. The potential role of senescence in the effects of radiation in the Hs578t cells was determined with the use of β-Galactosidase dye staining for senescence. It appears from these studies that autophagy need not to be cytoprotective in all breast cancer cell lines. Additional studies are in progress to effort to identify the factors that might distinguish between cytoprotective and non-cytoprotective autophagy.

SECTION 1: INTRODUCTION

1.1 Cancer

Cancer is a leading cause of death and accounts for about 13% of deaths worldwide (2008). (who.net). Treating cancer is very difficult due the existence of multiple mechanisms of resistance; in addition, tumors may metastasize to sites where radiation might not be practical and where drugs may have difficulty in gaining access. There are no prominent symptoms exhibited by many types of the cancers at their early stages, which makes them difficult to diagnose before the disease has metastasized.. The risk of developing cancer increases with age and about 77% of diagnosed cancers are in individuals 55 years and above. (cancer.org)

Cancer arises from abnormal growth of cells that have mutations in genes which control cell proliferation and survival. Both oncogenes and tumor suppressor genes can contribute to the development of cancer. Over expression of the oncogene, Her2/Neu, can cause cell transformation and plays an important role in tumorigenesis. The tumor suppressor gene p53 is essential in controlling abnormal cell proliferation and maintaining genomic integrity (Zhu & Bai, 2006, Vousden, 2002). Over half of the tumors examined show mutations in p53, a tumor suppressor gene (Tripathi et al 2004, Weinberg et al, 2000).

Tumors can be malignant or benign. Benign tumors do not invade other tissues and are rarely life threatening (Choe et al, 2009). Benign tumors are localized (Choe et al, 2009) whereas malignant tumors invade into other tissues and furthermore frequently have the

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capacity to metastasize and grow at distant sites (Holland-Frei Cancer Medicine. 5th edition).

1.2 Cancer Statistics

Excluding skin cancer, breast cancer is the most common malignancy among women, accounting for nearly 1 in 3 cancers diagnosed among women in the United States. (DeSantis, 2011). About 1 in 8 U.S. women will develop invasive breast cancer during her lifetime (U.S. Breast cancer statistics 2012). Breast cancer is the second leading cause of death in women in the United States.

1.3 Breast Cancer

Breast cancer is the leading cause of death in women in not only developed countries but also in developing countries (Porter, 2012). There are two forms of breast cancer which are commonly found, which are ductal and lobular. Ductal carcinoma is formed in the lining of a milk duct within the breast which carries milk from the breast to the nipple. Lobular carcinoma starts in the lobules of the breast where milk is formed (Mayo Clinic 2012). Approximately 70-75% of breast cancers are estrogen receptor positive, and which are therefore likely to respond to hormone therapy (Wax, 2010). Approximately 12-17% of women with breast cancer have triple negative type cancer. Triple negative breast cancer cells do not express the estrogen receptor, progesterone receptor or the Her2 gene. Triple negative cancers are aggressive and have a very poor prognosis. Triple negative breast cancers cannot be treated with hormone therapy or targeted therapy (Her2 targeted therapy) (Reis and Filho, 2010). BT-549, Hs578t and HCC1806 are examples of triple

negative breast cancer cell lines (atcc.org). The studies presented in this thesis were performed in Hs578t cells which are also mutant in p53 (Choi et al, 2010).

1.4 Current Treatments

Breast cancer is generally treated with surgery, chemotherapy and radiation therapy often in combination with hormonal therapy.

Surgery: Surgery is used as the primary treatment when cancer is localized. Surgeons can perform either a lumpectomy or mastectomy, depending on the progression of the disease. With a lumpectomy, only the tumor is removed with a small amount of surrounding tissues whereas with a mastectomy, removal of all the breast tissue is involved. Almost all patients with breast cancer have some therapeutic options for their surgery. (Dooley WC 1998). Surgery is the choice for treatment when the tumor is localized and has not metastasized although surgery is necessary to remove the primary tumor even when metastasis is likely to have occurred.

Chemotherapy: In chemotherapy, anti-neoplastic agents are used to treat cancer. Chemotherapeutic drugs generally are most effective against rapidly dividing cancer cells. However, resistance development is one of the major limitations of chemotherapy. (Longley et al, .2008). Chemotherapy is utilized in order to weaken and destroy cancer cells at the original site as well as cancer cells that may have travelled to other parts of the body. Cytotoxic chemotherapy in both advanced and early stage breast cancer is well-established. Depending on the molecular basis of cancer, chemotherapy can be personalized (Hussain SA 2010). Commonly used chemotherapy drugs in breast cancer are Taxol, Adriamycin, 5- Fluorouracil and Cyclophosphamide.

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Hormonal Therapy: Estrogen is the principal hormone involved in the development of breast tumors. (Smith & Dowsett, 2003). Hormone therapy is widely used to treat estrogen receptor positive breast cancer. If breast cancers are estrogen receptor positive, they can be treated with hormonal therapy in two ways, either by lowering levels of estrogen in the body or by blocking actions of estrogen on breast cancer cells. (American Cancer Society 2012). Two types of drugs are widely used to treat breast tumors by hormone therapy. They are Tamoxifen and Aromatase inhibitors. Tamoxifen inhibits estrogenic effects by acting as a competitive antagonist for estrogen at the estrogen receptor site (Smith & Dowsett, 2003). Aromatase inhibitors decrease estrogen synthesis in the breast tumor by inhibiting the aromatase enzyme that is responsible for the conversion of androgen to small quantities of estrogen in post-menopausal women (Smith & Dowsett, 2003).

Targeted Therapy: Targeted therapy is the most advanced approach to treat breast cancer. One of the agent which is widely used in such therapy is Trastuzumab which is the first approved targeted anti-cancer drug used in treating patients with HER-2 positive breast cancer. (Finn et al , 2012). Trastuzumab is a humanized monoclonal antibody which is capable of significantly improving recovery from metastatic breast cancer. Trastuzumab is generally given with conventional chemotherapeutic drugs such as Paclitaxel (Blackwell, 2008). Another targeted therapy approach involves anti angiogenic compounds (Current Pharmaceutical Design, Vol 13 Issue 5) targeting growth factors which are important component of cancer growth as well as invasion and metastasis. Bevacizumab is a humanized monoclonal antibody against the pro-angiogenic factor, which is vascular endothelial growth factor (VEGF) (Kamby et al; 2010). Bevacizumab

was approved by FDA to use in breast cancer treatment but it is no longer used as it did not show any significant effect and possesses risk of some serious side effects (FDA, 2011).

Radiation Therapy: Radiation therapy is a highly effective way to shrink tumors by killing cancer cells (American Cancer Society, 2012). Radiation therapy uses high power X-rays, particles or radioactive seeds to eliminate cancer cells. (ncbi.nlm.nih.gov). Ionizing radiation can cause loss of integrity of DNA and produces a wide variety of DNA damage (Masuda and Kamiya 2012). Patient can receive radiation externally by the use of a radiation beam or internally by delivering radiation sources inside the body (American Cancer Society, 2007).

Like chemotherapy, radiation therapy is toxic not only to the tumor, but to surrounding healthy cells. To reduce this toxicity, patients receive smaller, fractionated doses of radiation; fractionated radiation also serves to increase the likelihood that cancer cells are exposed to radiation at the points in the cell cycle when they are most vulnerable to DNA damage. Patients who receive most types of external-beam radiation therapy may receive one dose (a single fraction), or multiple doses of radiation a day, up to 5 days a week for several weeks. Patients generally receive a total of between 40Gy to 60Gy in 2Gy doses. (American Cancer Society, 2010).

Exposure to ionizing radiation (IR) induces single strand breaks (SSB), base damage and DNA- proteins cross links in the genomic DNA. (Balajee et al 2010). Among these, DNA double strand breaks (DSBs) are considered the most lethal form of DNA damage. They can be introduced by exogenous agents such as ionizing radiation (IR), topoisomerase poisons, radiomimetic drugs (e.g. bleomycin), and by cellular processes such as V (D) J

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recombination. (Miller 2009, Povirk 2006, Helleday 2007). DNA damage caused by ionizing radiation can either be direct or indirect. Indirect damage is caused due to ionization of atoms which leads to formation of hydroxyl (OH) or O_2 radicals (Hall & Giaccia, 2006).

1.5 Apoptosis

The term apoptosis was first introduced by Kerr, Wyllie and Currie in 1972. Apoptosis is a process of programmed cell death which is characterized by distinct morphological features such as membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation at 3'-OH terminal (Potten et al, 2001). Apoptosis is a normal physiological process and it plays an important role in embryogenesis and ageing (Debatin et al, 2006). Apoptosis can be activated by DNA damage, stresses such as γ-radiation or cytotoxic chemotherapy (Debatin et al, 2006).

The apoptosis process can follow one of the two main pathways, either Intrinsic or Extrinsic. Both pathways eventually lead to activation of caspases (Elmore, 2007). Caspases are the main components of the apoptosis process. So far, at least 14 caspases have been identified out of which 11 are found in humans cells (Shi, 2002). Caspases are generally divided into two types, initiators and effectors. Initiator caspases include Capases-2, -8, -9 whereas effector caspases include Caspase-3, -6, -7 (Shi et al, 2002). Initiator caspases are responsible for cleavage and activation of effector caspases. Effector caspases further break down different proteins which results in apoptosis (Kaufmann et al, 1999). Effector caspases such as Caspase-3, -6, -7 are capable of self-activation (Kaufmann et al, 1999).

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Extracellular death ligands such as FasL are responsible for activation of the extrinsic pathway. The death ligand FasL binds to death receptor such as Fas (Shi et al, 2002). Binding of death ligands to death receptors leads to formation of Ligand-Receptor complexes which further induces activation of cytosolic factors in action such as FADD and Caspase-8. Activation of Caspase-8 further activates Caspase-3 cleavage (Hengartner 2000, Shi et al, 2002).

The intrinsic Pathway is mediated by mitochondria. In the response to apoptotic stimuli, many proteins are released from the intermembrane space of mitochondria (Wang, 2001, Shi et al, 2002). BAX and BID are pro-apoptotic proteins which bind to the outer membrane of mitochondria and signal to release the inner content. Upon such release, cytochrome C forms a complex with ATP and Apaf 1 which in turn releases Caspase-9 (Debatin et al, 2006). The complex of Cytochrome C, Apaf-1 and ATP is called as Apoptosome which initiates caspase-3 cascade activation (Elmore, 2007).

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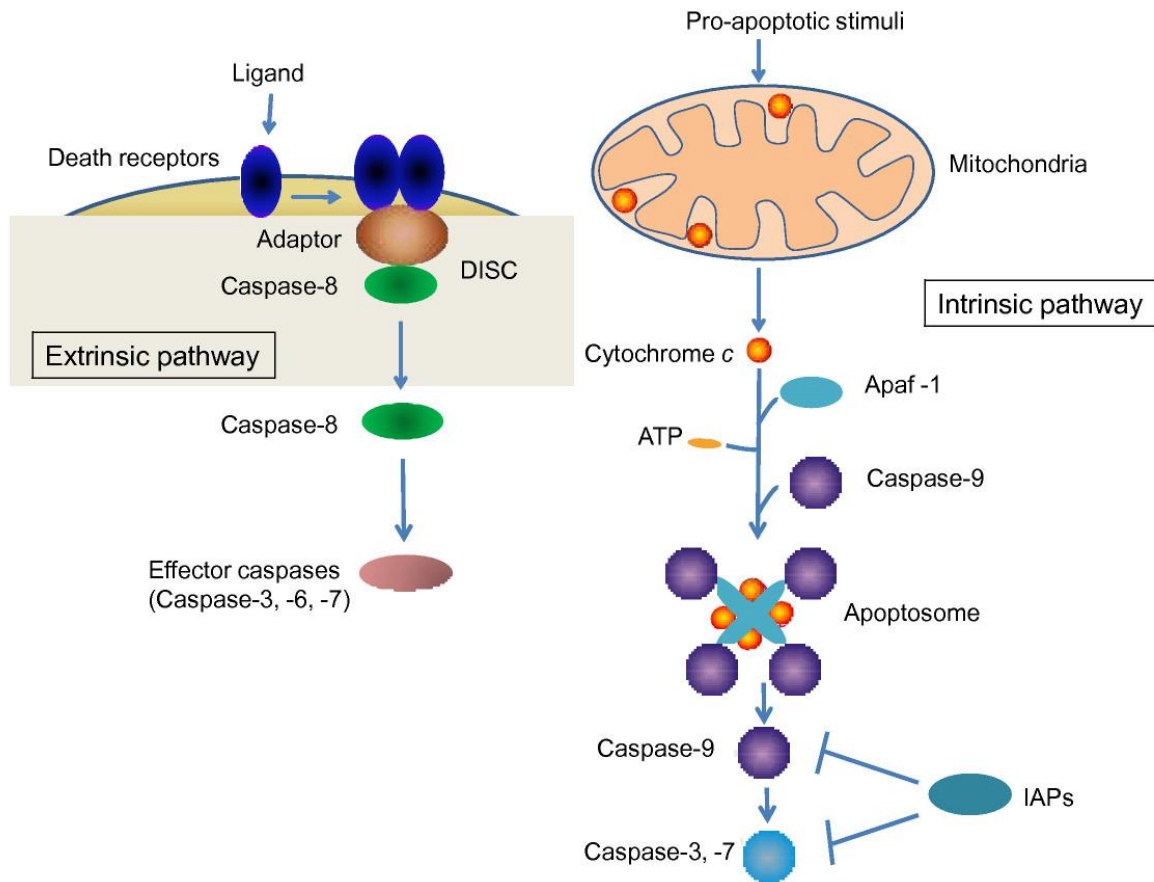
Figure 1.1 Extrinsic & Intrinsic Apoptotic Pathways (molecularbrain.com)

1.6 Necrotic Cell Death

Cell death is thought to occur primarily by two different modes, Apoptosis and Necrosis. Apoptosis is a programmed cell death whereas Necrosis is an unordered and accidental form of cell death (Farber, 2002). Necrotic cells exhibit different morphological features than that of apoptotic cells such as plasma membrane rupture, spillage of intracellular proteins and dilation of cytoplasmic organelles (Cookson et al, 2005). Induction of immune amplification is often shown by necrotic cells, which is in contrast to apoptotic cells (Kroemer et al, 2008).

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It was thought for a long time that necrotic cells do not exhibit any markers but in recent literature many markers have been shown to play a role in necrosis. RIP1, which is a Serine/threonine kinase receptor- interacting protein, is thought to play a role in initiation of necrosis (Vandenabeele et al, 2007). Irrespective of the stimuli, ROS (reactive oxygen species) and calcium play an important role in necrosis. ROS initiates DNA damage, loss of lipids and proteins which further causes loss of membrane integrity (Vandenabeele et al, 2008).

Death receptors such as the TNF Receptor-1 (TNF- RI) have shown the ability to initiate caspase-independent cell death. This form of necrotic death is due to the generation of ROS. This generation of superoxide formation is dependent on the NADPH oxidase which forms complexes with the adaptor molecules such as TRADD (Liu et al, 2008).

1.7 Mitotic Catastrophe

Mitotic catastrophe is generally defined as an abnormal form of mitosis which involves many morphological and biochemical changes leading to cell death (Zhivotovsky, 2008). Mitotic catastrophe is characterized by some distinct morphological features which are mainly micronucleation or multinucleation. Micronucleation is generally a product of faulty chromosome fragmentation. These fragments are the result of uneven distribution of chromosomal material between daughter nuclei. Multinucleation can result due to abnormal karyokinesis. (Vakifahmentoglu et al., 2008, Galluzzi et al., 2011). Biochemical signals which are involved in mitotic catastrophe are yet not clear but there are some processes which are activated for this phenomenon, which includes 1) activation of caspase-2, (Zhivotovsky et al., 2008, Galluzi et al., 2011) 2) long lasting

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activation of SAC (Spindle- assembly checkpoint) (Galluzzi, 2011) which prevents anaphase causing chromosome missegregation, 3) High levels of cyclin B1, (Galluzzi et al., 2011) and 4) induction of TP53 (Tumor Suppressor protein activity) (Vitale et al., 2011, Galluzzi et al., 2011). There are at least two subtypes of mitotic catastrophes. The first subtype occurs in a p53 independent manner where cells die during or near metaphase. In the second subtype, failed mitosis can lead to activation of mitotic catastrophe during activation of the polyploidy check point. This is partially p-53 dependent. (Galluzzi et al., 2011). It is still unclear if mitotic catastrophe requires caspase activation exclusively.

1.8 Senescence

In 1961, Hayflick first described the fundamental cell response of cellular senescence (Stewart et al, 2008, Hayflick & Moorhead., 1961). Replicative senescence is the phenomenon which prevents normal cells from multiplying indefinitely (Campisi,. 2000). Senescence is also induced by different stresses such as DNA damage by radiation (Stress induced, premature or accelerated senescence) and telomerase shortening (Ben-Porath, 2004). In stress induced senescence, one form of stress can be in the form of oncogene induces stress. Oncogenes can induce and initiate senescence which is referred to as 'Oncogene induced senescence' (Collado and Serrano, 2006). Activated oncogenes such as H-Ras play an important role in oncogene induced senescence (Neil et al, 2008). H-Ras is responsible for inducing cell cycle arrest when they are introduced alone. Oncogene induced senescence is dependent on the activation of the Raf-1/MEK/p38MAPK pathway (Mason, Jackson and Lin, 2004).

Senescent cells show a typical 'Senescent Phenotype' which involves changes in gene expression, growth arrest and generally resistance to cell death signals (Campisi., 2007).

Senescence growth arrest is regulated primarily by two tumor suppressor proteins, p53 and pRb. In the p53 pathway, where p53 is induced by stimuli such as ionizing radiation, activation of p53 is achieved by ATM/ATR and Chk1/Chk2 proteins (Ben- Porath, 2005). p21 is a very crucial protein in this pathway, acting as a transcriptional target of p53 and as mediator of p53 induced senescence. Another pathway involves p16 and phosphorylation of Rb and E2F expression. These two pathways work partially together but can also work independently as indicated in the review by (Campisi., 2007).
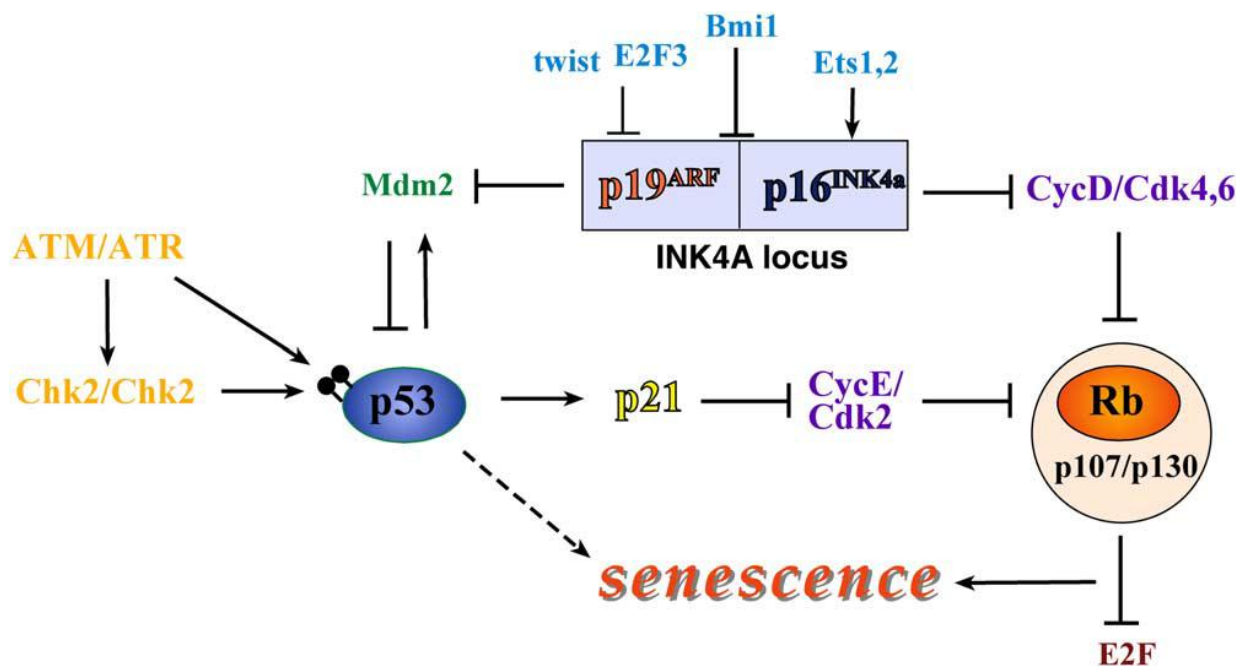
Figure 1.2 Senescent Pathways: Regulated mainly by p53, p21, Cdk2. (Ben- Porath, 2005).
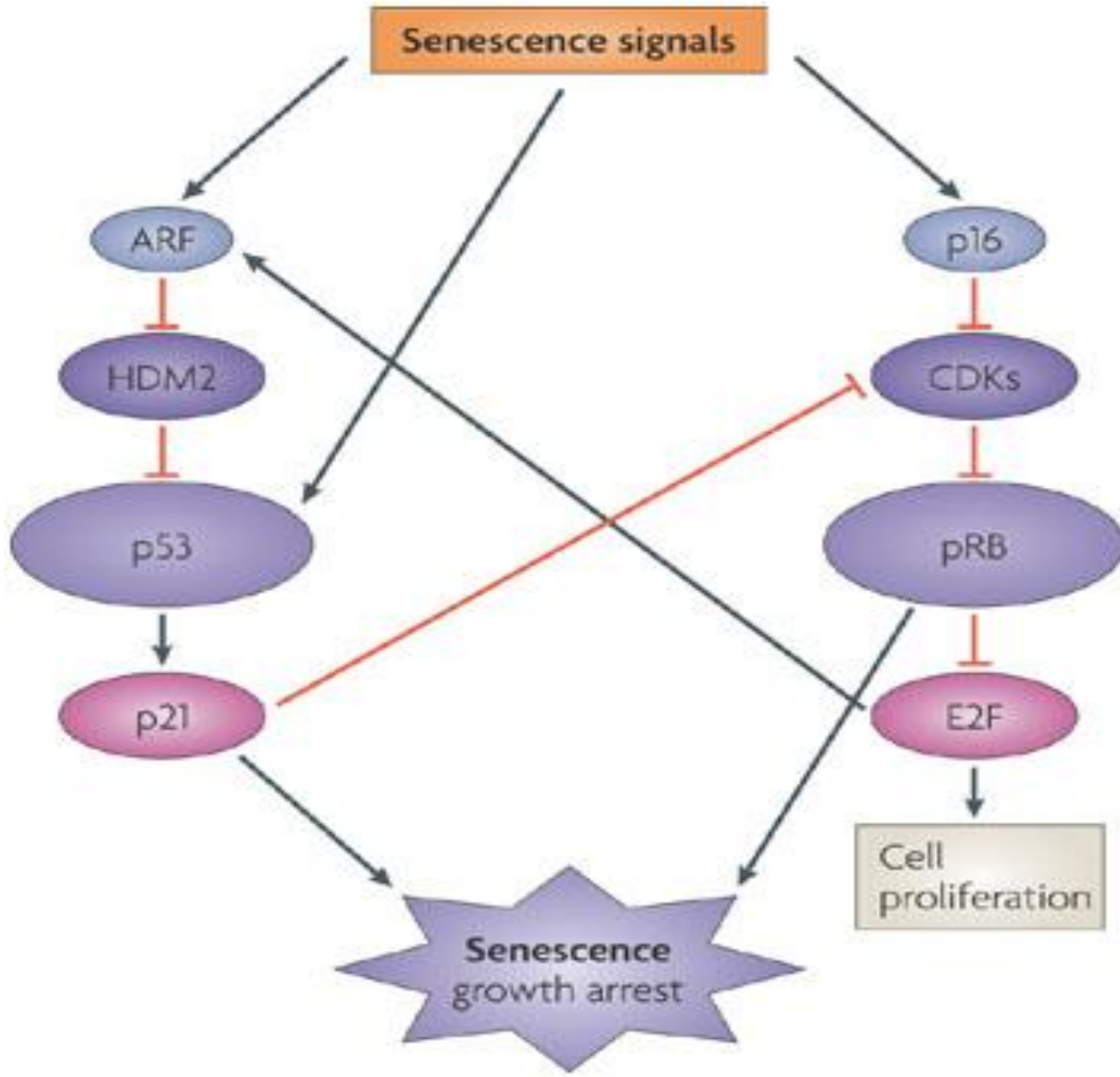
Nature Reviews | Molecular Cell Biology

Figure 1.3 Senescent Pathways: Regulated mainly by p53, p21, Cdk2. (Campisi et al, 2006)

1.9 Autophagy

Autophagy, a process that occurs in all eukaryotic cells is responsible for long lived proteins degradation as well as organelle turnover (Wen Wang and Klionsky, 2003). Autophagy is an evolutionary conserved process in which cellular organelles are

degraded. They are further reused and thus energy is conserved (N. Chen and Karantza-Wadsworth, 2009). Autophagy begins with separation of a portion of the cytoplasm and cell organelles by a unique isolation membrane. This isolation membrane extends itself to form a double membrane 'Autophagosome'. The autophagosome further fuses with lysosome to form a single membrane structure the 'Autophagolysosome'. Lysosomal ezymes are responsible for degradation of the content of the autophagolysosome (Mizushima, 2007). Multiple cellular and molecular events are required for the autophagy process. Several autophagy proteins are required for elongation and formation of the autophagic membrane. Upon various cellular stresses such as DNA damage or nutrient deprivation autophagy is activated. Various autophagy related genes (ATGs) are required for activation and elongation of the autophagosome. One of the protein which plays important role is Light chain protein (LC3) which is required for the stabilization of the autophagosome. Upon autophagy activation, LC3 I is converted to LC3 II by E-1 like enzymes ATG7 (Tanida et al., 2008). Another ubiquitin- binding protein, p62, plays an important role in the autophagy process. p62 binds to LC3 and is degraded, which indicates autophagic flux. Thus, upon autophagy induction p62 levels are decreased (Johansen et al., 2009). Upon the formation of the autophagolysosome (after fusion of autophagosome and lysosome), cellular contents are degraded and are used as substrates for synthesis of amino acids which are further used for the synthesis of important macromolecules (Geng and Klionsky, 2008).
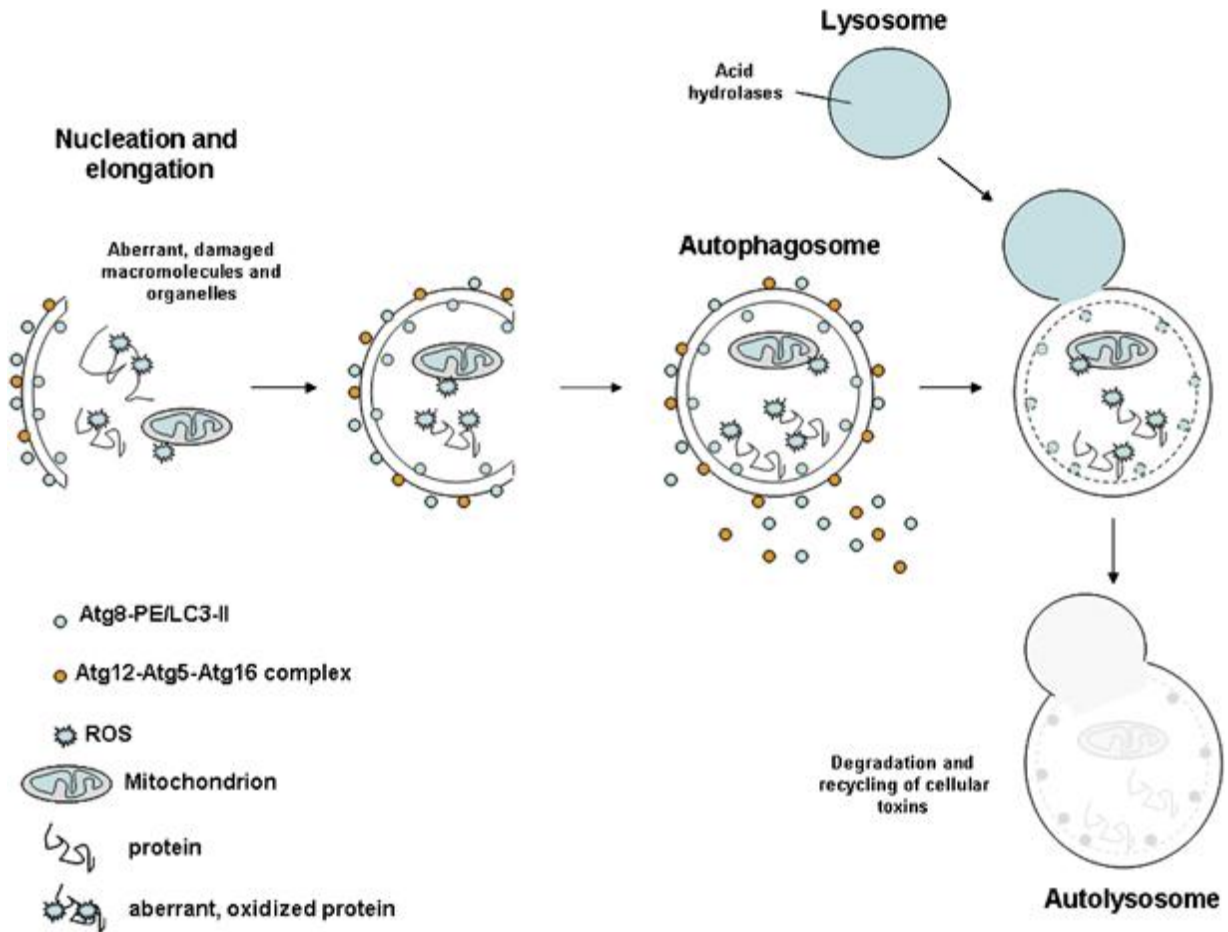
Figure 1.4 Autophagy process: Reference: T. Vellai (Autophagy genes & Ageing)

Autophagy is generally thought to be a cell survival mechanism. Upregulation of autophagy can work as a tumor cell survival mechanism under conditions such as stress (Eskelinen, 2011). There is also data which shows that autophagy also can lead to cell death when apoptosis is defective (Kroemer et al., 2005). Autophagy is induced upon radiotherapy and upon treatment with various cytotoxic agents (Koukourakis et al, 2009) and can act as mode of cell death or as a mechanism of resistance (Gewirtz et al, 2008). In order to demonstrate that autophagy is mediating cell death, prevention of death must be shown using pharmacological or genetic inhibition of autophagy (Shen et al, 2011).

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1.10 Previous Studies

Previously, our laboratory has demonstrated that vitamin D can enhance the response to radiation and analogues of vitamin D can be used to sensitize cells to radiation (Demasters et al, 2006). Radiosensitization caused by vitamin D was not mediated through apoptosis or mitotic catastrophe; instead autophagy was likely the mode of sensitization. (Demasters et al, 2006). In a recent paper, our laboratory has demonstrated the existence of both cytoprotective and cytotoxic autophagy in the same experimental system where autophagy induced by radiation alone was cytoprotective and upon treatment with radiation + vitamin D, cytotoxic autophagy mediated the effects in ZR-75-01 breast cancer cells (Wilson et al, 2012).

Although it has been shown that both MCF-7s and ZR-75-01 breast tumor cells can be sensitized to radiation upon autophagy inhibition, it is not necessarily the case that, all breast cancer cells exhibit sensitization upon autophagy inhibition. Studies performed on 4T1 cells in our laboratory established that genetic or pharmacological autophagy inhibition did not increase radiation sensitivity in vitro or in vivo (Bristol et al, 2012, under revision in press).

Recently the targeting of cytoprotective autophagy in order to overcome resistance has become a potential therapeutic approach (Kondo et al, 2005). A number of studies have been performed in cell culture and animal models in order to enhance sensitivity to treatment and clinical trials are in progress (Amaravadi et al, 2007, Jiang et al, 2010).

In the current work, all studies have been performed on Hs578t cells, which are a model of triple negative breast cancer. In previous studies of the effects of radiation on colony

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formation, Hs578t cells were established to be significantly less sensitive to radiation than MCF-7 cells (Bristol et al, 2012). Upon exposure to either radiation alone or 1,25 D3 alone, Hs578t cells did not undergo apoptosis; however, induction of apoptosis was observed when cells were treated with the combination of radiation and 1,25 D3. Autophagy induction as well as induction of autophagic flux was shown using acridine orange staining and p62 degradation respectively upon radiation (Bristol et al, 2012). Autophagic flux can be an indicative of completion of autophagy.

1.11 Hypothesis

Since preliminary studies demonstrated that autophagy was occurring in Hs578t cells, the current work was designed to determine if radiation induced autophagy is cytoprotective or cytotoxic. In cytoprotective autophagy, cell viability is reduced when autophagy is inhibited (Chiou et al, 2012, Bristol et al, 2012, Wilson et al, 2012). To evaluate if autophagy is mediating the cytotoxic effects of treatment, upon inhibition of autophagy cell number should be restored and cells should recover proliferative capacity. Interference with cytotoxic autophagy should be able to reduce radiation sensitivity (Wilson et al, 2012)

The following hypotheses were proposed:

1. Cytoprotective autophagy is a general response of breast cancer cells to radiation.

2. Inhibition of autophagy will sensitize breast tumor cells to radiation.

The following aims were established:

- To study if irradiation induces autophagy in Hs578t cells

- To evaluate if Hs578t cells are radiosensitized upon autophagy inhibition

- To determine what modes of growth arrest or cell death might be involved in the response to radiation in Hs578t cells.

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SECTION 2: MATERIALS AND METHODS

2.1 Materials

MEM Alpha medium containing L-Glutamine was obtained from Invitrogen (Grand Island, NY) Trypsin-EDTA (0.25% Trypsin, 0.53mM EDTA- 4Na) was obtained from Fisher. Fetal Bovine Serum was purchased from Hyclone Laboratories (Logan, UT). TUNEL Assay reagents (terminal transferase, reaction buffer, and $CoCl_2$ and fluorescein dUTP) were purchased from Roche (Manhein, Germany). Annexin PI and Propidium Iodide were purchased from BD Biosciences (San Diego, CA). Trypan Blue, Acetic Acid, Acridine Orange, Formaldehyde and DAPI reagent (6- Diaimidino 2- Phenylindole), were purchased from Invitrogen (Eugene, Oregon, USA). PBS (pH 7.4) was purchased from Gibco (Life Technologies, NY USA). X-Gal (5- bromo-4- chloro-3- for β-Galactosidase staining assay was purchased from Fermentas Life Sciences (Made in EU).

2.2 Cell Lines

Hs578t mammalian breast tumor cells were obtained from ATCC and kept under liquid nitrogen in 10% DMSO (Sigma Chemicals, St. Louis, MO) with 10% Fetal Bovine Serum (Serum Source International, Charlotte NC USA) until use.

2.3 Cell Culture & Treatment

Hs578t cells were grown from frozen stock in MEM-Alpha supplemented with 20% FBS and penicillin/streptomycin (0.5ml/100ml medium). Cells were cultured in T75 flasks (Cellstar) and were maintained at 37^0C under a humidified 5% CO2 atmosphere. Cells

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were passed at 80% confluence after washing once with 1X PBS (Gibco) harvested with 0.25% Trypsin-EDTA for 4-5 minutes and then deactivated with complete MEM Alpha medium. Cells were collected and centrifuged at 2000 rpm for 5 minutes (Centrifuge 5810 R ^{G}18.0). Medium was aspirated and fresh medium was added to the cell pellet. Cells were suspended in the media and 400µl of cell suspension was added in T75 flask with 10ml complete MEM Alpha media. Cells were plated and allowed to adhere to the plate overnight under incubation. All plates with cells were examined for bacterial and fungal infections prior to experiments.

2.4 Drug preparation & Treatment

Chloroquine (CQ) was obtained from Sigma (St. Louise, MO). A stock solution was prepared by suspending CQ in pure water. A stock concentration of 50mM was made and stored at -20^{0}C. 3-methyladenine (3MA) was obtained from Sigma (St. Louise, MO) and dissolved in PBS by heating. A stock solution of 150mM was prepared and refrigerated.

Hs578t cells were treated with a dose of 5 x 2Gy given over a period of 3 days. Chloroquine or 3MA were added 3 hours before radiation and cells were maintained in the presence of CQ or 3MA until radiation was completed. Media was replenished once treatment was terminated.

2.5 MTT Assay

The MTT assay was used to determine the effective dose of Chloroquine and 3MA for further experiments. For the MTT assay, cells were plated in 96 wells plate at a density of 5000 cells /well in 200µl of MEM Alpha medium. Cells were allowed to adhere to the plates overnight and then treated with different doses of Chloroquine and 3MA. CQ doses

used ranged from 2.5μM- 50μM and 3MA was tested over the range of 2.5mM – 25mM. Cells were incubated with drug for 72 hours; then media was then aspirated and cells were incubated with the MTT solution (2mg/ml PBS) in each well for 3 hours at 37^0 C. The MTT solution was removed, 100μl of autoclaved DMSO was added and plates were shaken for 10 min. In the MTT assay, the MTT (3-(4, 5- dimethylthiazol -2- yl)-2, 5- diphenyltetrazolium bromide reagent is reduced by enzymes in living cells to formazan. DMSO acts as solubilizing solution dissolving insoluble purple formazan product into a colored solution.

2.6 Cell Viability

Cell Viability was measured using Trypan Blue exclusion. Trypan Blue is an exclusion dye that does not stain cells which have an intact cell membrane. Cells were plated in triplicate at a density of 10,000 cells/well and were allowed to adhere overnight. Cells were then treated with CQ or 3MA for 3 hours and/or followed by 5x2Gy radiation. Media was replenished once irradiation was completed and cells were counted at various time points. Cells were incubated with trypsin (0.25% Trypsin-EDTA) for 5-10 min and stained with Trypan Blue (0.4% Trypan Blue). Cells were counted using a haemocytometer with phase contrast microscopy on Day 1, 3, 5 and 7 post radiations.

2.7 Clonogenic Survival Assay

The Clonogenic survival assay evaluates the ability of single cells to form colony. Cells were plated in triplicate typically at a density 100 cells/well. 10-14 days after treatment, cells were fixed with 100% methanol, air dried and stained with 0.1% v/v Crystal violet (Sigma 3886).

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2.8 Acridine Orange Staining Assay for Autophagy

The volume of the cellular acidic compartment is increased in autophagy and hence staining of the acidified autophagosome is used as an autophagy marker. (Paglin et al., 2001) Cells were plated and treated as described for the cell viability assay. At various time points (the same time points at which cells viability was determined), cells were treated with 0.4μM Acridine Orange (Invitrogen A3568) in medium and incubated for 15 minutes. Cells were then washed with 1X PBS, fresh media was added and fluorescent micrographs were taken using an inverted fluorescence microscope (Olympus). Increased numbers of acidic vesicular organelles were observed using at least three replicates per condition per time point.

2.9 Beta Galactosidase Staining Assay for Senescence

Senescence is a state of cellular growth arrest.(Serrano M. et al., 2007) In senescent cells the beta galactosidase enzyme is upregulated and cleaves the X-Gal substrate. Cells were plated and treated as indicated in the cell viability assay.

At various time points (again, the same times at which cells were counted for viability), cells were washed with 1X PBS, fixed with 2% formaldehyde and 0.2% Glutaraldehyde for 10 minutes followed by a wash with PBS. Cells were then incubated in a CO_2 free incubator with staining solution. A CO_2 free incubator is essential in order to maintain pH at 6. The staining solution or staining buffer for the beta galactosidase assay contains 20mg/ml X-Gal in dimethyl formaldehyde, 0.2M citric acid/Na phosphate buffer at pH 6, 100mM potassium ferrocynide, 100mM Potassium ferricynide, 5M NaCl, 1M MgCl2 in distilled water. The senescence signal can be observed as early as 2 hours after

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incubation but for best results overnight incubation was preferred. Cells were washed with PBS and images were captured using light microscopy.

2.10 DAPI staining Assay

DAPI (4'6, diamidino-2-phenylindole) is a fluorescent stain that binds tightly to DNA in A-T rich regions. It is used as a nuclear marker to detect DNA fragmentation. Cells were collected at specific time points and were centrifuged at 5000 rpm (G 18.0), for 5 minutes. Slides with these cells were made by spinning cells at 10,000 rpm for 5 minutes in a Shandon Cytospin 4 (Thermal Electron Corp). Slides were refrigerated until ready for staining. On the day of staining, slides were washed 2X with PBS for 5 minutes at room temperature. Cells were fixed on slides using a 1:2 dilution of acetic acid: ethanol at 20^0 C for 5 minutes. Slides were again washed with 1X PBS for 5 minutes. Slides were prepared with 10μl of a 1:1000 dilution of Vectashield. Coverslips were fixed to slides using clear nailpolish and micrographs were taken using an Olympus SC 35 Type 12 camera.

2.11 TUNEL Assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling)

The TUNEL assay is widely used to distinguish between apoptotic cells where DNA is fragmented and normal healthy cells. Fluorescein is inserted at the 3'-OH terminal of fragmented DNA with the help of the enzyme terminal transferase. The method of Gavrieli et al. 1992 was used and fragmented DNAs in cells undergoing apoptosis was detected using an In Situ Detection Kit (Roche 03 333 566 001). Cells that had been treated were examined at various time points by fixation on slides using Cytospin 4

(Shandon) and were stored at 4 0 C until ready for staining. For staining, cells were fixed by immersing in a 4% formaldehyde solution for 10 min at room temperature followed by 2X wash with PBS for 5 min each. Slides were then immersed in 1:2 mixture of Glacial acetic acid: Ethanol for 5 min at room temperature. 2X PBS washes were followed. Using an ImmunoPen (CaliBiochem, San Diago, CA) tight circles were drawn around cells. Cells were blocked then using 1mg/ml of Bovine Serum Albumin for 30 min at room temperature followed by 2 washes at room temperature with PBS for 5 min each. Cells were then incubated at 37^0C for one hour using an enzyme mix which contained 4μl of 5X reaction buffer, 0.2μl of Terminal Transferase, 2μl of 25 mM COCl2, 0.4μl of fluorescein-12-dUTP and 13.4μl water per sample. Slides were washed twice with PBS at room temperature, coverslips were placed on slides using clear nailpolish and micrographs were taken using an Olympus inverted microscope. All images presented are at the same magnification.

2.12 Western Blot Analysis

Cells were harvested and treated as for the cell growth survival assay. Cells were plated typically at a cell density of 50,000-100,000 cells per 10mm cell culture dish. After treatment, cells were washed with PBS and lysed using 500-600μl lysis buffer. (1:100 Phosphatase inhibitors and 1:50 protease inhibitor in M-PER lysis buffer) (Thermo Scientific # 78501). Protein concentrations were determined by the Bradford method using Bradford reagent (Bio- Rad 500-0205). Equal aliquots of 25μg were separated using 12% Mini PROTEAN Pre-Cast Gels (Bio Rad). Proteins were transferred onto PVDF membranes (soaked in methanol) using transfer buffer (200ml methanol, 100ml 10X tris/glycine & 700 ml water) (Bio Rad 161-0732). After protein transfer, the

membrane was blocked using a blotto mixture comprised of 5% skim milk powder and 0.1% Tween-20 made up in 1X PBS. After blocking the membrane was incubated with the primary antibody overnight followed by three washes with 1X PBS. The membrane was then incubated with the secondary antibody for 2 hours followed by a wash with 1X TBPS. The membrane was developed using West Femto Maximum Sensitivity Substrate (Thermo Scientific # 34095) and Premium Quality X-Ray films (PHENIX). Primary antibodies were diluted in blotto at a 1: 1000 dilution and secondary antibodies were diluted at 1:5000 dilutions. Primary antibodies were used anti-p62 (Santa Cruz SC-28359) and antiβ-action (Santa Cruz) was used. The secondary antibody was anti-mouse (Santa Cruz SC- 2005 Lot A2312).

2.13 FLOW Cytometry Analysis

2.13.1 FACS analysis using Annexin V – PI staining to determine apoptotic and necrotic cells

FACS analysis was performed in order to determine and quantify the extent of apoptosis and necrosis in Hs578t cells. For FACS analysis, treated cells were collected and labeled fluorescently using a FITC Annexin V apoptosis detection kit I (BD Biosciences Lot # 556547). Labeling was done by adding 500µl of binding buffer, 5µl Annexin V- FITC and 5µl Propidium Iodide to each sample. Samples were mixed gently and incubated in the dark for 15 min. Increased Annexin V- PI labeled cells were measured by Flow Cytometry and analyzed by BD FACS Canto II and BDS DIVA software. A minimum of 10,000 cells were counted within gated region.

2.13.2 FACS Analysis of Acridine Orange Staining to quantify the extent of Autophagy

Treated cells were collected at specific time points and suspended in 500µl PBS. Cells were then stained with Acridine Orange stain (Invitrogen A3568) in 1:10000 dilution in PBS for 10 min; cells were then analyzed using by BD FACS Canto II and BDS DIVA software. A minimum of 10,000 cells were counted for the each gated region.

SECTION 3: RESULTS

3.1 Effect of fractionated radiation on growth and survival of Hs578t breast tumor cells

Radiation is widely used as a primary treatment for breast cancer. Patients receive multiple small doses of radiation (2 Gy), up to 40 Gy of a total radiation dose; therefore the use of fractionated radiation is clinically relevant. Previous studies in our laboratory have demonstrated that a dose of 5×2Gy arrests breast cancer cells without killing cells and that autophagy inhibition can sensitize breast cancer cells to radiation (Bristol et al, 2012).

Cells were irradiated with a 5×2 Gy of radiation over a period of 3 days and viable cell number was determined using trypan blue exclusion. Cells were counted on Days 1, 3, 5 & 7 post-radiation.

Fig 3.1 indicates that irradiated cells undergo an apparent growth arrest without cell death at all days. Although cell numbers appear to decline between from Day 0 to Day 1, this decline is not significant.
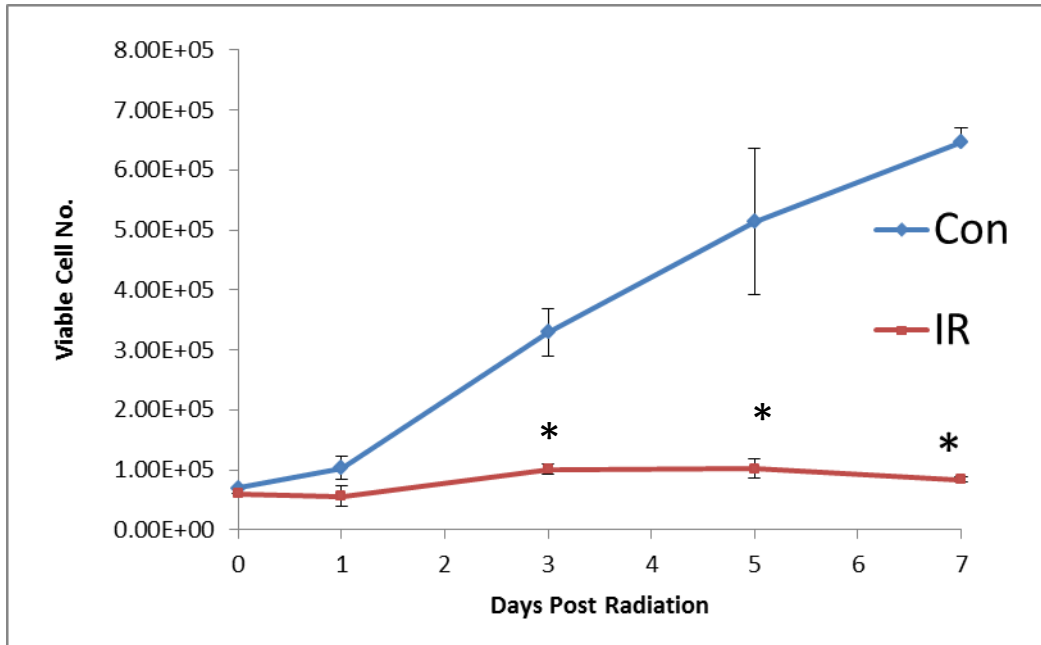
Figure 3.1 Effect of fractional radiation on viable cell number

Viable cell numbers were determined by Trypan Blue Exclusion. Irradiation dose= 5×2 Gy. Tukey. *≤0.05 as compared to control cells after One way ANOVA. Given results are representative of three experiments.

3.2 Induction of autophagy by radiation

3.2.1 Measurement of induction of autophagy using Acridine Orange staining

Previous studies in our laboratory have shown that upon radiation breast tumor cell lines such as MCF-7s undergo autophagy and upon inhibition of such autophagy, cells are sensitized to radiation. (Bristol et al, 2012). This approach can theoretically be used in order to increase the efficacy of the radiation therapy.

In order to study if irradiation induces autophagy in Hs578t cells, irradiated cells were stained with Acridine Orange and micrographs were taken at various time points using an inverted fluorescence microscope.

Acridine Orange staining is widely used to identify cells undergoing autophagy. Acridine orange is a lysomotropic dye which accumulates in acidic organelles in a pH dependent manner. Acidic organelles are present in all cells but they are accumulated in cells undergoing autophagy. At physiological pH, acridine orange is a green fluorescent molecule but within an acidic environment it becomes protonated and is trapped in acidic vesicles, and emits bright red fluorescence (Klionsky et al, 2007).

Irradiated cells clearly showed increased acridine orange staining and thus extensive induction of autophagy upon radiation as compared to control cells as indicated by Fig 3.2. To understand the extent of autophagy it should be quantified. Serum starved cells were used as positive control.

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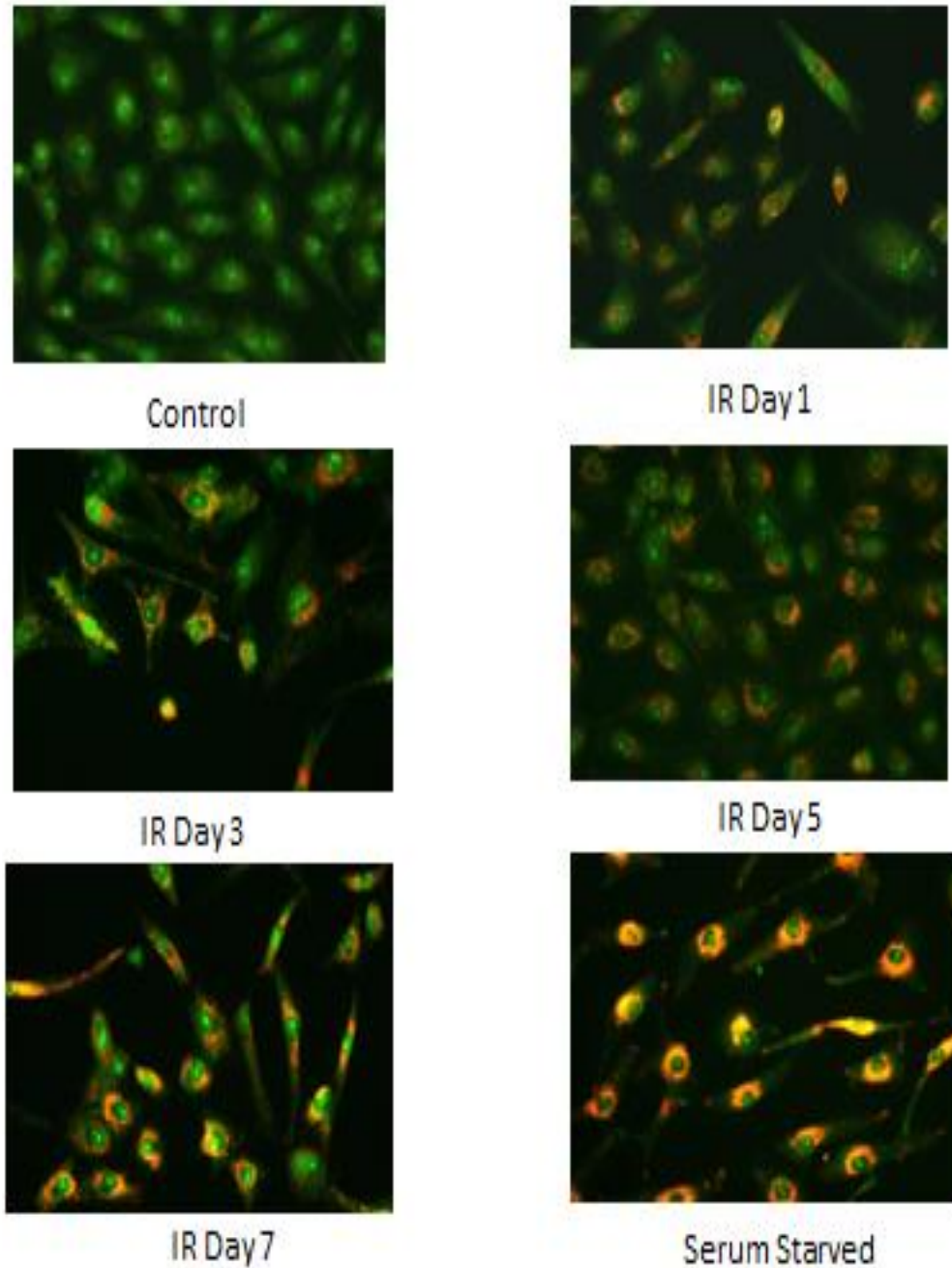
Figure 3.2 Induction of autophagy upon radiation

Cells were irradiated using fractionated radiation administered over the course of 3 days with a minimum 6 hour interval between two doses (5×2Gy). Cells were stained with Acridine Orange and observed under an inverted fluorescent microscope (20X) on Days 1, 3, 5 & 7 post radiation. Given images are representative of three experiments.

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3.2.2 Quantification of acidic vesicular organelles (AVOs) using Flow Cytometry

In order to quantify the extent of autophagy induced in irradiated cells, Flow cyometry analytical techniques were used. Cells were analyzed at the same time points as that of the viable cell count assay.

Fig 3.3 indicates that irradiated cells show a significant increase in accumulation of acidic vesicular organelles from day 3 to day 5 post radiation whereas the extent of autophagy is reduced by day 7.
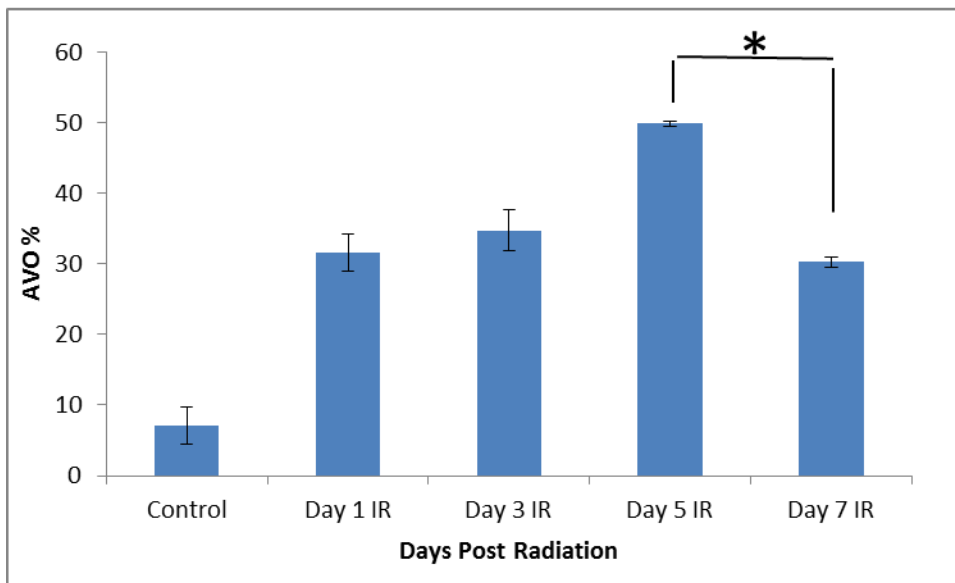
Figure 3.3 Quantification of radiation induced autophagy

Cells were irradiated with fractionated radiation (5×2GY) and media was replenished once radiation was completed. Cells were then analyzed using Flow cytometry on days 1,3,5 and 7 post radiation. ANOVA was used followed by Tukey, *p<0.05. The extent of autophagy is significantly increased over controls at all time points.

3.2.3 Degradation of p62 indicating autophagic flux upon radiation of Hs578t cells

A classic approach to understand if autophagy is going to completion is to monitor degradation of the p62 protein. p62 is a multifunctional- ubiquitin- binding scaffolding protein which is degraded with induction of autophagic flux and thus plays an important role (Johansen et al, 2009). LC3 acts as a receptor for p62. Ubiquitin protein p62 binds to LC3 in order to facilitate its degradation in autophagy. LC3 II is a microtubule associated protein which is converted from LC3I to LC3 II when autophagy is induced (Johansen et al, 2009). Thus, LC3 and p62 are both autophagy associated proteins and widely used as markers for autophagy.

Western blot analysis was performed to probe for p62. Serum starved cells were used as positive control. Fig 3.4 indicates irradiated cells showed almost complete degradation of the p62 protein as compared to control cells indicating that autophagic flux is occurring. Serum starved cells also show almost complete degradation of p62.

| Serum Starved | Control | IR | CQ 10µm | CQ25µm | IR + CQ (10µm) | IR + CQ (25µm) | |
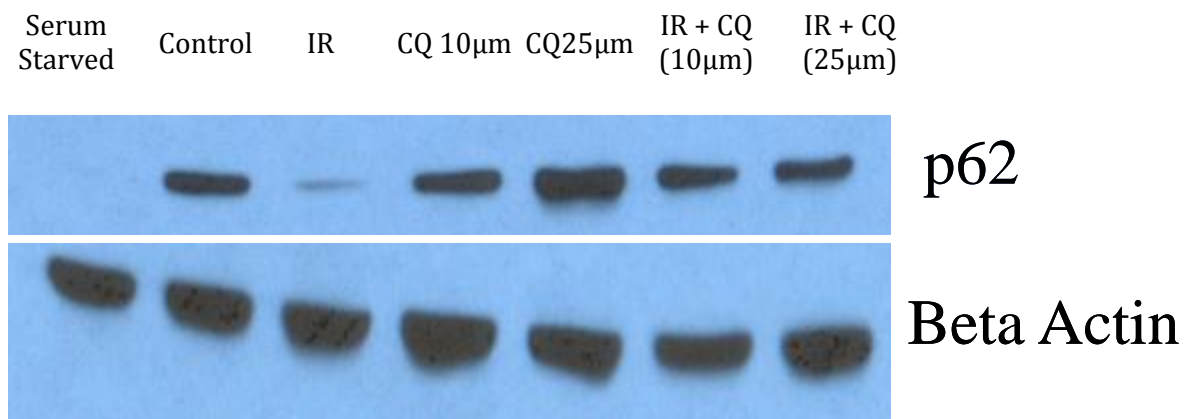
Figure 3.4 Assessment of p62 degradation by Western Immunoblot

Protein extracts were analyzed by western immunoblot analysis, Proteins collected on day 4 were analyzed using 10% gels. Actin was used as a loading control. N=2

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3.3 Inhibition of autophagy

3.3.1 Determination of CQ dose and 3MA dose

Previous studies in our laboratory have shown that autophagy inhibition can sensitize breast cancer cells to radiation (Bristol et al, 2012).

Autophagy inhibition can be achieved using pharmacological or genetic approaches. Pharmacological inhibitors such as Chloroquine and 3MA (3- Methyl Adenine) are commonly used to inhibit autophagy (Rodemann et al, 2011). Chloroquine is a lysomotropic agent which accumulates in the lysosome and interferes with the acidification of the lysosome. (Solomonand and Lee, 2009). It inhibits autophagy by interfering with the fusion of the lysosome with the autophagosome and inhibits formation of the autophagolysosome. 3MA is a PI3 kinase inhibitor (Bursch et al., 1996) that inhibits autophagy at a very early stage by interfering with the formation of the autophagosome.

In order to inhibit autophagy using the pharmacological agents CQ and 3MA. The MTT assay was used to determine the concentrations of autophagy inhibitors that would have limited toxicity. Cells were exposed to drug for 72 hours since irradiation is carried out for 3 days and were analyzed by measuring absorbance at 540nm. The MTT assay indicated that 3MA concentrations of 2.5mM and 5mM are relatively nontoxic. Thus, a 5mM dose (Fig 3.5) was selected for further treatments.

Likewise, cells were treated with CQ for 72 hours and cell viability was measured using the MTT assay and a concentration 25µM (Fig 3.6) was selected for further treatments.

32
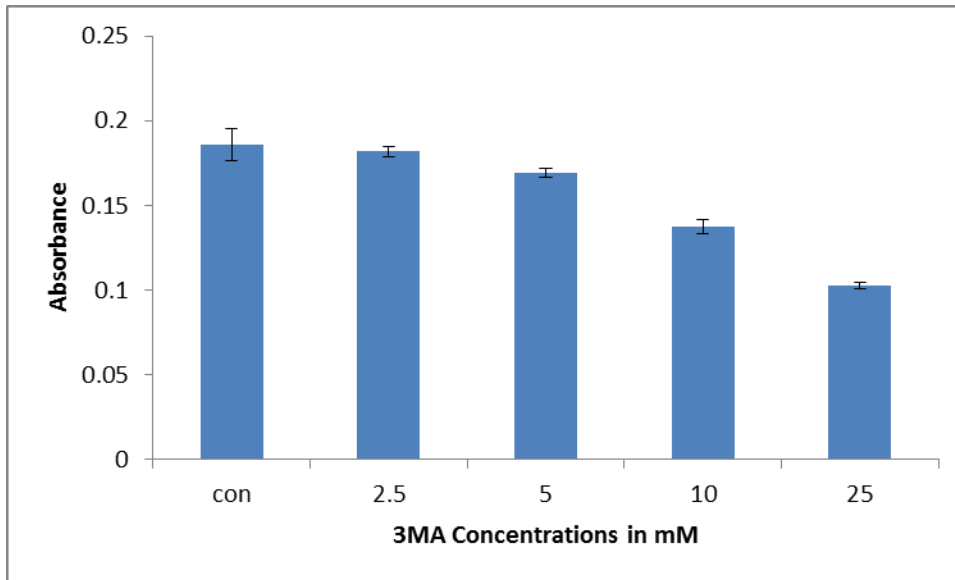
Figure 3.5 Determination of 3MA dose

Viability of cells after 72 hours of treatment with 3MA was measured using the MTT assay. Cells were plated in triplicate and exposed to increasing concentrations of 3MA. After 72 hours of incubation, 2mg/ml MTT was added to the plate for an additional 3 hours of incubation. The blue MTT formazan was then dissolved in 100µl DMSO. The absorbance was measured at 540nm using a multi plate reader. Cell viability was measured using the absolute absorbance at (540nm) n=3
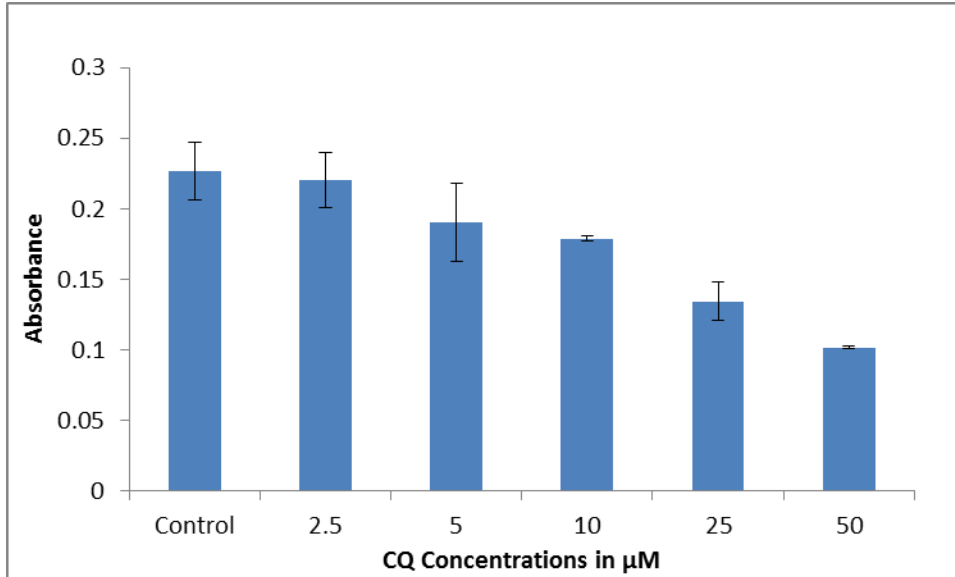
Figure 3.6 Determination of CQ dose

Viability of cells after 72 hours of treatment with CQ was measured using the MTT assay. Cells were plated in triplicate and exposed to increasing concentration of 3MA. After 72 hours of incubation, 2mg/ml MTT was added to the plate for an additional 3 hours of incubation period. The blue MTT formazan was then dissolved in 100µl DMSO. Wavelength 540nm. n=3

3.3.2 Autophagy induced by radiation can be inhibited by Chloroquine (CQ) 25µm

In order to determine if autophagy can be successfully inhibited by CQ, Acridine Orange staining was performed in Hs578t cells with radiation alone and with the combination of IR and CQ. Chloroquine is a lysomotropic agent. It is a basic compound which interferes

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with the acidity of the lysosome resulting in yellow colored vesicles when cells are exposed to acridine orange instead of the normal bright red staining in acidic compartments. Fig 3.7 shows that autophagy induced by radiation is inhibited by CQ treatment. This is confirmed in figure 3.4, where degradation of p62 induced by radiation was markedly decreased by CQ indicating successful inhibition of autophagy by CQ.
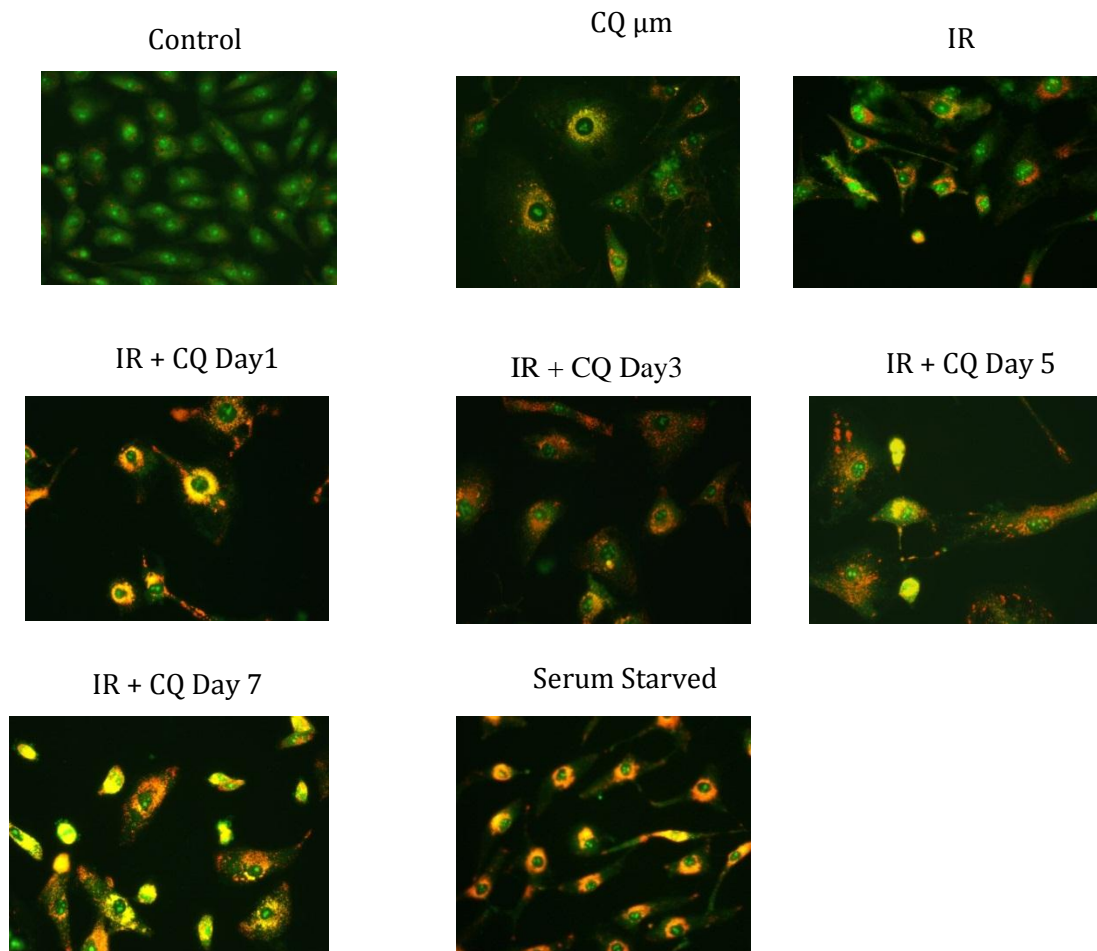
Figure 3.7 Inhibition of autophagy using CQ

Cells were pre-treated with CQ (Chloroquine) (25µm) 3hours prior to radiation (Radiation dose= 5×2Gy) and were treated with CQ throughout the irradiation period. Cells were incubated with Acridine Orange stain for 10 min. (1:10000). Images were captured using an inverted fluorescence microscope under 20X and are representative of three experiments.

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3.3.3 Autophagy induced by radiation can be inhibited by 3- Methyladenine (3MA) 5mM

Another pharmacological inhibitor, 3MA, was used to confirm the observations obtained in studies with CQ.

Hs578t cells were treated with 3MA for 3 hours prior to irradiation and were maintained in the presence of 3MA throughout the radiation period. 3MA acts at an early stage of autophagy and interferes with the formation of autophagosomes via inhibiting PI3K. Cells exhibit a different staining pattern when exposed to acridine orange in the presence of 3MA. Due to inhibition of the formation of the autophagosome, one will expect to see less acridine orange staining if autophagy is inhibited. When cells were treated with the combination of IR and 3MA, a decrease in acridine orange staining indicated that autophagy induced by radiation was inhibited which is shown in Fig 3.8. Serum starved cells were used as a positive control for acridine orange staining. So far, it can be said that irradiation induced autophagy can be successfully inhibited by using pharmacological inhibitors Chloroquine and 3-Methyladenine. Serum starved cells are used as a positive control, since starved cells induce high levels of autophagy to survive and generate metabolic precursors.

Control   3MA   IR

IR + 3MA Day 1   IR + 3MA Day 3   IR + 3MA Day 5

IR + 3MA Day 7   Serum Starved
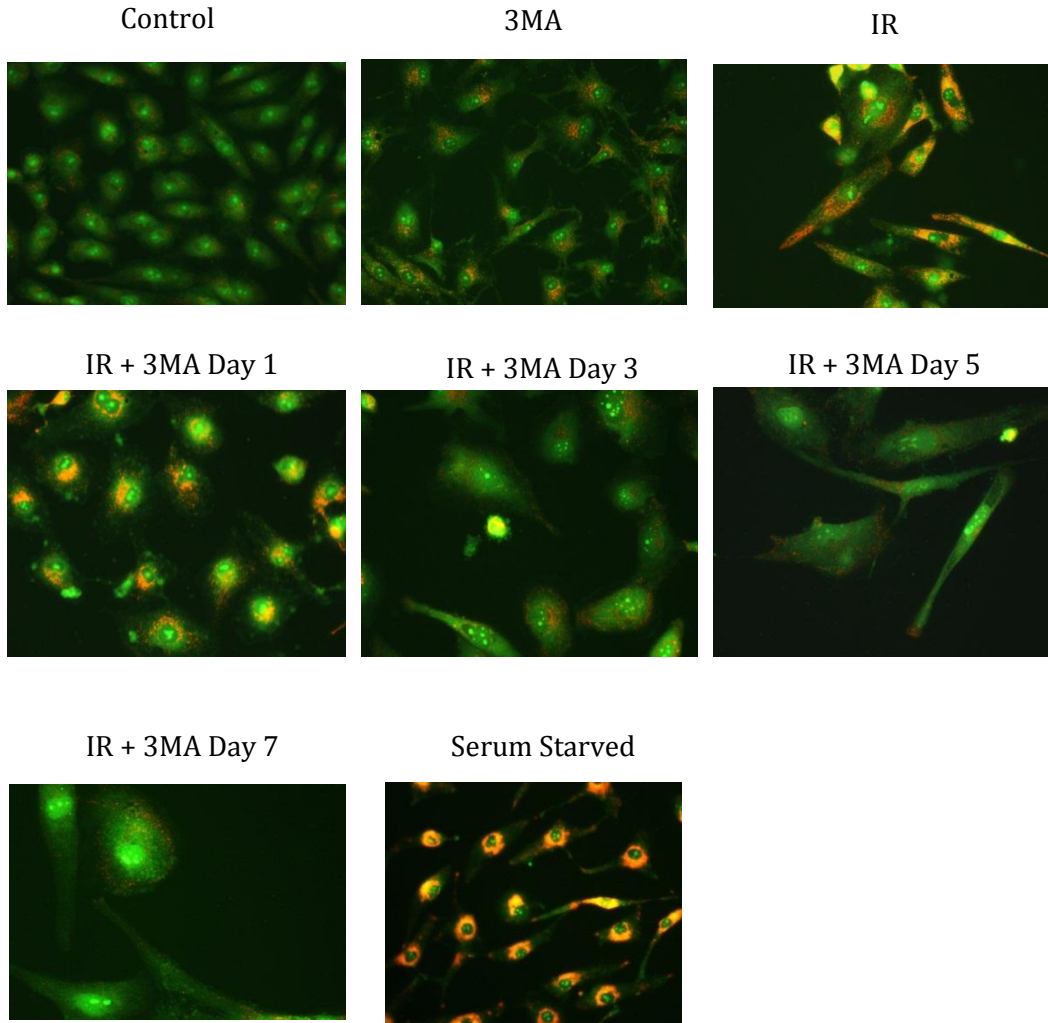
Figure 3.8 Inhibition of autophagy by 3MA (3-Methyladenine)

Autophagy was monitored using acridine orange staining. Radiation and combination of radiation and 3MA (3MA dose= 5mM) was used for treatment. Images were captured using an inverted fluorescence microscope under 20X and are representative of three experiments.

3.4 Effect of autophagy inhibition on radiation sensitivity of Hs578t breast tumor cells

3.4.1 Effect of the combination of IR and CQ on viable cell number

In order to determine if inhibition of radiation induced autophagy sensitizes Hs578t cells to radiation, cells were irradiated with and without Chloroquine (CQ). The impact of treatment was measured by determining viable cell number. As shown in Fig 3.9, only an apparent additive effect of radiation and chloroquine was observed. Cells treated with the combination of IR and CQ show only slightly more growth inhibition than IR treated cells. Sensitization of Hs578t cell would be expected to exhibit a robust response to combination treatment.
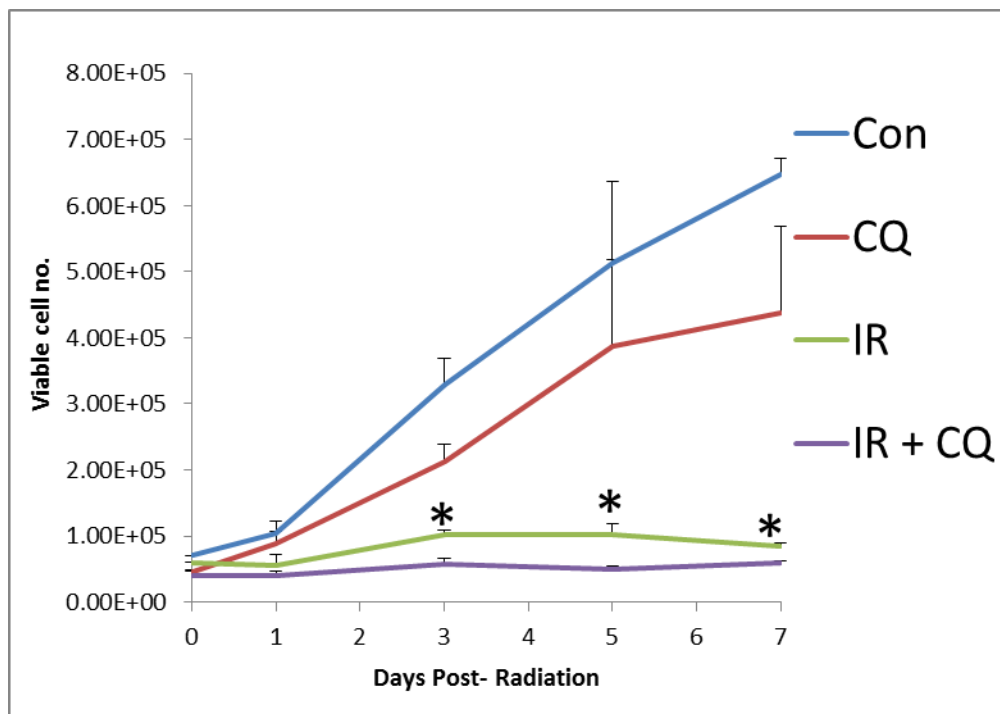
Figure 3.9 Analysis of the effect of inhibition of autophagy on radiation sensitivity using CQ

Viable cell number after each treatment was measured by Trypan Blue exclusion. Radiation dose: 5×2GY, CQ: 25µm. Results are from average of three experiments, each in triplicate. ANOVA was run followed by Tukey *≤0.05.

As shown in Figure 3.9, cells treated with CQ alone show about a 25% reduction in viable cell number as compared to control cells on day 5. Irradiated cells show about an 80% reduction in cell population. As a combined effect, CQ+IR treatment causes about a 95% reduction in the population indicating a lack of sensitization.

3.4.2 Effect of autophagy inhibition on colony formation ability of Hs578t cells

To further confirm the lack of sensitization of autophagy inhibition, a clonogenic survival assay was performed. A clonogenic survival assay is often used to measure the efficacy of radiation or chemotherapy treatment by observing the ability of single cells to form colonies and is thought to be sensitive. 100 cells were plated in each well and the ability of the cells to form colonies was measured after 10-14 days.
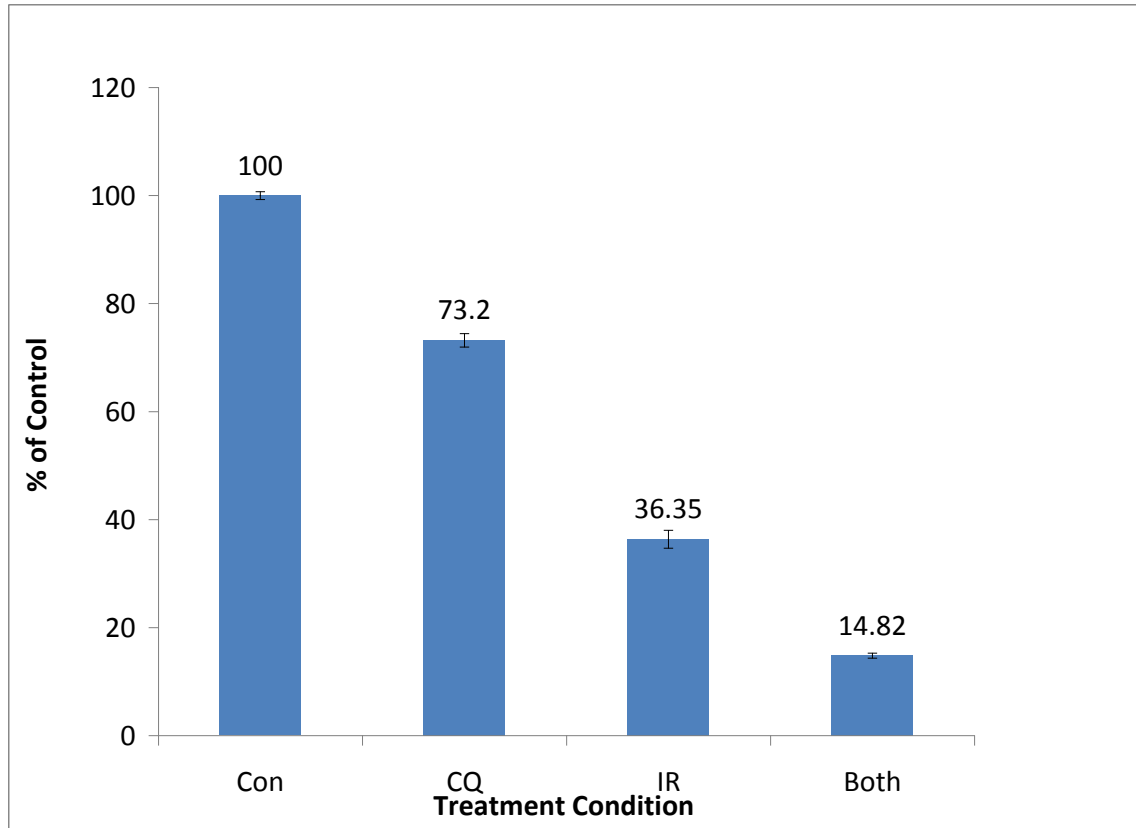
Figure 3.10 Clonogenic Survival Assay using CQ

Clonogenic survival assay was analyzed 14 days post treatment. CQ dose= 25μm, Irradiation dose= 5×2 Gy. N=2 with similar results.

The clonogenic survival assay Fig 3.10 also clearly reflects additive effects when Hs578t cells were treated with the combination of IR and CQ. In classic cytoprotective autophagy, cells use autophagy as a mechanism for cell survival and consequently when autophagy is inhibited, cells die (mostly by apoptosis). In Figures 3.9 and 3.10 Hs578t cells exhibited lack of sensitization upon autophagy inhibition indicating that autophagy is not cytoprotective in this system.

3.5 Effect of the autophagy inhibitor, 3MA (3-methyadenine), on the radiation sensitivity of Hs578t cells

3.5.1 Effect of combination of IR and 3MA on viable cell number

In order to confirm that if lack of sensitization occurs irrespective of the pharmacological inhibitor used, sensitivity studies were repeated with another pharmacological inhibitor 3-MA. The treatment conditions were essentially identical to those for CQ. Cells were pre-treated with 3MA 3 hours prior to irradiation and were treated with it throughout the irradiation period. Viable cell numbers were monitored using the trypan blue exclusion assay.

Fig 3.11 shows that cells treated with 3MA alone showed growth inhibition as compared to control cells but 3-MA alone did not exhibit excessive toxicity. Cells treated with irradiation showed a significant decrease in cell numbers compared to control cells with growth arrest. Cells treated with the combination of IR and 3MA exhibited a similar pattern of growth inhibition and growth arrest as seen with IR and CQ treatment. Lack of sensitization seemed to be independent of the pharmacological inhibitor.
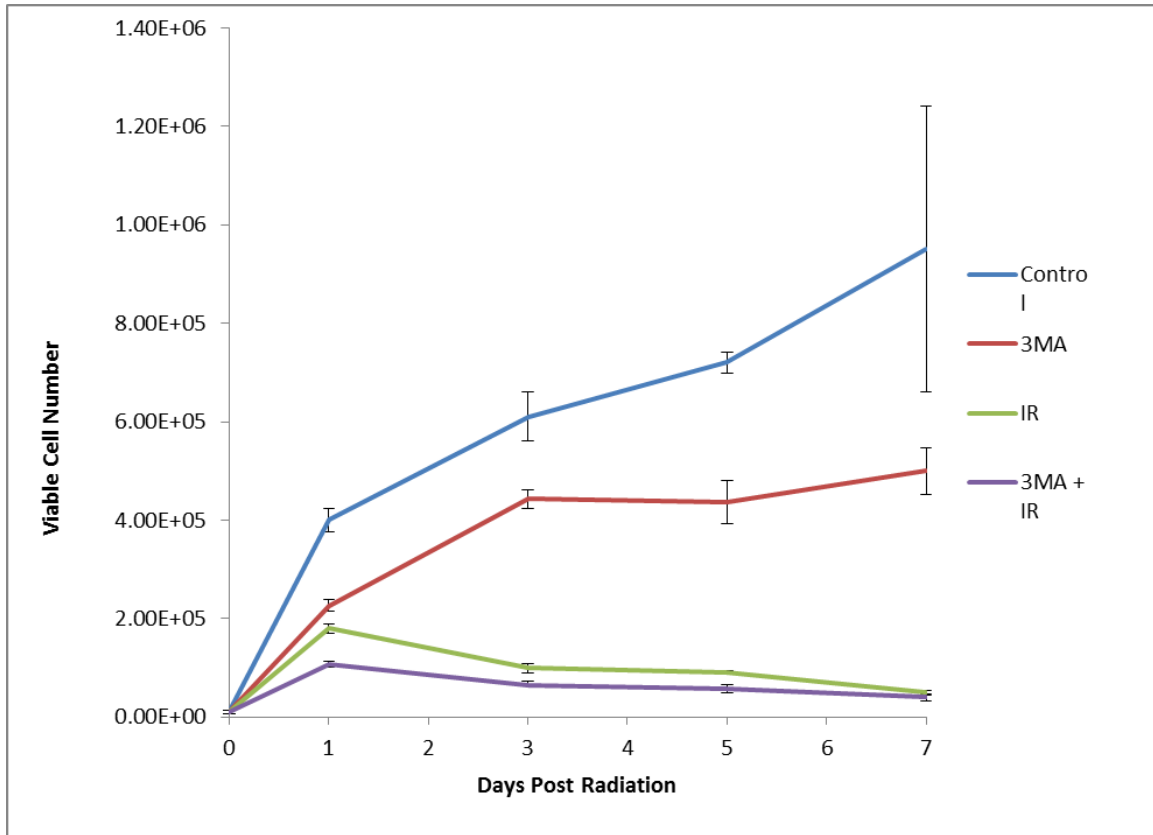
Figure 3.11 Analysis of the effect of inhibition of autophagy on radiation sensitivity using 3MA

 Viable cells were monitored using trypan blue exclusion. Radiation dose: 5×2GY, 3MA dose= 5mM, n=3 with similar results. 3MA dose= 5mM. No significance was observed when IR treated and IR + CQ cells were compared by One Way ANOVA.

3.5.2 Analysis of effect of 3MA on the colony formation ability of Hs578t cells upon radiation

To verify the results obtained from the cell viability assay when 3MA was used with IR in Hs578t cells, a clonogenic survival assay was performed. Fig 3.12 shows that cells treated with 3MA alone showed about 10% inhibition as compared to controls. Irradiated cells showed an approximately 50% reduction in colony formation. Cells treated with IR and 3MA combination showed complete lack of sensitization by exhibiting essentially the same extent of loss of clonogenic survival as that of IR alone.
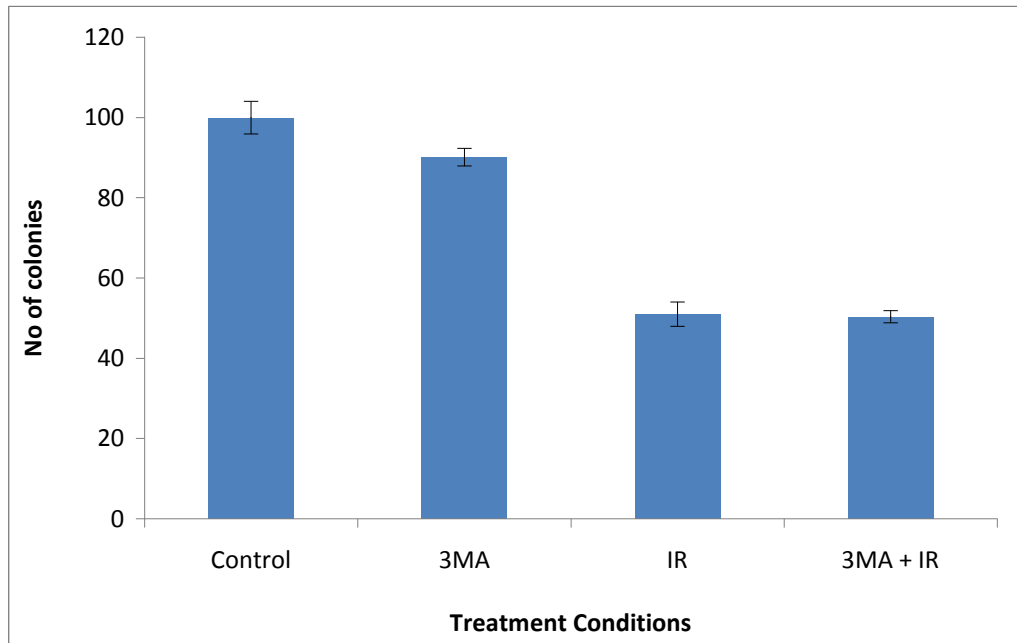

Figure 3.12 Clonogenic Survival Assay using 3MA

Clonogenic survival assay was analyzed on day 14 after treatment. Irradiation Dose= 5× 2Gy. N=2 with similar results.

3.6 Radiation induces minimal apoptosis in HS578t cells

DAPI and TUNEL are widely used assays to determine apoptotic cells. With the use of Vectashield, DAPI (4, 6- Diamidino-2-phenylindole) and TUNEL staining can be performed on the same slides without interference. In normal cells, DAPI staining shows a uniform nucleus with a clear margin in contrast to apoptotic cells where the margin is not clear and cell nucleus is distorted.

One primary characteristic of apoptotic cells is the fragmentation of DNA (Tschopp et al, 1993). In the TUNEL assay, fluorescent nucleotides are inserted at the 3'-OH end of fragmented DNA, which gives bright green fluorescence in apoptotic cells.

It has been observed that if radiation induced autophagy is cytoprotective, then upon inhibition of autophagy, cells become sensitized to radiation and die by apoptosis. (Jin H et al, 2011, Bristol et al., 2012). Another important aspect of this study was to check if radiation alone induces any apoptosis in Hs578t cells in order to study the mode of cell death.

Cells were irradiated and upon DAPI/TUNEL analysis, it was evident that Hs578t cells undergo minimal apoptosis upon radiation. Taxol (1µm for 24 hrs.) was used as a positive control for apoptosis as shown by Fig 3.14.

Irradiated cells exhibit lack of increased fluorescence indicating lack of induction of extensive apoptosis. A minimal apoptosis is detected at days 5 and day 7.

DAPI   TUNEL

Control

IR Day 1

IR Day 3

IR Day 5

IR Day 7
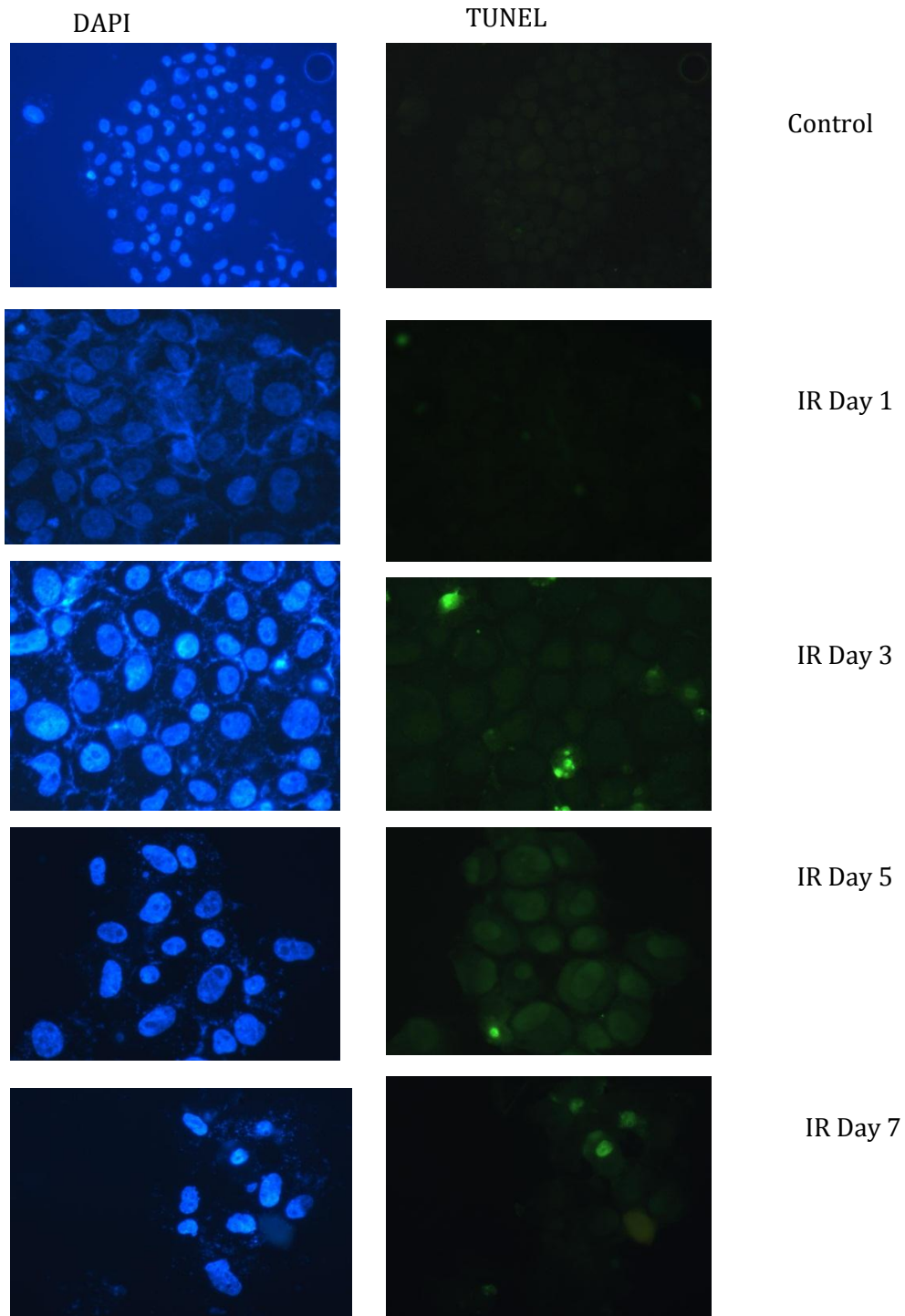
Figure 3.13 DAPI/TUNEL Analysis in irradiated Hs578t cells

Cells were fixed to the slides using a Cytospin and were stained for DAPI and TUNEL using 10 μm VectaShield in 1:10000 dilutions and Cell death detection kit (Roche). Radiation dose= 5X 2 Gy. N=3. Olympus Microscope was used

3.7 Effect of autophagy inhibition on the extent of apoptosis by TUNEL/DAPI

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Control Day 10		TAXOL
TUNEL IR	Day 1	TUNEL CQ+IR
TUNEL IR	Day 3	TUNEL CQ+IR
TUNEL IR	Day 5	TUNEL CQ+IR
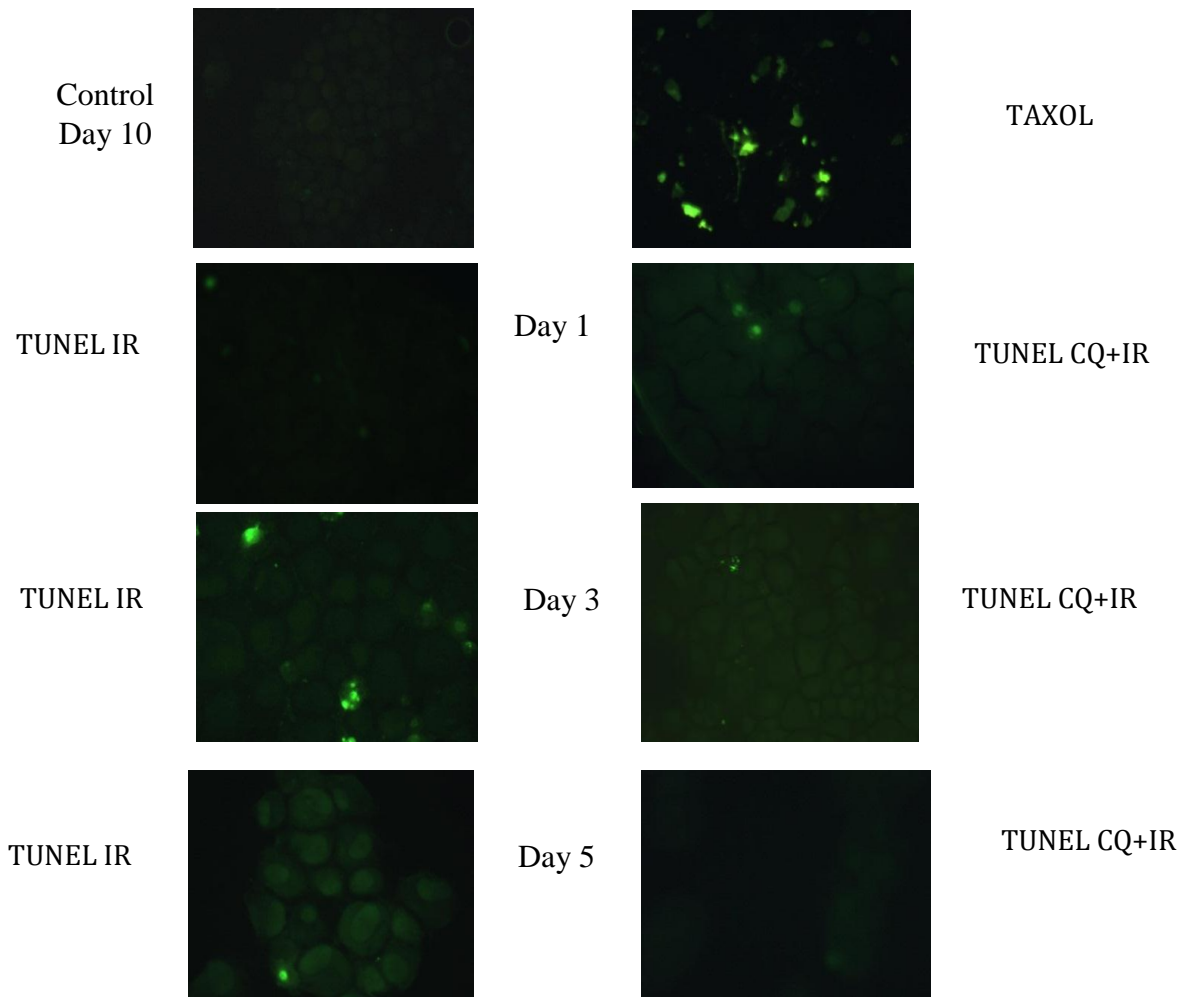
Figure 3.14 TUNEL Analysis for apoptotic cells with IR +CQ

Cells were fixed to the slides using a Cytospin and were stained for TUNEL using Cell death detection kit (Roche). Micrographs were taken using an Olympus Inverted Microscope. All images shown were taken at 20X and are representative of three experiments. CQ=25μm,

IR=5 X2 Gy

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Irradiated cells exhibited minimal apoptosis when analyzed using TUNEL/DAPI (Figure 3.13). Inhibition of autophagy using CQ did not increase the extent of apoptosis in the cells (Figure 3.14), which is consistent with the cell viability assay observations where it shows a growth arrest pattern. This indicates that autophagy inhibition is not promoting cell death by apoptosis which can be an indication that autophagy and it is unlikely to be cytoprotective in this experimental system.

3.8 Quantification of Apoptotic cells by Annexin V/PI Flow Cytometry

To confirm that apoptosis in Hs578t cells by IR alone as well as by IR + CQ is minimal, the extent of apoptosis was quantified using Annexin/PI assay by Flow Cytometry. Annexin/PI staining indicates the number of cells in early as well as in late apoptosis and necrosis. Annexin V stains cells which are in early apoptosis stage. A living cell has PS (Phosphotidylserine) in its inner membrane; during apoptosis, PS is translocated to the outer membrane and Annexin V has high affinity for PS, and cells in early apoptosis will stain with Annexin V. (Sawai et al, 2011, miltenyibiotec.com). On the other hand, PI cannot cross an intact cell membrane; during late apoptosis cells have compromised cell membranes and consequently PI can easily pass across the cell membrane and stains DNA by intercalating between bases. Necrotic cell are stained by both Annexin V / PI whereas viable cells are stained by none of these reagents. (researcher.nsc.gov.tw).

The extent of apoptosis is quantified by taking the sum of the two right quadrants which represent early and late apoptosis.

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	Annexin V
PI (Propidium Iodide)	
Q1 Necrosis	Q2 Late Apoptosis
Q3 Viable cells	Q4 Early Apoptosis

Annexin V

Figure 3.15 Each quadrant indicates a cell condition in AnnexinV/PI staining.
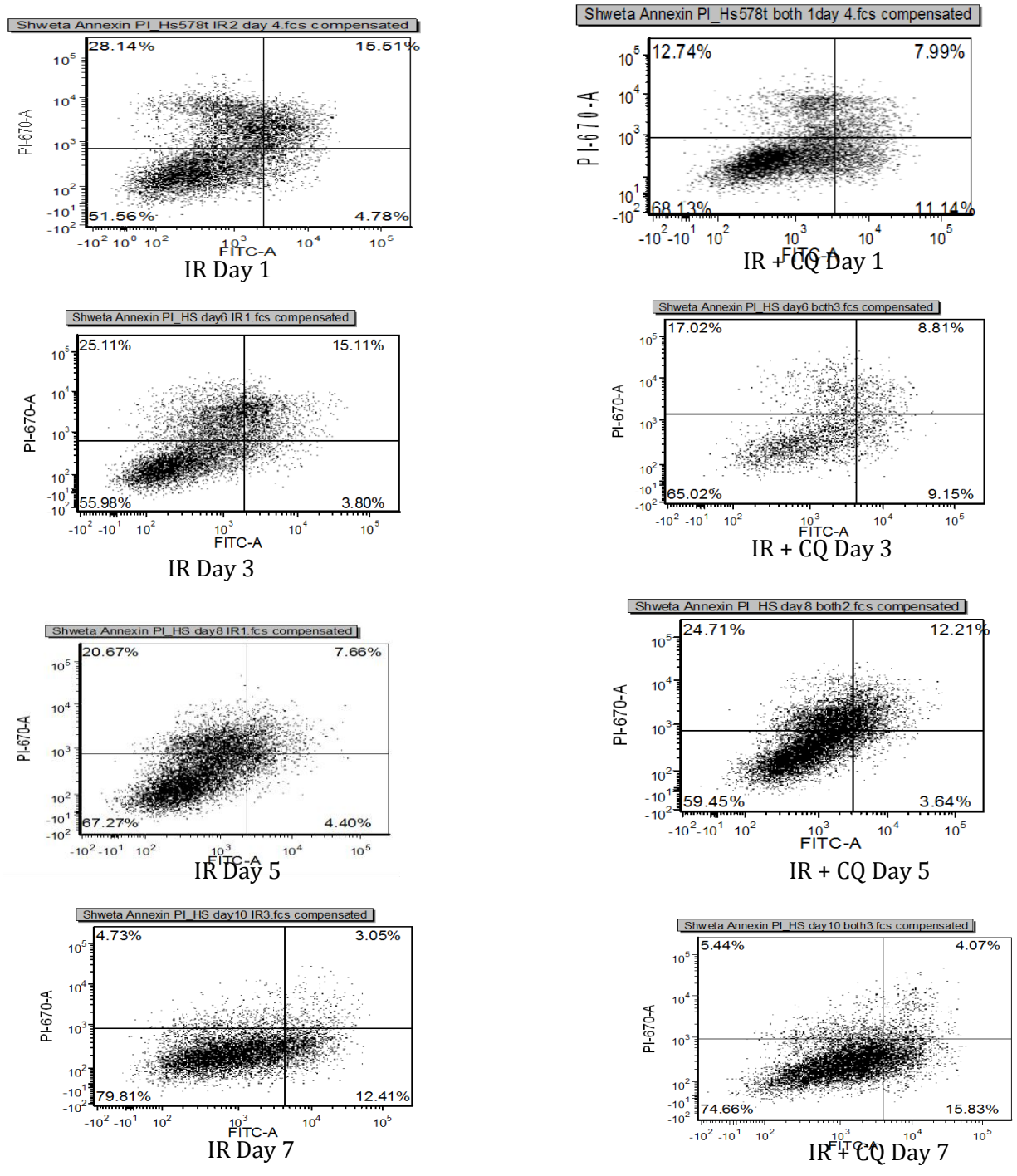
Figure 3.16 AnnexinV-PI staining in IR and IR+CQ treated cells.

Quantification of Annexin V/PI staining gave results expected based on the TUNEL/DAPI experiments. Irradiated cells showed about 15-18% of apoptotic cells whereas cells treated with the combination treatment of IR+CQ did not show any significant increase in percentage of apoptotic cells (Figure 3.16).

Table 3.1 indicates viable cell number is each quadrant

Table 3.1 Quantification of Annexin V/PI staining assay: Each Column represents average of three replicates of each condition

	Necrosis	Late Apoptosis	Early Apoptosis	Viable
Con 7	2.76±0.56	0.40±0.07	3.62±0.64	93.20±1.18
CQ 1	15.74±2.6	3.71±0.33	2.82±0.25	77.72±2.27
CQ 3	12.29±0.33	1.26±0.18	3.49±0.66	82.95±0.51
CQ 5	4.27±0.12	0.30±0.15	3.19±0.97	92.22±1.00
CQ 7	2.40±0.25	1.27±0.16	8.48±0.44	87.84±.34
IR 1	20.46±0.76	7.34±0.56	8.42±0.72	63.77±0.55
IR 3	17.84±0.74	7.39±0.83	6.21±0.47	68.54±0.98
IR 5	6.08±0.96	2.41±0.32	5.84±0.25	85.64±1.19
IR 7	1.66±0.27	1.26±0.19	10.18±0.22	86.89±0.25
IR+CQ1	13.73±0.95	3.64±0.64	11.57±0.59	71.05±0.93
IR+CQ3	14.24±1.37	8.47±0.49	11.37±.87	65.92±1.25
IR+CQ5	4.94±0.39	4.52±0.33	9.37±0.27	81.16±0.80
IR+CQ7	1.8±0.30	1.05±0.23	9.84±2.12	87.11±2.00

Both TUNEL/DAPI and Annexin V/PI staining confirmed that there is no significant increase in apoptotic cells when autophagy was inhibited in Hs578t cells, indicating the absence of sensitization. There is overlap between apoptosis and necrosis but Annexin V/ PI staining helps in distinguishing between cells which are early apoptotic and late apoptotic. Irradiated cells seem to be dying by necrosis on day 1 and Day 3 which can be explained as the initial response to radiation. Irradiation induces about 9 -15% of apoptosis on all treatment days but the percentage of apoptotic cells did not increase at any time points when they were treated with the combination of IR + CQ.

3.9 Irradiation of Hs578t cells promotes senescence

The assay of viable cell number (Figure. 3.1) indicates that radiation treatment results in growth arrest which might be an indicative of senescence. The breast tumor cells might be undergoing senescence as an association between autophagy and senescence has been shown by others as well as by our laboratory (Goehe et al, 2012 in press). Cellular senescence is a form of stable cell cycle arrest with active metabolism (Young et al., 2009). Beta-galactosidase staining is widely used assay to distinguish senescent cells from normal cells. In senescent cells, beta galactosidase is upregulated when these cells are treated with the chromogenic substrate X-gal; X-gal is cleaved by beta galactosidase and generates an insoluble blue compound at pH 6. (Toussaint et al., 2009)

Control	IR Day 1
IR Day 3	IR Day 5
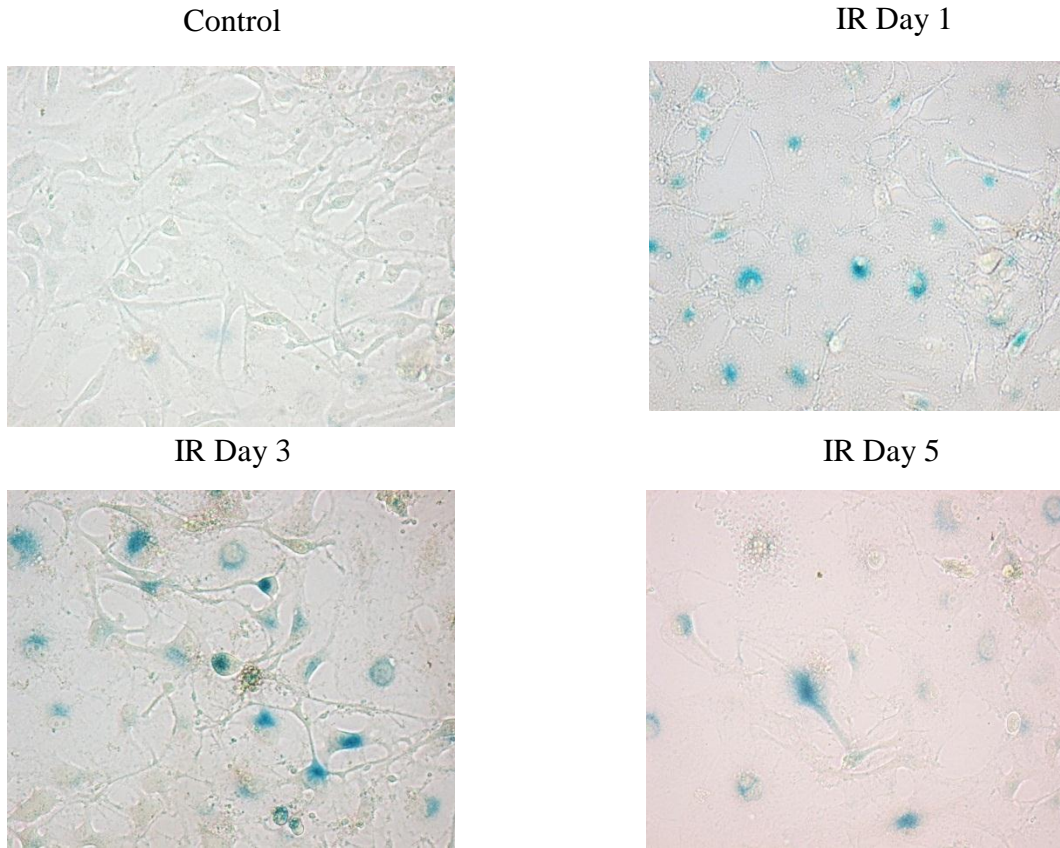
Figure 3.17 Radiation induces senescence in Hs578t cells

Irradiated cells were fixed and then stained on the indicated days and images were taken under normal white light microscope. Radiation Dose= 5×2Gy.

Irradiated cells clearly show blue staining from Day 1 to Day 5 post radiation confirming the presence of senescent cells as shown in Fig 3.17. Thus, it can be said that fractionated radiation induces senescence in Hs578t cells. Irradiation induced senescence was reduced by day 5 which establishes that autophagy and senescence might be related to each other. Irradiation induced autophagy is also reduced by day 7 which was shown using flow cytometry (Figure 3.3).

3.10 Effect of autophagy inhibition on senescence induced by radiation

Studies were performed to assess if there is any relationship between autophagy and senescence induced by radiation by determining whether autophagy inhibition altered senescence. The β-galactosidase assay was performed on cells treated with the combination of IR+CQ.

Figure 3.18, indicates that Hs578t cells treated with IR+CQ did show reduced amount of beta galactosidase staining suggesting the possibility of a link between irradiation induced autophagy and senescence; that is when autophagy was inhibited, senescence was reduced.

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IR   IR + CQ

Day 1

Day 3

Day 5

Control   Adriamycin
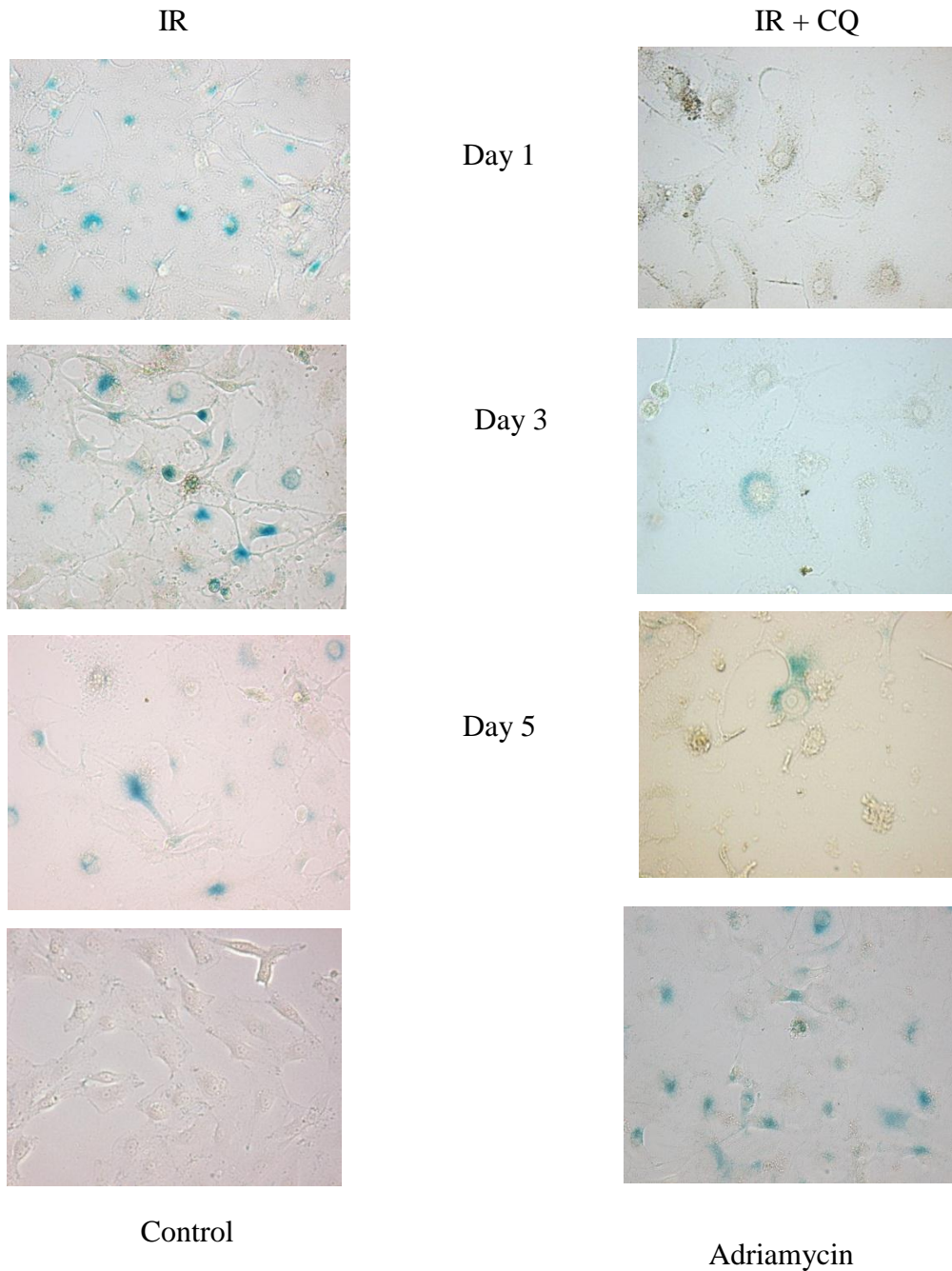
Figure 3.18 Impact of autophagy inhibition on senescence in Hs578t cells using CQ.

All micrographs were taken under white light inverted microscope. Radiation dose= 5×2Gy. 20X, n=3. Cells treated with ADR (Adriamycin) were used as positive control.

3.11 Assessment of effects of 3MA (another autophagy inhibitor) on senescence

In previous studies, 3MA has been used to inhibit autophagy. Assessment of the effect of 3MA on senescence was performed using Beta-Galactosidase staining. 3MA seems to have the same effect as that of CQ on the extent of senescence. Although 3MA did not reduce extent of beta galactosidase staining as early as day 1, but from day 3 onwards it inhibited the amount of senescence in Hs578t cells.

	IR	IR + 3MA
Day 1		
Day 3		
Day5		
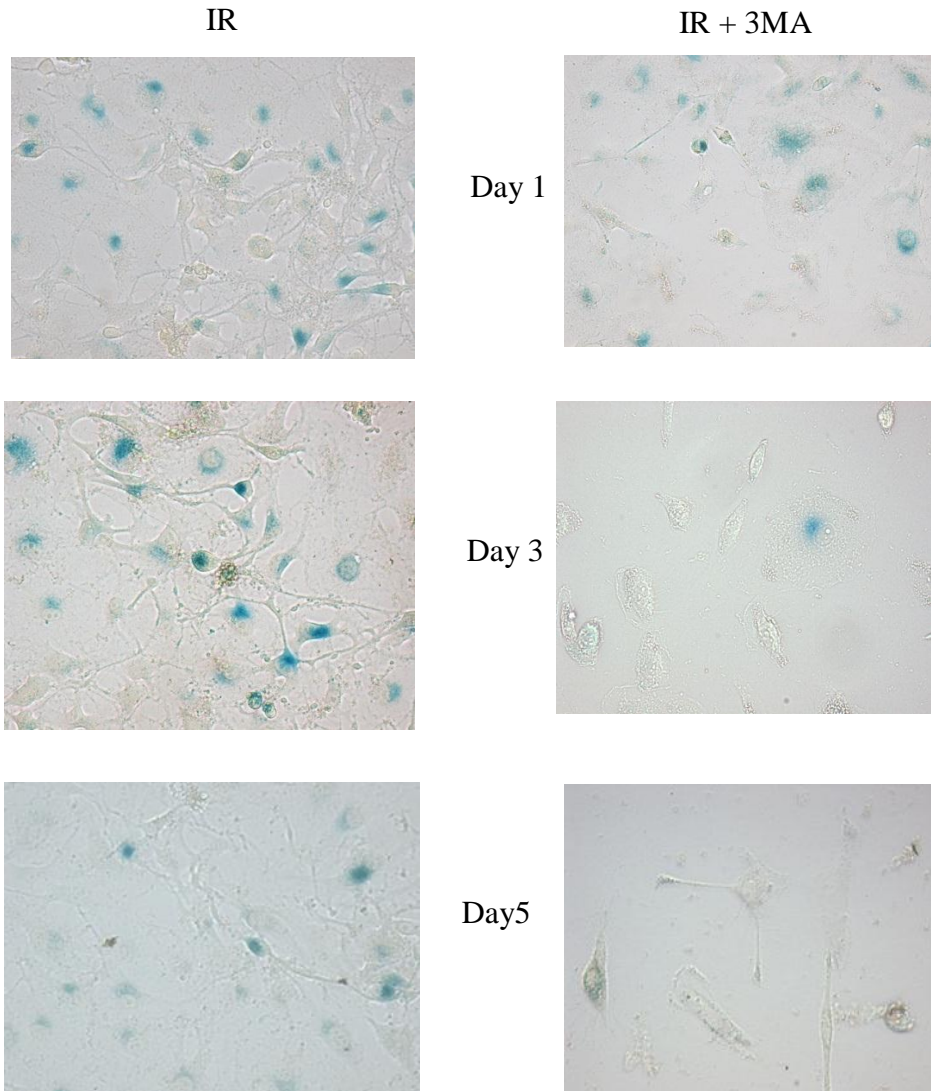
Figure 3.19 Impact of autophagy inhibition on senescence in Hs578t cells using 3MA

3MA dose= 5mM. Radiation Dose= 5× 2Gy.

3.12 Impact of single dose of radiation on Hs578t cells

Hs578t cells do not appear to be sensitized to radiation upon inhibition of autophagy in contrast with other breast cancer cell lines such as MCF-7s. Another approach was taken to determine if a single dose of radiation sensitize cells to radiation upon autophagy inhibition. Cells were irradiated with a 4Gy single dose of radiation.
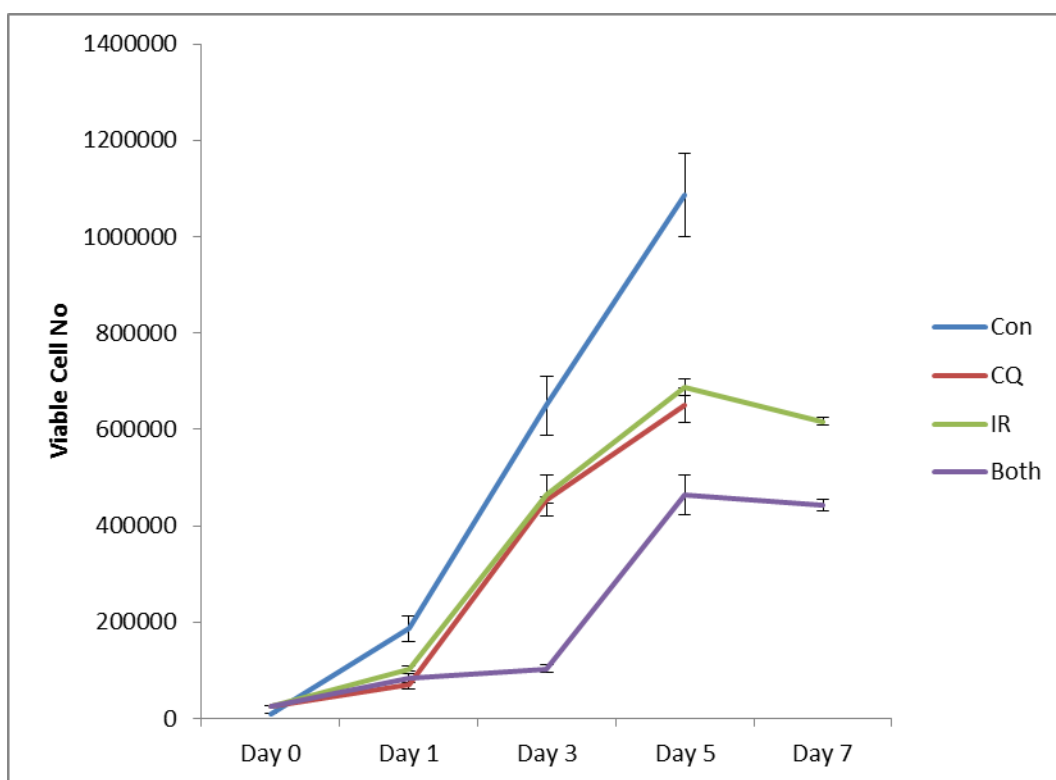
Figure 3.20 Effect of single dose of radiation on cell survival when autophagy was inhibited. CQ dose= 25µm. Radiation Dose= 4Gy.

The same treatment paradigm was followed as fractionated dose. Viable cells were counted on respective time points using trypan blue staining. This experiment was performed to determine whether the radiation dose influenced susceptibility to autophagy

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inhibition and sensitization. As shown in Fig 3.21, when cells were treated with single 4Gy dose of radiation as compared to 5× 2Gy, growth was slowed but the cells did not actually arrest until between day 5 and day 7. Again, the combination treatment failed to indicate sensitization, but only an additive effect of IR + CQ was observed.

3.13 Effect of single dose of radiation on colony formation ability of Hs578t cells

When cells were treated with a single dose of 4 Gy radiation dose, the clonogenic survival assay showed in Fig 3.21 indicated complete lack of sensitization by CQ but only additive was shown.
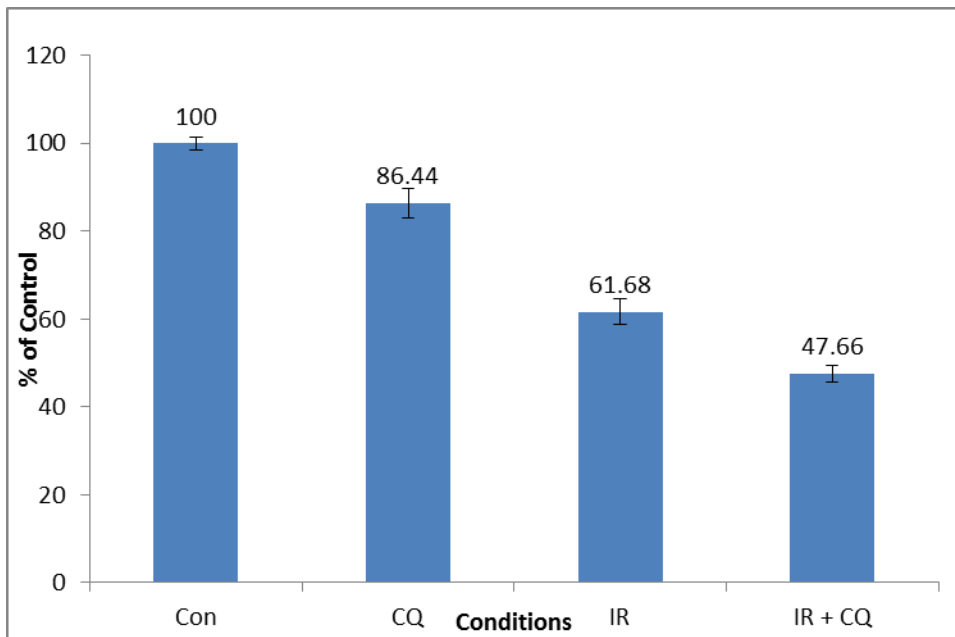
Figure 3.21 Clonogenic assay with single 4GY dose of radiation. CQ dose= 25µm.

SECTION 4: DISCUSSION

Radiation therapy is widely used to treat various cancers. Irradiation causes extensive DNA damage and kills cancer cells (Masuda & Kamiya, 2011). The development of resistance and recurrence of disease are the primary limitations of radiation therapy. In order to overcome these limitations, combination therapy can be a beneficial approach.

Upon irradiation, it has been found that breast tumor cells undergo autophagy (Rodemann et al, 2011). Autophagy is an evolutionary conserved catabolic process in which cytoplasm and other cellular organelles are recycled and reused (Przyklenk et al, 2010). Autophagy can serve as a defense mechanism to protect cells against DNA damage or it can act as a mode of cell death; however there is controversy as to the role of cytotoxic autophagy in chemotherapy and radiotherapy (Eileen White et al, 2010).

In cytoprotective autophagy, autophagy acts as defense mechanism. Autophagy can be inhibited using different pharmacological or genetic approaches. CQ (Chloroquine), 3MA (3- Methyladenine) and Baf (Bafilomycin) are agents that are commonly used to inhibit autophagy pharmacologically (Rodemann et al, 2010, Zisterer et al, 2012). The combination of irradiation with an autophagy inhibitor can sensitize breast cancer cells to radiation (Bristol et al, 2012). Sensitization is a condition of supra additive effect where the total effect of two agents is greater than the additive impact of each of those agents individually (P.269-270,2nd edition, advances in radiation oncology in Lung cancer, Brady L.W., Heilmann H.P., Molls.M. Nieder.C.) Sensitization of cells can be used as an approach to increase the effectiveness of treatment to tumor cells that develop resistance.

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Previously it has been shown in our laboratory that MCF-7 and ZR-5 breast tumors can be sensitized to radiation when treated with an autophagy inhibitor such as Chloroquine (Bristol et al, 2012 and Wilson et al, 2012).

About 12-17% of breast cancers are triple negative breast cancers (Reis & Fliho, 2010). Triple negative breast cancers do not respond well to established cancer treatments. Hs578t is a triple negative breast cancer cell line (Lehmann et al, 2011). Previous studies in our laboratory confirmed that Hs578t cells are relatively resistant to irradiation compared to other breast cancer cell lines such as MCF7 cells (Bristol et al, 2012). Our studies were performed using Hs578t cells to determine if sensitization to radiation could occur using a combination treatment of irradiation with an autophagy inhibitor (CQ).

When Hs578t cells were irradiated with fractionated doses of 5x 2Gy, the irradiated cells showed a slight decrease in viable cell number but the primary response was growth arrest. Irradiated cells also demonstrated autophagy which was inhibited using CQ (Chloroquine) and 3MA. When Hs578t cells were treated with the combination of IR and CQ, there was no increase in sensitivity but merely an additive effect of IR and CQ indicating that autophagy may not be cytoprotective. Cells were arrested throughout the period. These observations were further confirmed using another autophagy inhibitor, 3-MA, which also exhibited lack of sensitization.

To check if autophagy inhibition exerts any effect on the extent of apoptosis, DAPI/TUNEL staining was performed on the cells treated with the combination of CQ and IR. Autophagy inhibition did not show any significant increase in cell death by apoptosis. The lack of increased apoptosis was further confirmed by using flow cytometry analysis with Annexin V/PI staining.

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Irradiation induced minimal apoptosis. Annexin/PI staining by Flow cytometry did show that the extent of apoptosis in irradiated cells was approximately between 15-18% whereas in control cells it was approximately between 5-10%. This clearly indicates that irradiated cells do not undergo apoptosis to a great extent. If autophagy is a pro-survival response then one might expect that upon inhibition of autophagy cells will die. (Bristol et al, 2012).

In our studies, Hs578t cells show minimal apoptosis when exposed to radiation alone and furthermore apoptosis does not increase with autophagy inhibition. Cells also show lack of sensitization with merely additive effects using autophagy inhibitors. Irradiated Hs578t cells clearly show an increase in autophagic markers such as acridine orange staining as well as an increase in autophagic flux which was shown by p62 degradation. If autophagy is apparently not cytoprotective, then it might be playing a role in mediating cytotoxic effects of irradiation. Autophagy would be considered as being pro-death where inhibition of autophagy protects the cells from the radiation treatment (Gewirtz et al, 2009).

Cytotoxic autophagy where cells die by autophagic cell death without involvement of apoptosis or necrosis remains an unclear phenomenon, where debate continues if autophagy itself acts as a mode of cell death or just leads to some other form of cell death. Nevertheless, cytotoxic autophagy has clearly been indicated in some experimental systems such as in a study by Freundt et al, autophagic cell death has been reported which was induced by zVAD in limited number of cell types (Codogno et al, 2011). Such autophagy should demonstrate cell death occurs without apoptosis and suppression of autophagy pharmacologically or genetically should be able to rescue cell number. In the

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current system, cells are undergoing senescence and are not dying by apoptosis but at the same time cells are not rescued upon autophagy inhibition, indicating the absence of cytotoxic autophagy.

The time course study of cell viability demonstrated a sustained growth arrest pattern, which can be an indication of senescence. To further asses the possibility of senescence, irradiated cells were analyzed for beta galactosidase staining. Irradiated cells clearly showed induction of senescence.

Our laboratory has generated data that senescence and autophagy can be functionally dependent on each other (Goehe et al, in press). In Young et al (2009), it was reported that autophagy might be contributing to oncogene induced senescence. To verify if autophagy and senescence might be related in our experimental model, we investigated whether irradiation induced senescence has any relation with autophagy. Cells were again analyzed for beta galactosidase staining using the combinations of IR+ CQ and IR+ 3MA. After treatment with the autophagy inhibitors, senescence was apparently decreased. This can be indicative that in Hs578t cells, autophagy and senescence might be closely related responses. Understanding the relationship between autophagy and senescence is important in this system in order to understand the mechanism behind resistance to radiation.

Several experimental systems including cellular and animal models as well as clinical trials are in progress in order to study if sensitivity to chemotherapy or radiation treatment can be increased by using the autophagy inhibitor chloroquine or hydroxychloroquine (Kelekar et al, 2007, Amaravadi et al, 2007).

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Recently in our laboratory, it has been shown that 4T1 cells are not sensitized to the effects of radiation when autophagy was inhibited either in vivo or in vitro (Bristol et al, 2012, in press).

Another possibility of p53 status also should be taken into considerations, Hs578t do show different responses upon radiation which is not same as that of another breast cancer cell lines such as MCF-7s. MCF-7s are p53 wild type cells whereas Hs578t are p53 mutant cells which might be one of the basis for the different responses.

Thus it can be said that cytoprotective autophagy may not be a universal response of breast tumor cells upon radiation. Since autophagy can play a role in tumor prevention or tumor survival studying the nature and role of autophagy in cancer is an important aspect of cancer research.

SECTION 5: FUTURE EXPERIMENTS

In order to further study the role of autophagy in Hs578t cells, the effect of autophagy inhibition on radiation sensitivity using genetic approaches should be performed. To inhibit autophagy, atg5 or atg7 can be silenced using siRNA techniques. Loss of function of these genes essential for autophagy should result in autophagy inhibition.

Such genetic inhibition should rescue cells if autophagy is cytotoxic and promote cell death if autophagy is cytoprotective. If genetic inhibition does alter the response to radiation in a significant way, then it can be concluded that pharmacological inhibition was nonspecific whereas genetic inhibition is specific.

Effects of pharmacological inhibition suggested that cytoprotective autophagy may not be a general response in all breast tumor cells. In our studies, CQ (Chloroquine) and 3MA were used as pharmacological inhibitors. 3-MA is PI3 kinase inhibitor which acts on class I as well as class III (Liu J et al, 2012). 3MA is known to act on both classes of enzymes. Effects of 3MA can be thus nonspecific as it can act on class I which inhibit mTOR and induce autophagy instead of inhibiting autophagy (Shen et al, 2011).

In order to confirm that autophagy and senescence are correlated, quantification of beta galactosidase activity will be determined with irradiated cells treated with the combination treatment IR + CQ and IR + 3MA.

Time course studies for p62 degradation as well as for p21 induction will help to establish the relationship between autophagy and senescence.

Controversy in the literature continues whether autophagy is pro-survival or pro-death or can exist together. Our laboratory is currently interested in knowing what is the

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difference between cytotoxic and cytoprotective autophagy. Our laboratory also focuses on the relationship between autophagy and senescence and if they are dependent on each other. A clear distinction between pro-survival and pro-death autophagy would be helpful in order to understand the multiple roles of autophagy.

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VITA

Shweta Moreshwar Chakrdaeo was born in Mumbai, India and is a citizen of India. She grew up in Kalyan, Maharshtra. She completed her undergraduate degree in Pharmacy from University of Mumbai in 2008. She joined Mega Lifesciences Pvt. Ltd as a brand executive and handled three neutraceutical brands in India. She joined Department of Pharmacology and Toxicology in January 2011 for master's program. She has been working in Dr. Gewirtz's laboratory as a master's student.