RADIATION RESPONSES OF CHEMORESISTANT ADENOCARCINOMA CELLS: FROM MOLECULAR MECHANISMS TO NEW REVERSAL STRATEGIES

Lidiya Luzhna
Bachelor of Science, Prycarpathian National University, Ukraine, 2007

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ABSTRACT

Breast cancer is a major cause of cancer-related death among women throughout the world. Treatment of breast cancer often fails due to the development of resistance to both chemo- and radiotherapy.

The aim of this study was to analyze and compare the response to radiation of MCF-7 breast adenocarcinoma cells and MCF-7 cells that are resistant to doxorubicin (MCF-7/DOX). The results presented in this thesis show that drug-resistant MCF-7/DOX cells survive high doses of radiation exposure better than MCF-7 cells. Moreover, the chemo- and radioresistance of MCF-7/DOX cells share common molecular mechanisms and loss of sensitivity to radiation in chemo-resistant cells may be explained by alterations in their DNA methylation profile.

The results of experiments presented in this thesis may, therefore, serve as a first step for future analysis of tumour resistance to radio- and chemotherapy and for the development of novel epigenetic strategies for reversal of breast cancer resistance to cytotoxic treatment regimens.

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LIST OF ABBREVIATIONS

5-azaC – 5-azacytidine

Apaf-1 – apoptotic protease activating factor 1

ASO – antisense oligonucleotides

ATM – ataxia telangiectasia mutated

ATP – adenosine-5'-triphosphate

ATR – ATM-Rad3-related

Bad – Bcl-2 antagonist of cell death

Bax – Bcl-2 –associated X protein

Bcl-2 – B-cell lymphoma 2

BER – base excision repair

bp – base pair

BRCA1 – breast cancer gene 1

BRCA2 – breast cancer gene 2

BSA – bovine serum albumin

DABCO – 1,4-diazabicyclo[2.2.2]octane

DAPI – 4', 6-diamidino-2-phenylindole

dATP – deoxyadenine triphosphate

dCTP – deoxycytidine triphosphate

dGTP – deoxyguanine triphosphate

DHAC – dihydro-5-azadcytidine

DMEM – Dulbecco's Modified Eagle's Medium

DNA-PK – DNA-dependant protein kinase

DNMT – DNA methyltransferase

DSB – double strand break

DTT – dithiothreitol

dTTP – deoxythymidine triphosphate

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

ER – estrogen receptor

FANCF – Fanconi anemia complementation group F

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

GST – glutathione-S-transferase

HDAC – histone deacetylase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER2 – human epidermal growth factor receptor 2

HR – homologous recombination

IAP – inhibitor of apoptosis proteins

IGF-IR – insulin-like growth factor-I receptor

Ig G – immunoglobulin G

IR – ionizing radiation

MCF-7 – Michigan Cancer Foundation cells. The trailing seven refers to the number of attempts that were required to establish stock of cells

MDR – multiple drug resistance

MGMT – O6-methylguanine DNA methyltransferase

miRNA - microRNA

MMR – mismatch repair

MRP – multidrug resistance protein

MTD – maximum tolerable dose

MUC1 – mucine-1

NER – nucleotide excision repair

NF-kB – nuclear factor kappa beta

NHEJ – non-homologous end-joining

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PCR – polymerase chain reaction

Pgp – P-glycoprotein

PI – propidium iodide

PI3K – phosphatidylinositol-3-kinase

PKB – protein kinase B

PP2A – protein phosphatase 2A

PS - phosphatidylserine

PVDF – polyvinylidene difluoride

Rb - retinoblastoma

SAH – S-adenosyl-homocysteine

SAM – S-adenosyl-methionine

SDS – sodium dodecyl sulphate

SEM – standard error of the mean

TG2 – tissue transglutaminase

TGFβ – tumour growth factor beta

Top2A – topoisomerase 2 alpha

VEGF – vascular endothelial growth factor

CHAPTER 1: GENERAL INTRODUCTION

BREAST CANCER AND BREAST CANCER TREATMENT OPTIONS

Breast cancer develops as an uncontrolled growth of breast cells that often form malignant tumors. Breast cancer is one of the major clinical challenges throughout the world (Russo, et al., 2000). It has become the second leading cause of cancer-related deaths among North American women and the leading cause of death among women aged 35 to 55 years (Schairer et al., 2004; Widschwendter and Jones, 2002). According to Canadian Cancer Statistics (2009) breast cancer accounts for up to 22,700 new cases among Canadian women in 2009 with total deaths of 5,400 (www.cancer.ca/statistics). The histological origin of breast cancer can be either lobular that begins in the milk producing glands or ductular when a tumor originates in ducts that carry milk from the glands to the nipple (www.breastcancer.org).

Numerous clinical studies identified several main breast cancer risk factors (Pike et all., 1993). About 5 – 10 % of breast cancers are hereditary, caused by mutations in breast cancer susceptibility genes BRCA1 and BRCA2 which normally prevent uncontrolled cell growth. Other genes which, when mutated, can be associated with breast cancer are: the DNA repair gene ATM, the tumour suppressor gene p53, the cell growth regulator PTEN, and the human epidermal growth factor HER2 (Russo et al., 2000). Estrogen (E_2) is a well-known hormonal breast carcinogen. Women with elevated E_2 levels are considered to be a high-risk group for breast cancer development (Bernstein and Ross, 1993). Other breast cancer risk factors include age, exposure to ionizing radiation, increased alcohol consumption, postmenopausal obesity and nulliparity (Russo and Russo, 2004).

The main treatment approaches are based on a patient's risk level and stage of cancer. For the majority of patients, surgery followed by radiation and chemotherapy is a standard treatment procedure (Guarneri and Conte, 2004; Veronesi, 1990). A breast cancer treatment plan is a doctor's/patient's choice made from the whole menu of treatment options available now.

Surgery is usually the first line of attack against breast cancer (Arriagada et al., 1996). Systemic therapy is a form of treatment that affects the whole body through the bloodstream. Hormonal therapy and chemotherapy are commonly used types of systemic therapy (National Cancer Institute: www.cancer.gov). Hormonal or anti-estrogen therapy is used against hormone-receptor-positive breast cancer and is based on blocking the action of estrogen (www.breastcancer.org), while chemotherapy is based on the usage of drugs to kill cancer cells (www.breastcancer.org/treatment/chemotherapy). Radiation therapy, or radiotherapy, is a highly targeted way of cell killing using a high-energy beam of ionizing radiation to damage DNA in cancer cells (www.breastcancer.org/treatment/radiation).

CHEMOTHERAPY AND CHEMORESISTANCE

TYPES OF CHEMOTHERAPY

Chemotherapy is based on the usage of one drug or a combination of drugs to kill cancer cells. In contrast to surgery or radiotherapy, it does not have a local effect but targets different types of cells and organs as a chemodrug is transported throughout the body by blood. Depending on treatment goals, there are at least three types of chemotherapy treatment: a. neoadjuvant chemotherapy is used to reduce the

size of tumour before surgery or radiotherapy (Kuremsky et al., 2009); b. adjuvant chemotherapy when drugs are used to remove residual cancer cells remaining in the body after other treatments (Winter-Roach et al., 2009); c. combination chemotherapy that is used to destroy cancer by itself or in combination with other treatments (Baum et al., 2002).

The most common chemotherapy agents for breast cancer are:

- alkylating agents that bind to DNA and break links between DNA strands (melphalan, cyclophosphamide, cisplatin);
- anticancer antibiotics that inhibit topoisomerase which induces DNA breaks (doxorubicin, daunomycin, dactinomycin);
- drugs derived from plants that damage mitotic spindle (taxanes, vinblastine);
- antimetabolites that inhibit enzymes participating in DNA and RNA synthesis (methotrexate, 5-azacytosine) (Stavrovskaya, 2000).

ANTHRACYCLINES AS ANTI-CANCER AGENTS

Amongst the agents listed above, anthracyclines have the widest spectrum of activity in cancers, and only a few cancers (e.g., colon cancer) do not respond to them (Weiss, 1992). Doxorubicin was one of the first two anthracyclines with high toxicity in mammalian cells *in vitro* and in *vivo* (Barranco et al., 1973; Lomovskaya et al., 1999).

Doxorubicin hydrochloride is used for treatment of a wide variety of cancers such as cancer of the bladder, breast, head and neck, liver, lung, blood (leukemia), lymph (lymphoma), ovaries, pancreas, prostate, stomach, testis, thyroid and uterus and

mesothelioma, multiple myeloma, neuroblastoma and sarcoma. (www.chemocare.com/bio/doxorubicin.asp). Doxorubicin was isolated from the bacterium *Streptomyces peucetius var. caesius* (Lomovskaya et al., 1999). Cells in the S phase of the cell cycle are the most sensitive to the cytotoxic action of doxorubicin. The cytotoxic activity of doxorubicin is based on the DNA intercalating property of this drug. Doxorubicin creates a complex with DNA by intercalating its chromophore group in GC-rich sequences, while the groups in the rings form hydrogen bond with the base pairs above and below the intercalation site (Manfait et al., 1982). Such complexes interfere with polynucleotide synthesis. The main target for doxorubicin is the enzyme topoisomerase 2α (Top2A) that is important for unwinding DNA for transcription and replicaton. Mechanistically, doxorubicin intercalates DNA and inhibits the progression of Top2A. Functionally, it stabilizes the Top2A complex after it breaks the DNA chain, thus preventing DNA resealing and thereby blocking replication (Fornari et al., 1994; Fortune and Osheroff, 2000; Frederick et al., 1990). Inhibition of topoisomerase II by doxorubicin is accompanied by the formation of free radical-mediated double-strand breaks in DNA. Cytotoxicity of oxygen free radicals formed by doxorubicin action is secondary to lipid peroxidation of cell membrane lipids (Fornari et al., 1994). In addition, several studies reported the ability of doxorubicin to inhibit the viral, bacterial, and mammalian DNA-dependent DNA polymerase and bacterial RNA polymerase (Momparler et al., 1976).

Doxorubicin and epirubicin (differing chemically from doxorubicin only in the spatial orientation of one hydroxyl group) are frequently used in the curative-intent adjuvant and palliative treatment of metastatic breast cancer (Dean-Colomb and Esteva, 2008; Fornari et al., 1994). Although, doxorubicin is among the most active

agents in breast cancer treatment, many women experience a relapse. Furthermore, approximately half of women with metastatic breast cancer fail to respond to doxorubicin, and the majority of those showing initial benefits subsequently demonstrate acquired clinical resistance, as demonstrated by tumor growth despite ongoing anthracycline therapy (Dean-Colomb and Esteva, 2008; Fornari et al., 1994). Chemoresistance can be broadly defined as protection of cancer cells against chemotherapy.

MULTIPLE DRUG RESISTANCE (MDR)

General principles of MDR

Because mechanisms of chemoresistance are multifactorial and tumour cells develop many ways to eliminate effects of chemotherapy drugs, resistance often develops to a broad spectrum of drugs and cells acquire multiple drug resistance (MDR) (Ling, 1997; Stavrovskaya, 1999). MDR is a phenomenon that contributes to virtually half of breast cancer deaths and still, unfortunately, remains uncontrollable. Therefore, the problem of MDR is actively researched (Gonzalez-Angulo et al., 2007). There are two main goals in studying MDR: a) understanding the mechanisms of MDR in breast cancer cells; b) reversal of the MDR phenotype to form drug-sensitive cells.

Overall, MDR is a multi-factorial phenomenon involving numerous mechanisms (Lehnert, 1996; Shishova and Chekhun, 2000; Szakăcs et al., 2006). At present, several major mechanisms are being investigated for their involvement in acquired chemotherapeutic drug resistance in cancer cells. These include: decreased uptake of water-soluble drugs which require transporters to enter cells (Gottesman, 2002); various cellular changes such as alterations in cell cycle and signal

transduction pathways; increased repair of DNA damage; reduced apoptosis; altered drug metabolism that diminishes the capacity of cytotoxic drugs to kill cancer cells (Hickman, 1996; Pommier et al., 2004; Stavrovskaya, 2000); increased energy-dependent efflux of hydrophobic drugs (Gotteman, 2002; Gottesman and Ling, 2006); and increased DNA tolerance to DNA- damaging drugs through the inactivation of DNA repair pathways (Karran, 2001).

Efflux pumps and chemoresistance

A decrease in drug accumulation can result from either a decreased drug influx or an increased drug efflux. Obviously, both are connected to changes in the plasma membrane. Drug-resistant and drug-sensitive cancer cells often have different lipid composition (Simon and Schindler, 1994), but the main difference is the presence of energy-dependant pumps which exclude cytotoxic substances from cells. The two main ATP-dependant pumps that are currently characterized are a P-glycoprotein (Pgp-MDR) and a multidrug resistance-associated protein (MRP) (Kars et al., 2006; Ling, 1997). Pgp-MDR1 and MRP belong to the superfamily of ABC (ATP-Binding Cassette) transporters which transport different substrates ranging from inorganic ions to polysaccharides, proteins and drugs (Higgins, 1995). Both proteins are expressed in normal tissues at low levels. Their expression levels are significantly increased in cells with acquired chemoresistance (Chekhun et al. 2007; Thottassery et al., 1997). Pgp is a large transmembrane glycoprotein encoded by the MDR1 gene. It consists of two subunits and includes six hydrophobic transmembrane segments and two ATP-binding domains. Substrate binding sites (e.g. anti-cancer drugs) are located in the transmembrane domains or under the membrane (Bosh and Croop, 1996). The drug molecule binds to a specific site on MDR1 and is transported from the cell after ATP

hydrolysis. There are some recent suggestions that this protein may function as a flipase which moves substances out of the cell (Higgins et al., 1997).

The MRP protein is encoded by the MRP1 gene and causes resistance of cancer cells to drugs identical to that one mediated by MDR1. The functional activity of MRP is different from MDR1 as it requires cellular glutathione for drug effluxing (GS-X pump) (Borst et al., 1997; Deeley and Cole, 1997; Morrow and Cowan, 1990). Glutathione interacts via its thiol with a chemo drug. A drug and thiol conjugate is water-soluble and less toxic. Moreover, it can be excluded from the cell by GS-X pumps (Stavrovskaya, 2000). Numerous studies indicate that increased levels of glutathione and its isoenzyme GSTπ are frequently found in chemoresistant cells (Tew, 1994).

DNA repair and chemoresistance

The cytotoxic effect of many anti-cancer drugs is based on the ability of these drugs to cause DNA damage. Therefore, the MDR phenotype can be associated with the ability to remove potentially lethal DNA lesions. Several studies indicate an increased DNA repair potential in MDR cells. The main DNA repair pathways involved in chemoresistance are: nucleotide-excision repair (NER) which removes DNA adducts produced by some therapeutic anti-cancer drugs, base excision repair (BER) that removes more subtle forms of DNA damage such as oxidized/reduced bases, chemically modified bases and small adducts, and the single-strand and double-strand break repair pathways (Karran, 2001). Paradoxically, inactivation of mismatch (MMR) repair is accompanied by tolerance to the cytotoxic effect of some drugs (Buermeyer et al., 1999). In MDR cells, potentially lethal DNA lesions are often not

excised from DNA but instead become persistent and uncoupled from cell death (Karran, 2001). Changes in DNA repair in chemoresistant cells can be explained not only by elevated amounts of proteins which repair DNA but also by increased levels of some proteins involved in recognizing DNA injury and recruiting repair complexes to damaged sites (Chu, 1994).

Apoptosis and chemoresistance

A common feature of drug-resistant cancer cells is that they exhibit a profound resistance to apoptosis. Examples of numerous alterations that cause resistance to apoptosis in advanced cancers are: activation of pro-survival signal transduction pathways mediated by Ras, PI3K/Akt, or NF-kB; inactivation of apoptotic pathways due to mutation or silencing of p53, pRb, Bax, Bad, Apaf-1, caspase-8 genes or overexpression of pro-survival proteins such as Bcl-2, IAP, and FLIP, and others (Fesik, 2005). It has recently been found that drug-resistant cancer cells isolated from metastatic sites expressed very high levels of a multifunctional protein, tissue transglutaminase (TG2), compared to the parental drug-sensitive cell line from which they were derived. TG2 is known to associate with integrin proteins and promote stable interactions between cells and the extracellular matrix, resulting in increased cell survival, cell migration and invasion. TG2 also causes activation of the nuclear transcription factor-kB (NF-kB) (Verma and Mehta, 2007). The anti-oncogene PTEN can also influence drug resistance. PTEN is a tumor suppressor and a broad spectrum phosphatase which negatively regulates the phosphatidylinositol 3'-kinase (PI3'K) PKB/Akt signalling pathway. It was shown that PTEN-deficient cells demonstrated resistance to some cytotoxic agents (Stambolic et al., 1998).

Doxorubicin resistance

As mentioned above, doxorubicin is one of the most widely used drugs for treatment of different types of cancer, including breast cancer. But it is also a well known fact that cancer cells quickly develop resistance to doxorubicin. Furthermore, resistance to the maximum tolerable dose (MTD) of doxorubicin was quickly and readily obtained in mammary tumours in mice with conditionally mutated p53 and Brca1 tumour suppressor genes (Rottenberg et al., 2007). Tolerance to doxorubicin includes various mechanisms, some of which are well studied and described. It is known that doxorubicin-resistant cells such as MCF-7 breast adenocarcinoma cells are Pgp-MDR1 and MRP1 positive. Such cells express both MDR1 and MRP1 genes which code for ABC transporters involved in export of doxorubicin out of the cell before it can reach its target (Kars et al., 2006). An interesting fact about doxorubicin resistance mediated by Pgp is cross-resistance to taxanes because other biochemical doxorubicin resistance mechanisms do not affect taxane sensitivity (Rottenberg et al., 2007). As an inhibitor of topoisomerase II, doxorubicin stabilizes the DNA-topoisomerase complex which under normal circumstances is easily disassembled. In doxorubicin-resistant cells, the activity and quantities of this enzyme are reduced. Mutations in the topoisomerase II gene could also be a cause of drug resistance and elevated expression of tissue transglutaminase (TG2) in doxorubicin resistant cells was also reported (Verma and Mehta, 2007). Moreover, doxorubicin resistant cells have reduced susceptibility to DNA single- and double-strand breaks, and DNA damage is more prolonged and might be lethal in sensitive cells, while it is repairable in doxorubicin resistant cells (Goldenberg et al., 1986).

GST is also implicated in resistance of cancer cells to doxorubicin (Batist et al., 1986). Elevation of anionic GST has been described in human MCF-7 cells

resistant to doxorubicin, and in some cases, GST activity was 50 % higher in drug-resistant cells compared to sensitive lines (Deffie et al., 1988). All of the above is the evidence that resistance to doxorubicin is a multifactorial phenomenon. Such multifactorial resistance to doxorubicin may explain cross-resistance of doxorubicin-resistant cells to other compounds that in some cases are not even structurally related such as actimomycin D, daunorubicin, mitoxantrone, colchicines and etoposide (Deffie et al., 1988).

RADIATION THERAPY AND RADIORESISTANCE

Radiation therapy is the use of ionizing radiation to destroy cancer cells; it has been practiced in cancer therapy for more than a hundred years (Camphausen and Lawrence, 2008). Radiotherapy has become a recognized treatment modality for breast cancer patients. It is considered mandatory for patients undergoing conservative surgery and is appropriate for women at high risk of recurrence after mastectomy (Pierce et al., 2000).

Radiotherapy works by damaging the DNA of cells (Cuzick, 2005). Radiation induces a variety of DNA lesions such as damage to nucleotide bases, cross-linking, DNA single- and double-strand breaks (Little, 2000). The limitation of radiotherapy is that solid tumour cells often become deficient in oxygen after radiation exposure. This means that such tumours can outgrow their blood supply causing hypoxia (Harrison et al., 2002). In order for radiation to form DNA-damaging free radicals, the presence of oxygen is crucial. Under hypoxic conditions, cancer cells can be 2 to 3 times more resistant to radiation. Because oxygen is a strong radiosensitizer,

much research has been devoted to the development of blood substitutes that carry increased oxygen (Harrison et al., 2002).

In general, each type of cancer has different radiosensitivity (Nunez et al., 1996). Breast cancers are ranked as from moderately radiosensitive to radioresistant, therefore requiring significantly higher doses of radiation (45-60 Gy) to achieve radical cure than many other tumor types. The total dose is devided into 1.8-2 Gy fractions per day for several weeks (Tutt and Yarnold, 2006). Undifferentiated breast cancer cells generally reproduce faster and have lower capacity to repair sub-lethal damage caused by ionizing radiation in comparison to healthy differentiated cells. Therefore, fractionation allows normal cells to recover, while tumour cells are less efficient in repair between fractions (Pedraza Muriel, 2002).

There are two types of radiation therapy applied in breast cancer treatment: external and internal radiotherapy. External beam radiation is the most common radiation given after surgery. In this technique, a special X-ray machine called a simulator delivers a beam of high-energy radiation to the area of cancer. Internal radiation or brachytherapy is a less common form of radiation treatment after lumpectomy. During brachytherapy, small pieces of radioactive material called seeds are placed at the tumor area of the breast and the seeds emit radiation into the surrounding tissue (www.breastcancer.org/treatment/radiotherapy).

Radiotherapy can be used as a primary treatment, or it can be combined with surgery, chemotherapy or hormone therapy (Koukourakis et al., 1999). In about 70 % of breast cancer cases, radiotherapy reduces the risk of relapse (Cuzick, 2005). At least half of patients who develop recurrences after surgery and radiotherapy have invasive cancer with a high risk of metastasis and death (Boyages et al., 1999). In

patients who develop disease recurrence, the actual resistance mechanisms to radiotherapy remain unclear but the biology of radioresistance in breast cancer cells includes various extra-nuclear and intra-nuclear factors (Jameel et al., 2004).

Some extra-nuclear factors that cause resistance to radiation:

1) The insulin-like growth factor-I receptor (IGF-IR) is a tyrosine kinase that regulates cell growth, differentiation, transformation and apoptosis (Peretz et al., 2001). The levels of IGF-IR and its substrate are elevated in ER-positive breast tumours and can be linked with increased radioresistance and cancer relapse (Bartucci et al., 2001). Some experimental studies on primary breast tumours reported that expression of IGF-IR directly influenced radioresistance, and high levels of IGF-IR correlated with increased tumour recurrence after radiotherapy (Turner et al., 1997).

2) The phosphatidylinositol 3-kinase (PI-3K) pathway regulates cell progression and cell proliferation and inhibits apoptosis. The main effector of this pathway is a serine/threonine protein kinase PKB that initiates S phase and the G2-M transition of the cell cycle (Shtivelman, 2003). There are evidences that PI-3K/PKB activity contributed to resistance of human breast cancer cells to ionizing radiation. Inhibition of the pathway radiosensitized breast cancer cells (Liang et al., 2003).

3) Epidermal growth factor (EGF) – an extracellular factor which also controls cell growth and proliferation. Wollman et al. demonstrated that adding EGF to MCF-7 cells prior to irradiation caused radioresistance of cells by increasing the fraction of S-phase cells and glutathione levels (Wollman et al., 1994).

4) Human epidermal growth factor receptors (HERs) play a role in mammalian growth and development (Earp et al., 2003). Some current data show that overexpression of EGFRs is widely correlated with resistance to radiotherapy and

other forms of adjuvant therapy (Wallman et al., 1994). It has been shown in different studies that HER inhibitors affect cellular responses to ionizing radiation inducing apoptosis, cell cycle arrest and influencing DNA repair (Sartor et al., 2003). Liang et al. showed that using Trastuzumab (Herceptin), an antibody against HER2, sensitized breast cells to radiotherapy (Liang et al., 2003).

5) Vascular endothelial growth factor (VEGF) is an important mediator of endothelial cell proliferation, survival and angiogenesis. It is thought that there is a link between VEGF overexpression and radioresistance (Gupta et al., 2002). VEGF is found to be upregulated under hypoxic conditions, and failure of radiotherapy, as mentioned previously, is often associated with tumour hypoxia (Manders et al., 2003; Wachsberger et al., 2003).

Some intracellular factors that affect radioresistance:

1) p53 mutations are the most common genetic alterations in human breast carcinoma which are associated with pure prognosis and chemo/radioresistance due to the absence of p53-dependant apoptosis (Marchetti et al., 2003). The mechanism of radioresistance development in p53 mutants is believed to be due to loss of control over cell cycle, DNA repair and apoptosis. There is a possibility of retention of radiation-induced defects in the progeny of irradiated cells (Mazurik and Moroz, 2001).

2) BRCA1 and BRCA2 genes are responsible for about 80-90 % of hereditary breast cancer (Thull and Vogel). They function as tumour suppressor genes that play an important role in homologous recombination DNA repair and NER. Xia et al. found upregulation of HR and increased radioresistance when wild type BRCA2 was transfected into a BRCA2 defect tumour cell line (Xia et al., 2001), while Abbot et al.

showed that human cancer cells containing mutated BRCA1 are hypersensitive to ionizing radiation (Abbott et al., 1999).

3) Telomeres are functional elements of eukaryotic chromosomes responsible for maintaining stability of chromosomes, and they play an important role in cellular response to DNA damage. McIlrath et al. revealed a seven-fold reduction in telomere length in radiosensitive cells in comparison with radioresistant cells, thus concluding that telomere length can be used as a marker of radiosensitivity (McIlrath et al., 2001).

Despite the presence of numerous studies on mechanisms of radioresistance, it is still not known how to best distinguish breast cancer patients who would positively respond to radiation treatment from those who would develop radioresistance (Jameel et al., 2004).

Radiotherapy is usually combined with anthracycline chemotherapy, and some chemoradiation studies confirm local control and survival benefits of such treatment (Chakravarthy et al., 2000). Tumour cells of hematopoietic or lymphatic origin frequently undergo apoptosis after combined treatment with irradiation and doxorubicin (Ling et al., 1993; Skladanowski and Konopa, 1993; Zaleskis et al., 1994). In contrast, breast cancer is relatively refractory to cell death after either irradiation or chemotherapy (Fornari et al., 1996). There is even evidence that cell killing and growth inhibition occur only transiently after combined chemo- and radiotherapy, and extensive cell-killing tumours even re-grow after irradiation. Waldman et al. indicate that cells with intact G1 demonstrate a prolonged cell cycle arrest but not apoptosis in response to treatment. This permits re-growth of a sufficient number of breast cancer cells which repopulate the breast (Waldman et al., 1997). The

absence of apoptosis is associated with chromosomal instability, which can further substantiate radioresistance (Morgan et al., 1996).

Anthracyclines are frequently used as radiosensitizers, but their use often results in unacceptable levels of normal tissue toxicity and cardiac toxicity. This means that changes in chemo- and radiotherapy administration are needed to reduce cancer mortality and treatment side effects and increase the treatment efficacy (Cuzick, 2005).

DNA DAMAGE AND REPAIR

The cytotoxic effect of many anticancer drugs, such as doxorubicin and ionizing radiation, relies on the ability of these agents to damage DNA. Interestingly, different patients have different levels of sensitivity to drugs and radiotherapy. Usually, before treatment decision making, it is important to check radio- and chemo-sensitivity of breast cancer cells in the laboratory (Girinsky et al., 1992). The main indicator of radio- and chemosensitivity of breast cancer cells is the rate of DNA damage (usually double-strand breaks) and DNA repair capacity (Kelland et al., 1988).

The most common radiation and doxorubicin-induced forms of damage are double-strand breaks in DNA. About 40 DNA double-strand breaks are induced in a cell for each 1 Gy (Kanaar et al., 1998; Rich et al., 2000). Doxorubicin induces DNA breaks by blocking the function of cellular Top2α. There are several features that make DSBs difficult to repair. As both strands of DNA are broken, the broken ends can dissociate and interact with breaks at other sites that might lead to translocations

and deletions. The ends of breaks have sustained damage to bases that need to be replaced. In a single-strand break, such bases can be easily replaced using the opposite complementary strand as a template, while in DSB such template is unavailable (Tutt and Yarnold, 2006).

The response to radiation and doxorubicin-induced DNA damage includes recognizing DSBs and further recruiting a highly regulated signal transduction cascade that regulates changes in cell cycle progression and chromatin modifications around sites of DNA DSBs. Unrepaired DSB could lead to apoptosis (Rich et al., 2000). Therefore, recognition of DSB is a sensitive and rapid mechanism. Some data suggest that sensing mechanisms may distinguish between DNA damages that could be repaired and those which require a wider response, such as cell cycle checkpoint activation (Bradbury and Jackson, 2003). Initial sensing molecules are unknown, but the main model of DNA damage response suggests recruitment of proteins of the PI3-kinase-like family to damaged sites and phosphorylation of histones around DSBs. The second phase includes signal transducers which recruit repair proteins to the site of damage (Tutt and Yarnold, 2006).

A key component in DSB recognition and DNA repair is histone H2AX which becomes rapidly phosphorylated in response to DNA double-strand breaks (Banath et al., 2004). H2AX is a variant of histone H2A. Each nucleosome contains 2 to 20 % of H2AX depending on the cell type; overall, it is estimated that every fifth nucleosome includes an H2AX molecule (Bonner et al., 2008).

Immediately upon DSB formation after exposure, PI3K-like kinases, including ataxia telangiectasia (ATM), ATM-Rad3-related (ATR) and DNA-dependant protein kinase (DNA-PK), phosphorylate H2AX and other DNA repair

proteins (Bonner et al., 2008). It is believed that phosphorylation occurs within one minute after exposure to radiation. The maximum number of phosphorylated H2AX (γH2AX) molecules can be reached in 0-30 minutes after exposure and disappear slowly over time due to the rejoining of DNA breaks (Sedelnikova and Bonner, 2006). The presence of one γH2AX focus represents one physical break; therefore γH2AX has become the most popular indicator of DNA damage level. Using an antibody against γH2AX reveals the discrete number of DSBs. When double-strand breaks are repaired, foci disappear (Pilch et al., 2003). However, some studies indicate that γH2AX foci remain elevated even after most DSBs have been rejoined, suggesting that γH2AX removal may be dependant on some other mechanisms that follow DNA repair (Kinner et al., 2008). Now there are two known mechanisms of γH2AX removal: dephosphorylation of γH2AX and removal of γH2AX from the chromatin (Bonner et al., 2008). Chowdhury et al. have proved that dephosphorylation is performed by protein phosphatase 2A (PP2A) (Chowdhury et al., 2005).

Overall, the initial detection of DSB involves localization of Nijmegen breakage syndrome protein at the site of DSB and two other proteins (MRE11 and Rad50) which activate ATM (Kang et al., 2005). ATM activation means dissociation of homodimers by phosphorylating each other. Functions of ATM are the following: a) chromatin modification by phosphorylation of histone H2AX (described above); b) recruitment and regulation of both NHEJ and homologous recombination (HR); c) regulation of the cell cycle at G1/S via p53 and MDM2 proteins and at G2/M via BRCA1 and CHK1; d) regulation of apoptosis through CHK2 and p53 (Bakkenist and Kastan, 2003; Shiloh, 2003; Taylor et al., 1975). The second phase of repair includes the work of signal transducers CHK1 and CHK2. CHK1 involves BRCA1 (controls

G2/M checkpoint) into the process, confirming that DNA damage is repaired before cell division resumes. CHK2 not only regulates G1/S and G2/M checkpoints but also helps make a choice between different repair pathways (Tutt and Yarnold, 2006). The end of the cascade involves the actual repair proteins.

The main pathways of DSBs repair are: non-homologous end-joining (NHEJ) and Rad51-dependant homologous recombination (HR) (Kanaar et al., 1998). Each of them has different consequences on cell survival and genome stability. NHEJ is usually active during G0/1 and an early S phase when DSB repair does not depend on the presence of a template. NHEJ does not restore the original sequence, and in fact, it is highly error- prone, but it does restore the physical integrity of DNA. The actual joining of the two ends of the broken DNA requires such proteins as Ku70/Ku80, DNA-PKcs, XRCC4 and ligase 4 (Takata et all., 1998; Tutt and Yarnold, 2006; Wang et al., 2001).

Homologous recombination accounts for about 10% of DSBs repair in the mammalian genome and occurs during S and G2 phases of the cell cycle (usually in less differentiated cells) and requires the presence of a sister chromatid template. A homologous sequence of a sister chromatid provides highly accurate repair. This process involves an exchange of DNA single strands between homologous chromosomes (Jackson, 2002). Lesions having been identified, several proteins begin to associate with DNA. These proteins, including RAD52, RAD54 and BRCA2, act as mediators and ultimately lead to the presence of RAD51—a protein responsible for conducting a homology search (Powell and Kachnic, 2003; Tutt et al, 2001; Venkitaraman, 2002).

From what we described above, we can briefly conclude that radiosensitive cells develop more DSBs per unit dose, and the repair process is slower than in resistant cells. It can be directly linked to the confirmation of DNA which varies during the cell cycle and establishes the choice of DNA repair pathway.

EPIGENETICS OF DRUG RESISTANCE

Presently, three hypotheses – genetic, karyotypic and epigenetic – have been proposed to explain the mechanisms of acquired cancer drug resistance (Baker and El-Osta, 2003; Biedler and Spengler, 1994; Duesberg et al., 2007; Fojo, 2007; Glasspool et al., 2006; Iwasa et al., 2006; Roberti et al., 2006; Teodoridis et al., 2004). 'Genetic' is defined as a heritable change in the DNA sequence, according to which the occurrence of random drug-induced mutational events leads to the formation of drug-resistant cells from sensitive cells (Biedler and Spengler, 1994; Iwasa, 2006). 'Epigenetic' refers to the information contained in the chromatin rather than in the actual DNA sequence (Jaenisch and Bird, 2003) which is mediated via short RNAs. According to this hypothesis, the induction of epigenetic changes results in resistance to cytotoxic drugs (Baker and El-Osta, 2003; Glasspool et al., 2006). The absence of convincing evidence that genetic changes have a role in acquired clinical resistance following anti-cancer therapy undermines the genetic hypothesis (Glasspool et al., 2006).

In contrast, a number of studies have indicated substantial alterations of epigenetic elements in drug-resistant cancer cells, including changes in DNA methylation, histone modification and short RNA patterns (Fojo, 2007; Glasspool et

al., 2006; Teodoridis et al., 2004). The 'karyotypic' hypothesis that refers to abnormalities in the number, form or structure of chromosomes (Duesberg et al., 2007; Fojo, 2007; Glasspool et al., 2006; Iwasa et al., 2006; Teodoridis et al., 2004) is closely related to the epigenetic hypothesis since epigenetic changes are necessary precursors of karyotypic changes (Matarazzo et al., 2007; Wilson et al., 2007). Therefore, karyotypic changes may be considered as a consequence of a progression of epigenetic alterations and may serve as indirect evidence for the importance of epigenetic dysregulation in the acquisition of cancer drug resistance.

Epigenetics is "*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*" (Russo et al., 1996). In eukaryotic cells, epigenetic changes include DNA methylation and histone modifications (Hirst and Marra, 2008).

Cytosine DNA methylation was the first epigenetic alteration identified; and currently it is the most widely studied epigenetic mechanism. It is crucially important for the normal development, cell proliferation and proper maintenance of genome stability of a given organism (Baylin 2005; Baylin and Ohm, 2006; Jaenisch and Bird 2003).

DNA methylation occurs at the C5 position of cytosines that precede guanines and form the so-called CpGs (Bird, 2002; Weber and Schubeler, 2007). Approximately 1 % of all cytosines in the genome are methylated, which corresponds to 70-80 % of all CpGs (Ehrlich et al., 1982; Weber and Schubeler, 2007). CpGs are not randomly distributed in the genome but form CpG rich regions called "CpG islands" (Antequera and Bird, 1993). Methylation of DNA is a transfer of a methyl group from the methyl donor S-adenosyl-methionine (SAM) to the carbon 5 position

of cytosine which is catalyzed by DNA methyltransferase enzymes (DNMT), DNMT1, DNMT3a and DNMT3b (Szyf et al., 2004; Teodoris et al., 2004). DNMT1 functions primarily to maintain a methylation pattern after DNA replication (Vertino et al., 2002), while DNMT3a and DNMT3b act mainly as *de novo* methyltransferases establishing methylation patterns during development (Chen et al., 2003). CpG island methylation is associated with gene silencing (Jones et al., 1998). Loss of DNA methylation at CpGs was the first epigenetic change identified in cancer cells (Feinberg and Vogelstein, 1983).

In breast cancer, there are two changes in a methylation pattern established: global hypomethylation and regional hypermethylation of promoter regions of certain genes (Szyf et al., 2004). There have been proposed three mechanisms to explain the contribution of DNA hypomethylation to cancer development: (a) an increase in genomic instability; (b) reactivation of transposable elements; and (c) loss of imprinting (Esteller, 2008). Hypomethylation could be possibly involved in activation of tumour promoting genes and pro-metastatic genes. Interestingly, along with global hypomethylation, cancer cell genomes are characterized by hypermethylation of the CpG islands localized in the active regions of tumour suppressor genes and microRNA genes (Hirst and Marra, 2008). Inactivation of tumour suppressor genes through hypermethylation of CpGs also plays a significant part in the process of carcinogenesis.

Undoubtedly, changes in DNA methylation are not isolated events, and they occur in the context of global chromatin deregulation and altered histone modification levels (Jaenisch and Bird, 2003; Weidman et al., 2007). Histone modifications, including acetylation, methylation, phosphorylation and ubiquitination, are important in the

transcriptional regulation (Jenuwein and Allis, 2001; Kovalchuk and Baulch, 2008; Weidman et al., 2007). Many histone modifications are stably maintained during the process of cell division. The acetylated histone tails lose their positive charge, reducing the affinity to negatively charged DNA,and leading to the relaxed chromatin packaging. Therefore, histone acetylation is linked to transcriptional activation, while histone deacetylation is an opposite repression event (Jenuwein and Allis, 2001). Histone methylation can result in different transcriptional consequences depending upon the type of residue affected (Cheung and Lau, 2005; Saha et al., 2006). Histone H3 lysine 9 methylation is associated with chromatin compaction and gene silencing, while histone H3 lysine 4 and lysine 27 methylation results in transcription activation and chromatin relaxation. Additionally, histone residues can be mono-, di- and tri-methylated, adding an enormous complexity to the yet unexplored histone code (Cheung and Lau, 2005; Saha et al., 2006; Weidman et al., 2007). It was recently shown that tumors undergo a massive loss of tri-methylation at lysine 20 of histone H4, which was suggested to be a universal marker of malignant transformation (Fraga et al., 2005; Tryndyak et al., 2006a; Tryndyak et al., 2006b; Fraga et al., 2005).

Phosphorylation is another important histone modification (Sedelnikova, 2003). One of the best-studied modifications is phosphorylation of histone H2AX. H2AX is a member of the H2A histone family. It becomes phosphorylated at serine 139 (γH2AX) as one of the earliest cellular responses to double strand breaks (DSBs) (Pilch et al., 2003; Rogakou et al., 1998; Sedelnikova et al., 2003). γH2AX accumulates in the nucleus forming γH2AX foci; a direct correlation has been found between H2AX phosphorylation and the number of DSBs resulting from radiation (Celeste et al., 2003a). γH2AX is crucially important for the repair of DNA strand

breaks and for the maintenance of genome stability (Celeste et al., 2003b). Finally, epigenetic control can also be mediated by small regulatory RNAs (Bernstein and Allis, 2005).

STRATEGIES FOR REVERSING DRUG RESISTANCE

Drug resistance, no matter whether it is intrinsic or acquired, is the main problem hindering the success of chemotherapy. Conventional drugs have limited potential, but it seems that if drug resistance can be overcome, the spectrum of activity of drugs will be extended. Targeting a weak component in the drug response pathway could possibly reverse resistance cells back to their sensitive status.

There are numerous ways of targeting chemoresistance (Fojo and Bates, 2003). Each of them is based on either genetic or epigenetic strategies which can revert a malignant phenotype or enhance drug sensitivity, and such strategies are based on the knowledge about mechanisms of drug resistance. One of the first approaches for overcoming drug resistance was targeting ABC transporters (Tsuruo et al., 1983). ABC-mediated chemoresistance is based on reduction of intracellular drug levels. It leads to the reduction in the amount of drug that can reach the target and consequently to the reduced cytotoxicity. Such type of resistance effects almost every existing drug; therefore, the inhibitition of drug efflux could improve drug activity and block multi-drug resistance. The first strategy to inhibit Pgp-MDR1 relied on the identification of agents which compete with drugs for the pump. Such compounds are not cytotoxic; they act as inhibitors and could be easily identified because Pgp-MDR1 could bind a vide variety of hydrophobic compounds (Ford and Hait, 1990). However,

Pgp is also expressed in several other tissues (liver, kidney, and colon) and plays an important physiological role in the excretion of toxins. Therefore, it is necessary to achieve a specific targeting of Pgp exclusively in cancer cells.

Another strategy to overcome Pgp activity includes the use of hammerhead ribozymes against the MDR1 gene and MDR1-targeted antisense oligonucleotides (ASOs) (Dassow et al., 2000; Huesker et al., 2002). Hammerhead ribozymes are oligonucleotides with endoribonucleolytic cleavage activity (Irie et al., 1997), and they cleave specific mRNA molecules encoding genes responsible for drug resistance (Holm et al., 1994). The ability to target specific cells is considered to be the advantage of ribozymes. Gao et al., (2007) showed that reversal of drug resistance in breast adenocarcinoma cells can be achieved by anti-mdr1 ribozyme regulated by a tumor-specific MUC-1 promoter. In this case, the expression of ribozymes was limited to only breast cancer cells and did not affect normal cells. ASOs do not possess cleavage activity, but they recruit endonuclease RNAse H which destroys target mRNAs. Both ribozymes and ASOs are susceptible to degradation and can be recycled, so they can affect more mRNAs.

A different strategy to modulate multidrug resistance is depletion of glutathione levels. Due to its reactivity and high intracellular concentrations, GSH is implicated in resistance to numerous drugs including anthracyclines (Lai et al., 1991). It has been shown that GSH depletion increased the sensitivity of MRP1-expressing but not MDR1-expressing cells, suggesting that GSH participates in MRP1-mediated anthracycline resistance (Benderra et al., 2000).

Targeting apoptosis can also modulate drug resistance. Apoptosis is the outcome of interactions between pro- and anti-apoptotic regulators. The process of

apoptosis is very complex; therefore, there are many potential target sites for intervention. Suppression of proteins inhibiting apoptosis is approached by using antisense oligonucleotides (ASOs). ASOs are small 16-24 bp oligonucleotides composed of short sequences of single-stranded DNA complimentary to certain mRNAs (Tamm, et al., 2001). ASOs form heteroduplexes with mRNAs releasing RNAse H which destroys the mRNA strand.

Such strategy was applied to target Bcl-2 overexpression, which is associated with chemoresistance (Klasa et al., 2002). ASOs have also been used to target TGFβ (Fakhrai, 1999) and glucose transporters (Chan et al., 1999) aimed to increase the rate of apoptosis upon chemotreatment.

Chemoresistance is often acquired during treatment (Perez-Placencia and Duenas-Gonzalez, 2006). Therefore, chemotherapy should be studied at a global genomic and epigenomic level. It is already known that cancer cells do have multiple genetic defects such as deletions, duplications, translocations, etc., as well as epigenetic aberrations which include numerous alterations in DNA and histone modifications (Baylin and Ohm, 2006; Weinstein, 2000). Because multi-drug resistance is driven by so many mechanisms described in previous paragraphs, it would require changes in expression of a very large number of genes. In this regard, it is very hard to explain a chemoresistant phenotype by genetic changes, instead epigenetic changes could rapidly affect expression of multiple genes shortly after chemotherapeutical treatment (Glasspool, et al., 2006).

When considering prospects for the reversal of drug resistance, it becomes obvious that in contrast to genetic defects, epigenetic alterations are reversible and therefore may be much easier to target. Furthermore, the role of epigenetic control of

gene expression in chemoresistance is highlighted in many studies; therefore, many new approaches to reverse drug resistance are based on altering the cellular epigenetic status (Brown and Strathdee, 2002).

Many aberrantly methylated genes were found to be involved in determining chemoresistance. For example, methylation of DNA repair genes such as MGMT and FANCF leads to inactivation of DNA repair and provides chemosensitivity, whereas methylation of pro-apoptotic genes such as MLH1 and APAF1 would confer resistance (Esteller et al., 2000; Glasspool et al., 2006).

It has been shown by Gloria and colleagues that the MDR1 promoter gene is highly hypermethylated in drug sensitive MCF-7 human breast cancer cells and, therefore they lack the expression of MDR1, while an adriamycin (doxorubicin)-resistant variant of the same cells (MCF-7/ADR) showed a low methylation status of MDR1 and high levels of Pgp-MDR1 protein (David et al., 2004). Similar evidence for the role of hypomethylation of the MDR1 promoter in the development of chemoresistance was provided by Chekhun and colleagues. They have reported a significant decrease in methylation of promoters of MDR1, glutathione-S-transferase (GSTπ), O6-methylguanine DNA methyltransferase (MGMT) and urokinase (Upa) genes in doxorubicin resistant MCF7/DOX cells as compared to sensitive NCF-7 cells (Chekhun et al., 2006).

Other classes of epigenetic modifying agents include inhibitors of DNA methyltransferases (DNMT). An inhibitor of DNMT1, 5-aza-2'deoxycytidine (decitabine), forms irreversible covalent bonds with DNMT1 after its incorporation into DNA and induces degradation of DNMT1 (Christman, 2002). Several other

derivatives of 2'-deoxycytidine such as 5-azadcytidine (5-azaC), arabinosyl-5-azacytosine (fazarabine) and dihydro-5-azacytidine (DHAC) are demethylating agents that have been proposed to have anti-tumour properties since they can activate expression of epigenetically silenced genes (Arnold, et all., 2003). It has been shown that the addition of these agents can induce sensitivity of cells to cisplatin, epirubicin and temozolomide (Plumb et al., 2000). 5-azadcytidine was also proved to re-express the MLH1 gene by its promoter methylation. MLH1 is a key component of mismatch repair, and as it was mentioned previously, loss of MMR results in resistance to cisplatin and doxorubicin in breast cancer cells (Plumb et al., 2000). Pakneshan and colleagues showed that the addition of a methylating agent S-adenosyl-methionine (AdoMet) can hypermethylate and inhibit expression of urokinase (uPA) which is expressed in highly invasive cancer cells (Pakneshan et al., 2004).

These studies confirm the possibility of targeting epigenetic modifications to concur multiple chemoresistance mechanisms. It could be expected that agents targeting DNA methylation would revert the epigenetic status and therefore induce sensitivity of breast cancer cells to chemotherapy agents, including doxorubicin. Taken together, chemoresistance can be reversed by epigenetic therapy either by reversing hypermethylation of tumour suppressor genes or by achieving hypermethylation of protooncogenes.

The main agents that could cause changes in histone modification patterns of drug-resistant cells are those that inhibit histone deacetylases (HDACs). HDAC inhibitors can reactivate genes involved in cell cycle control or apoptotic signalling, thereby inhibiting proliferation (Donadelli et al., 2003). In addition, treatment with HDAC inhibitors can sensitize cancer cells to irradiation (Camphausen et al., 2004).

In sum, from the existing literature we have learned that:

- Doxorubicin is an anthracycline drug frequently used in curative-intent adjuvant and palliative treatment of metastatic breast cancer. Although doxorubicin is among the most active agents in breast cancer treatment, many patients will experience a relapse.
- Drug resistant cancer cells often fail to respond to cytotoxic radiotherapy and develop a multi-drug resistant phenotype; however, the data on radiation responses of chemoresistant tumors is contradictory. On the one hand, some studies suggest significant benefits of chemo- and radiotherapy combination for management of breast cancer. On the other hand, there is an evidence that chemotherapy as "induction therapy before radiotherapy" has no significant additive anti-tumor effects.
- Breast tumors tend to resist and reoccur after the aforementioned treatments. Acquired treatment resistance is a multi-factorial phenomenon involving multiple mechanisms and processes including: decreased uptake of drugs; alterations in cell cycle and signal transduction pathways; increased repair of DNA damage; reduced apoptosis; increased efflux of hydrophobic drugs; and DNA damage tolerance. Nevertheless, the exact nature and mechanisms of radiation responses of chemoresistant tumor cells still remain obscure.

PRESENT STUDY: OBJECTIVES AND HYPOTHESES

The main goal of the current thesis is to investigate molecular mechanisms of radiation responses of MCF-7 breast adenocarcinoma cells resistant to doxorubicin and propose a strategy to modify/improve their radiation sensitivity.

Guiding Hypothesis:

Based on evidence from the existing literature, **we hypothesize** that doxorubicin-resistant MCF-7 cells (MCF-7/DOX) are much more resistant to radiation exposure than their drug-sensitive analogues (MCF-7 cells). We think that differences in radiation responses are due to different susceptability of MCF-7 and MCF-7/DOX cells to radiation-induced DNA damage and apoptosis. We suggest that the latter phenomenon is epigenetically mediated via a different DNA methylation status of MCF-7 and MCF-7/DOX cells. Therefore, we predict that modifying the epigenetic status of MCF-7/DOX cells will alter their radiation responsiveness.

The following experiments were conducted to achieve the goal and test the proposed hypotheses:

Experiment 1: To analyze molecular mechanisms of radiation resistance in doxorubicin-resistant breast adenocarcinoma cells.

Experiment 2: To test whether epigenetic therapy aimed to modify the DNA methylation status will improve radiation sensitivity of MCF-7/DOX cells.

The outcomes of these experiments are presented as chapters of this thesis.

CHAPTER 2: MOLECULAR MECHANISMS OF RADIATION RESISTANCE IN DOXORUBICIN-RESISTANT BREAST ADENOCARCINOMA CELLS

Chapter 2 has been submitted in its entirety:

Luzhna L, Golubov A, Pogribny I and Kovalchuk O. Molecular mechanisms of radiation resistance in doxorubicin-resistant breast adenocarcinoma cells. DNA Repair (in review)

ABSTRACT

Positive response to breast cancer treatment is largely dependant on the successful combination of anticancer treatment modalities such as chemo- and radiation therapy. Unfortunately, chemotherapy resistance occurs frequently. Furthermore, drug resistant tumors can become unresponsive to the use of other antitumor therapies, and often fail to respond to radiation therapy. The molecular mechanisms underlying the radiation responses of chemoresistant cells and tumors are not well understood.

In the present study, we analyzed the effect of ionizing radiation on MCF-7 human breast adenocarcinoma cells and their doxorubicin resistant variant MCF-7/DOX. The results demonstrate that drug resistant MCF-7/DOX cells were less susceptible to radiation-induced DNA damage and apoptosis. This was evidenced by lower levels of γH2AX foci upon irradiation and altered levels of DNA repair proteins, including pATM, KU70 and RAD51. Additionally, MCF-7/DOX drug-resistant cells harbored DNA polymerases with significantly lower fidelity. In summary, our study revealed that drug-resistant MCF-7/DOX cells have high DNA repair potential and low fidelity of DNA polymerases seemingly sacrificing specificity and efficiency to gain higher survival potential. In the long run this may lead to an increased probability of accumulation of mutations and further to the development of even more pronounced resistance phenotype.

Therefore, this study may provide a roadmap for the analysis of the roles of DNA repair function and effectiveness and apoptosis in response to radiation, chemotherapy and combinations of both treatment modalities.

INTRODUCTION

Systemic chemotherapy is an important breast cancer treatment modality and its effectiveness has significantly improved over the past decade (Guarneri and Conte, 2004). Notwithstanding, the development of cancer cells resistant to chemotherapeutic agents is a major clinical obstacle in the successful treatment of breast cancer (Lehnert, 1996; Szakacs et al., 2006). Understanding the mechanisms underlying drug resistance development and predisposition is critical to saving lives.

Overall, acquired drug resistance is a multi-factorial phenomenon, involving multiple mechanisms and processes (Lehnert, 1996; Szakacs et al., 2006; Fojo, 2007; O'Driscoll and Clynes, 2006) including: decreased uptake of drugs (Gottesman, 2002); alterations in cell cycle and signal transduction pathways (Pommier et al., 2004; Stavrovskaya, 2000); increased repair of DNA damage (Karran, 2001); reduced apoptosis (Pommier et al., 2004; Rixe and Foho, 2007; Hickman, 1996); increased efflux of hydrophobic drugs (O'Driscoll and Clynes, 2006; Gottesman, 2002; Stavrovskaya, 2000; Gottesman and Ling, 2006; Modok et al., 2006); and DNA damage tolerance (Karran, 2001). Resistance to individual chemotherapeutic agents usually occurs through alterations in the targets for these drugs, but broad resistance can also occur affecting the utility of a variety of diverse and unrelated antitumor drugs with different chemical structures and different mechanisms of action (O'Driscoll and Clynes, 2006; Gottesman and Ling, 2006; Coley, 2008; Gonzalez-Angulo et al., 2007; Petrelli and Giordano, 2008). Apoptosis avoidance is one of the key mechanisms underlying multiple drug resistance phenotype (Pommier et al., 2004; Rixe and Fojo, 2007; Hickman, 1996; Chekhun et al., 2007).

Doxorubicin is an anthracycline drug frequently used in curative-intent adjuvant and palliative treatment of metastatic breast cancer (Dean-Colomb and Esteva, 2008). Although doxorubicin is among the most active agents in breast cancer treatment, many patients will experience a relapse. Furthermore, approximately half of metastatic breast cancer patients will fail to respond to doxorubicin entirely, and the majority of those showing initial benefit will subsequently demonstrate acquired clinical resistance, as demonstrated by tumor growth despite ongoing anthracycline therapy (Dean-Colomb and Esteva, 2008). Importantly, it has also been reported that drug resistant cancer cells may fail to respond to cytotoxic radiotherapy and may develop a multi-drug resistant phenotype (Ozols et al., 1988; Shimm et al., 1988; Belli, 1989; Lehnert et al., 1989; Lehnert et al., 1990; Alaoui-Jamali et al., 1992; Miller et al., 1992; Zhang et al., 1992; Lehnert et al., 1994). However, the data on the radiation responses of chemoresistant tumors is contradictory. For instance, some clinical studies suggest significant benefits of combination of chemo- and radiotherapy for management of breast cancer (Liang et al., 2003). On the other hand, there is proof that chemotherapy as "induction therapy before radiotherapy" has no significant additive anti-tumor effects (Koukourakis et al., 1999). Breast tumors tend to resist and reoccur after the aforementioned treatments (Gewirtz, 2000). The exact nature and mechanisms of radiation responses of chemoresistant tumor cells remain obscure.

One of the key features of cancer cells resistant to therapeutic agents is their associated resistance to apoptotic cell death (Pommier et al., 2004). Chemoresistant cells and tumors harbor a strong capacity to withstand and avoid apoptosis upon chemotherapy treatment (Pommier et al., 2004; Reinhold et al., 2003). Ionizing

radiation (IR) exposure is known to induce apoptosis in exposed cells, yet little is known about the status of IR-induced apoptosis in drug resistant cell lines.

In this study we analyze the cellular and molecular mechanisms of radiation responses in MCF-7 breast adenocarcinoma cells and their derivative line that is resistant to doxorubicin (MCF-7/DOX). Here we for the first time show that MCF-7/DOX cells while harboring an elevated potential to repair radiation-induced DNA damage also have a significantly decreased fidelity of DNA polymerases and low levels of radiation-induced apoptosis.

MATERIALS AND METHODS

Cell lines and cell culture conditions

MCF-7 and MCF-7/DOX multidrug-resistant human breast adenocarcinoma cell lines were previously developed and described (Chekhun et al., 2007; Kovalchuk et al., 2008). Cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM /F-12) with 2.5 mM L-Glutamine, without HEPES and Phenol Red (HyClone, Logan, UT), supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), in the presence of antibiotics 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO) in 5% CO2 atmosphere at 37 ºC. Cells were harvested for analyses by trypsinization (Chekhun et al., 2007; Kovalchuk et al., 2008).

Irradiation conditions

Cells were irradiated at the confluency of 60% in DMEM. Two radiation doses (0.5 Gy and 5 Gy, 90 kV, 5 mA) were applied in order to check the cellular radiation

responses. Un-irradiated cells served as control. Cells were harvested 30 minutes, 24 hours and 48 hours after irradiation. All cells were tested in triplicate. Experiments were independently reproduced twice.

Immunofluorescence

For immunocytochemical analysis, cells were grown on Lab-Tek chambered 2-well slides (Nulge Nunc International Corp., Naperville, IL) and irradiated. After irradiation, the cells were fixed in 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 70% ethanol at -20 °C and washed in PBS containing 0.1% TRITON-X100. Blocking was done in 8% BSA in PBS for 1 hour. For immunocytochemical detection, cells were incubated for 2 hours at room temperature using the following antibodies: anti-γH2AX (Ser 139) rabbit antibodies (1:100, Cell Signaling Technology Inc., Danvers, MA), anti-RAD51 rabbit antibodies, anti-pATM, and anti-KU70 mouse antibodies (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Afterwards, cells were rinsed and incubated with 1:500 diluted secondary antibodies (goat anti-rabbit IgG Alexa Fluor 488, goat anti-mouse IgG Alexa Fluor 546 and goat anti-mouse IgG Alexa Fluor 488, Invitrogen Molecular Probes, Eugene, OR) at 37 ºC for 1 hour. Cell nuclei were counterstained with 0.1 mg/mL 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich Chemical Co., St. Louis, MO) for 30 minutes at 37 ºC. Slides were mounted with anti-fade fluorescence medium prepared from 1,4-diazabicyclo[2.2.2]octane (DABCO), polyvinyl alcohol and glycerol and analyzed with Zeiss epifluorescent microscope.

The number of γH2AX foci per cell was counted in at least 400 cells from each cell group, as previously described (Sedelnikova and Bonner, 2006). γH2AX levels were represented as the mean ± SE; $P \leq 0.05$.

Western immunoblotting

The cells from culture flasks were harvested, washed in PBS, lysed and sonicated in 0.2 mL of 1% sodium dodecyl sulphate (SDS) on ice. The lysates were cleared by centrifugation, and the supernatants collected and boiled at 95 ºC for 5 minutes. Protein content was determined with the Bradford protein determination assay (BioRad, Hercules, CA). Equal amounts of lysate protein (20 µg/10 µL) were subsequently run on 10-12% SDS-polyacrylamide gels and transferred to PVDF membranes (GE Healthcare, Baie d'Urfé, Québec).

Western immunoblotting was conducted with well-established protocols (Kovalchuk et al., 2008; Tryndyak et al., 2006). Membranes were incubated with antibodies against goat anti-polymerase iota, mouse anti-polymerase epsilon (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-polymerase beta and rabbit anti-polymerase delta (1:500 dilution, Abcam Inc., Cambridge, MA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and the ECL Plus immunoblotting detection system (GE Healthcare, Piscataway, NJ). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Unaltered PVDF membranes were stained with Coomassie Blue (BioRad, Hercules, CA) and the intensity of the Mr 50 000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software and normalized to both actin and the Mr 50 000 protein band, which gave consistent results (values relative to Mr 50 000 are plotted).

Analysis of DNA polymerase fidelity in MCF-7 and MCF-7/DOX cells

The DNA polymerase fidelity assay allows to determine activity of polymerases on damaged DNA and the quality of repair synthesis (Gening et al., 2004). The assay employs a FAM-labeled 15bp primer as a component of the substrate. Its oligonucleotide can be revealed on a gel. In the assay, different deoxyribonucleotides are added to the reaction mixture to check the ability of polymerases to incorporate the correct and incorrect dNTPs into the template. Any increase in primer weight upon incorporation would indicate higher DNA polymerase activity, while a decrease is associated with exonuclease activity. The efficiency of misincorporation is associated with changes in DNA polymerase fidelity.

Substrate (template/primer complex). In order to produce the substrate for assay, FAM-labeled 15bp primer was annealed with 30bp template (both were PAGE purified).

Template: AG030-PAGE

5’ - TCATCGAGCATGATCACGTCGTGACTGGGA – 3’

Primer: AG031-PAGE

5’ – FAM – TCCCAGTCACGACGT – 3’

The reaction was performed in 1 M Tris-HCl, pH 8.0, β-mercaptoethanol, BSA (100× NEB), 100 µM primer and 100 µM template, incubated at 95 ºC for 5 minutes and slowly cooled down at room temperature.

Cell extracts. MCF-7 and MCF-7/DOX control and irradiated (harvested 24 hours after 5 Gy X-ray treatment) cells were harvested, washed in 10 mL of 1x PBS, resuspended and sonicated in 200 µL of PBS (350 W, 5 x 10 min) and centrifuged at 4

ºC for 10 minutes, 14,000 g. Total protein concentration in samples was determined by Bradford Assay (BioRad, Hercules, CA).

DNA polymerase fidelity assay was carried out according to Gening and colleagues (Gening et al., 2004). The reaction was performed at 37 ºC for 15 minutes and quickly frozen afterwards. Reaction mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT, 70 µg of the tested lysate protein, template/primer complex and 2 mM dNTP. When the reaction was stopped, 5 µL of each sample was mixed with 10 µL of loading buffer (95% formamide, 50 mM EDTA, 0.05% bromophenol blue), incubated at 95 ºC for 3 minutes and cooled down on ice. Reaction products were separated in 20% polyacrylamide gel in the presence of 8 M urea in Tris-borate buffer at 750 V. Afterwards PAGE gels were scanned using Typhoon 9410 imager (excitation 488 nm, emission filter 520 BP 40, PMT 620 V, resolution 50 µm).

Annexin V assay

For early detection of apoptosis, Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) was used according to the manufacturer's protocol. Cells were grown on 75 cm^2 cell culture flasks and irradiated as previously described (section 2.2). The analysis was performed 24 and 48 hours after radiation exposure. Cells were harvested, washed with PBS, resuspended in 1X binding buffer, stained with Annexin V and propidium iodide for 15 min at 25 ºC in the dark and analyzed by flow cytometry within one hour at the Flow Cytometry Core Facility (University of Calgary, Calgary, AB). The results were represented as percentage of gated Annexin V positive cells.

Statistical analysis

Statistical analysis was performed using MS Excel 2007 and JMP5 software packages.

RESULTS

Radiation-induced apoptosis in MCF-7 breast adenocarcinoma cells and their drug-resistant counterpart MCF-7/DOX cells

In this study, we characterized and compared the responses of the MCF-7 breast adenocarcinoma line and its doxorubicin-resistant variant (MCF-7/DOX) (Chekhun et al, 2007; Kovalchuk et al., 2008) to ionizing radiation (IR) *in vitro*. IR exposure is known to induce apoptotic cell death in irradiated cells. Early apoptosis is characterized by various changes in the cellular plasma membrane, the main of which is the translocation of phosphatidylserine (PS) from the inner layer to the surface of the membrane. Annexin V possesses a high affinity to PS and this allows for early detection of apoptotic changes (Vermes et al., 1995). Here we analyzed IR-induced apoptosis using an Annexin V assay.

Figure 2.0 shows that MCF-7 cells began to undergo early apoptosis 24 hours after irradiation (Figure 2.0). We found a 2- and 2.5-fold increase in Annexin V positive cells 24 hours after exposure to 0.5 and 5 Gy of X-rays, respectively. The percentage of MCF-7 cells in early apoptosis returned to control level by 48 hours, however, the number of dead cells increased at this time point (Figure 2.0). Such changes may indicate that cells which were undergoing early apoptosis 24 hours after irradiation were dead by 48 hours. In contrast, MCF-7/DOX resistant cells showed

apoptotic response only 48 hours after treatment with the high IR dose (5 Gy). The 9.1-fold increase in Annexin V positive cells was reached 48 hours after X-ray treatment of MCF-7/DOX cells (Figure 2.1).

Radiation-induced DNA damage in MCF-7 and MCF-7/DOX

Next, we analyzed the mechanisms associated with such significant differences in IR-induced apoptotic response in MCF-7 and MCF-7/DOX cells. IR is a potent DNA damaging agent capable of inducing cross linking, nucleotide base damage and, most importantly, single and double strand breaks (DSBs), which are well-known inducers of apoptosis (Little, 2000; Huang et al., 2003). Therefore, we analyzed the levels of IR-induced DNA damage in MCF-7 and MCF-7/DOX cells by detecting γH2AX foci, a well accepted indicator of DNA strand breaks (Bonner et al., 2008).

Figure 2.2 shows that both IR doses, 0.5 Gy and 5 Gy, led to the formation of γH2AX foci in MCF-7 and MCF-7/DOX cells. However, MCF-7/DOX cells were much less sensitive to IR than MCF-7 cells (Figure 2.2). Specifically, irradiation of MCF-7 cells caused significant 2.6 and 8.5 times increases in the levels of γH2AX foci, from 3.14±0.22 foci per cell in control to 8.23±0.53 and 26.70±1.02 foci per cells, 30 minutes after 0.5 Gy and 5 Gy treatments, respectively (Figure 2.2 A). γH2AX foci induced by 0.5 Gy of X-rays disappeared 48 hours after irradiation, indicating efficient DNA repair. While application of 5 Gy of X-rays led to persistent elevation of γH2AX foci as detected 48 hours after exposure.

In MCF-7/DOX cells radiation exposure led to significant 1.9 and 6.0 times increases in the levels of γH2AX foci, from 1.83±0.2 foci per cell in control to 3.49±0.15 and 10.9±0.44 foci per cell after 0.5 Gy and 5 Gy treatments, respectively

(Figure 2.2 B). The levels γH2AX foci significantly decreased 24 and 48 hours after irradiation.

Most importantly, the levels of γH2AX foci in MCF-7 cells were in all cases significantly different from the levels seen at the corresponding time-points in MCF-7/DOX cells (Figure 2.2).

DNA repair machinery in MCF-7 and MCF-7/DOX cells

Apparent differences in the levels of IR-induced DNA damage between MCF-7 and MCF-7/DOX cells have logically led us to question how the resistant cells repair the DNA lesions. In mammalian cells, two mechanisms exist to repair DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ) (West, 2003; McGlynn and Lloyd, 2002; Helleday, 2003; Hoeijmakers, 2001).

The key component for both mechanisms is a serine/threonine specific protein kinase – ATM. Phosphorylation of ATM is necessary for DSB repair (Bonner et al., 2008; Chowdhury et al., 2005). Therefore, we analyzed the level of phosphorylated ATM (pATM) in MCF-7 and MCF-7/DOX cell lines after irradiation.

Overall, the level of pATM was higher in MCF-7/DOX cells (Figure 2.3). Interestingly, the subcellular localization of the protein was different in MCF-7 and MCF-7/DOX cell lines (Figure 2.4). For example, in MCF-7 cells, pATM was detected as nuclear foci (Figure 2.4). The number of pATM nuclear foci in MCF-7 cells increased after irradiation. The dynamics of pATM expression was similar to that of γH2AX (Figure 2.2 A).

In MCF-7/DOX resistant cells, no pATM foci were observed, and the protein was localized in both nucleus and cytoplasm. Yet, the general level of pATM in MCF-7/DOX cells was higher than in the MCF-7 cells (Figure 2.3 and Figure 2.4).

With evidence of different levels of γH2AX and pATM in MCF-7 and MCF-7/DOX cells, we then asked if HR or NHEJ-related proteins were differentially induced in these cell lines after irradiation. RAD51 is a key protein essential for repair of DSBs via HR in mammals (Lundin et al., 2003). KU70 is a key participant in the NHEJ pathway to repair DSBs (Hoeijmakers, 2001; Jin and Weaver, 1997).

Immunocytochemistry was performed to analyze the levels of RAD51 and KU70 in MCF-7 and MCF-7/DOX cells after irradiation. We found that the expression level of RAD51 increased after irradiation in both cell lines (Figure 2.5), but the highest level was observed in MCF-7/DOX cells after exposure to 5 Gy of X-rays (Figure 2.5).

Interestingly, MCF-7 cells expressed relatively high levels of KU70 prior to irradiation and an abundant amount of the protein was found after exposure (Figure 2.6). Contrarily, KU70 levels were almost undetectable in the un-irradiated MCF-7/DOX cells, and only exposure to 5 Gy of X-rays resulted in a noticeable up-regulation of KU70 levels (Figure 2.6).

Analysis of fidelity and expression of DNA polymerases in MCF-7 and MCF-7/DOX cells

All types of DNA repair involve re-synthesis of DNA to replace damaged strands. To uncover any correlation between the dynamics of induction and repair of IR-induced DNA damage, we studied the fidelity of the DNA polymerase pool in the cell lysates from MCF-7 and MCF-7/DOX (Figure 2.7). Because doxorubicin resistant MCF-7/DOX cells manage to deal with DNA damage faster than MCF-7 cells, we hypothesized that DNA polymerases may be more active in the resistant cells. We

analyzed the DNA polymerase fidelity in the MCF-7 and MCF-7/DOX (Gening et al., 2004).

dNTPs were added to the mixture containing the template and extracts of unirradiated or irradiated MCF-7 or MCF-7/DOX cells and the incorporation patterns were analyzed. According to the template sequence (see section Materials and Methods), the next nucleotide to be inserted was dGTP. In the case when only dGTP was in the reaction mixture, we obtained a 16bp gel band with higher intensity in MCF-7/DOX, moreover, the band corresponding to irradiated MCF-7/DOX had the highest intensity (Figure 2.7). The observed difference may be explained by higher DNA polymerase activity or an increased amount of polymerases in resistant cells.

Furthermore, as shown in figure 2.7, MCF-7/DOX had higher level of misincorporation of dATPs which means that DNA polymerase specificity or fidelity is lower in the drug-resistant cells. We did not observe incorporation of dTTP and dCTP. Therefore, we concluded that ATP is the most common wrong nucleotide to be inserted instead of GTP by the low fidelity polymerases to continue synthesis in MCF-7 cells. When adding both dGTPs and dATPs or all dNTPs to the samples, we obtained 16- and 17bp bands and completed synthesis respectively (Figure 2.7). In all the cases the activity of the polymerases was higher in the MCF-7/DOX resistant cell line.

At the same time, we observed more intensive DNA cleavage in MCF-7 cells due to a significant exonuclease activity. The excision of wrong nucleotides by exonucleases reduces mismatches. The control sample did not contain any dNTPs and no bands with a weight higher than 15bp were observed (Figure 2.7). The negative control contained all dNTPs and EDTA (to inactivate all metal-using enzymes). In

such conditions, the activity of exonucleases was lower and the intensity of all the bands was the same. Both controls indicated that there were no endogenous oligonucleotides observed on the gels (Figure 2.7).

In sum, we concluded that irradiated and un-irradiated MCF-7/DOX cells exhibited significantly higher processivity and significantly lower fidelity of polymerases.

This may be due to higher levels of polymerase expression. We therefore analyzed the levels of polymerases δ, ε , β and ι in MCF-7 and MCF-7/DOX cells.

The expression level of both polymerases δ and ε were found to be higher in MCF-7 cells (Figure 2.8). Furthermore, the level of DNA polymerase δ was slightly increased in doxorubicin-resistant MCF-7/DOX cells after radiation exposure.

Two other polymerases – β and ι were highly expressed in MCF-7/DOX. While polymerase β level was much lower in MCF-7 cells, than in MCF-7/DOX, polymerase ι was not detected at all (Figure 2.8). DNA polymerase ι was recently discovered as a polymerase that catalyses error-prone DNA synthesis. It promotes replication of damaged DNA by misincorporating deoxynucleotides opposite DNA lesions (Bebenek and Kunkel, 2004; Shcherbakova et al, 2003).

The altered levels of DNA polymerases may explain, at least in part, the higher processivity and lower fidelity seen in MCF-7/DOX cells.

DISCUSSION

Relapse risk in breast cancer is largely dependent on the combination of anticancer treatment modalities. Anthracycline chemotherapy is increasingly used for

treating locally advanced breast cancer and hormone-resistant metastatic breast tumors (Guarneri and Conte, 2004; Gonzalez-Angulo et al., 2007).

Unfortunately, resistance to chemotherapy occurs frequently (Gonzalez-Angulo, et al., 2007). Drug resistant tumors often become unresponsive to the use of other antitumor therapies, acquire multidrug resistance and often fail to respond to radiation therapy (Stavrovskaya, 2000). Frequently, the use of chemotherapy drugs as radiation sensitizers fails due to unknown reasons (Liang et al., 2003; Koukourakis et al., 1999). Overall, the data on the radiation response of drug resistant cells is scarce.

Doxorubicin is widely used in curative-intent adjuvant breast cancer therapy (Fornari et al., 1994). Mechanistically, doxorubicin, an anthracycline antibiotic, intercalates DNA and inhibits the progression of the enzyme topoisomerase 2α (Top2A) (Fornari et al., 1994). Functionally, it stabilizes the Top2A complex after it has broken the DNA chain, preventing DNA resealing and thereby blocking replication (Fortune and Osheroff, 2000). Therefore, because doxorubicin treatment leads to induction of strand breaks, we hypothesized that cells exposed to doxorubicin for a prolonged time could develop mechanisms to effectively repair DSBs and therefore avoid drug-induced apoptosis. These mechanisms may consequently help drug resistant cells to withstand the effects of other treatment modalities that induce DNA strand breaks as the main mechanism of their cell-killing action.

Therefore, in this study we assessed the levels of IR-induced apoptosis in MCF-7 and MCF-7/DOX cells. We noted that drug resistant cells were significantly less susceptible to IR-induced apoptosis than their resistant counterparts. Aiming to explain this apparent discrepancy in the levels of IR-induced apoptosis, we studied formation and repair of DNA DSBs, as seen through the induction of γH2AX foci in

MCF-7 and MCF-7/DOX cells after IR exposure. Importantly, the background number of γH2AX foci in untreated MCF-7 cells correlated with previous data from other investigators (Sedelnikova and Bonner, 2006).

γH2AX foci appear in the nuclei within 1 minute after irradiation and reach their maximum number by 30 min to 1 hour. Afterwards, the number of γH2AX foci reduces due to repair processes (Bonner et al., 2008; Banath et al., 2004). Our assay showed that non-resistant MCF-7 cells are more radiosensitive (Figure 2.2). MCF-7 cells were not able to completely repair DNA damages after high dose (5 Gy) treatment and even after 48 hours the residual amount of foci was very high. In contrast, drug-resistant MCF-7/DOX cells did not accumulate a lot of damage after low dose (0.5 Gy) treatment. The maximum number of foci was observed 30 minutes after 5 Gy X-ray exposure and was significantly lower than the number of foci detected in MCF-7 cells at this dose. Moreover, all DNA damage in the drug resistant MCF-7/DOX cells was repaired by 48 hours (Figure 2.2). Currently, it is thought that γH2AX recruits proteins to repair DNA damage, and γH2AX is dephosporylated after repair is complete (Chowdhury et al., 2005). Therefore, we assume that the faster foci disappear, the higher the DNA repair activity in the cells.

DSBs can be repaired by two major mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) (Hoeijmakers, 2001; Hoeijmakers, 2001; Jeggo and Lobrich, 2006). HR allows cells to use the undamaged sister chromatid or the homologous chromosome as a template for repair and thus is considered error-free (West, 2003; McGlynn and Lloyd, 2002; Helleday, 2003; Hoeijmakers, 2001; Hoeijmakers, 2001). The error-free HR is controlled by RAD51 protein (West, 2003; Lundin, 2003; Baumann and West, 1998; Dudas and Chovanec,

2004). RAD51 binds to single-stranded DNA and forms a nucleoprotein filament that catalyses homology searching, strand pairing, and strand exchange (Baumann and West, 1998; Benson et al., 1998). NHEJ is a fast, yet error-prone process of linking broken DNA ends together without reference to accurate base pairing (Hoeijmakers, 2001; Hoeijmakers, 2001). This DNA repair mechanism is most common in mammalian cells and requires a DNA-binding component – a heterodimer of KU70 and KU80 proteins (Hoeijmakers, 2001; Jin and Weaver, 1997). A crucial signalling component for both pathways is the protein kinase ATM. ATM coordinates DNA repair by phosphorylating the downstream proteins involved in the actual repair (Goodarzi et al., 2003). Activity of ATM is increased two- to three-fold after exposure to IR (Goodarzi, 2004). Such an increase in activity of ATM is thought to occur due to autophosphorylation of serine-1981 (Goodarzi, 2004). Our study showed that MCF-7 and drug-resistant MCF-7/DOX cell lines have different profiles of the aforementioned DNA repair proteins. Although both cell lines exhibited elevated levels of pATM, RAD51 and KU70 after exposure, the initial level of these proteins were different (Figure 2.3, Figure 2.4, Figure 2.5 and Figure 2.6). We found that MCF-7 cells express higher level of KU70 which is a key protein for NHEJ, while in doxorubicin-resistant MCF-7/DOX cells the elevation of RAD51 could contribute to the HR-mediated DNA repair. Why MCF-7 and MCF-7/DOX cells display different preference to error-free and error-prone DSB repair strategies remains unknown, but triggering certain steps of preferred repair pathways may improve chemo- and radiotherapy response. Our data are in agreement with previous reports showing higher DNA repair potential of drug resistant cells (Shimm et al., 1988; Belli, 1989; Lehnert et al., 1990; Lehnert et al., 1994; Harris, 1985).

All types of DNA repair involve re-synthesis of DNA to replace the damaged strand. Therefore, DNA polymerases play key roles not only in DNA replication, but also in DNA repair processes (Hoeijmakers, 2001; Hoeijmakers, 2001). Specifically, high fidelity and processivity of polymerases is crucial for faithful DNA replication and preventing the accumulation of mutations. Indeed, efficient repair of DNA synthesis depends upon the proper functioning of DNA polymerases. Eukaryotic cells have 15 polymerases that belong to several families (Bebenek and Kunkel, 2004). Members of the B-family of polymerases include the major eukaryotic DNA polymerases α, δ, ε (Bebenek and Kunkel, 2004; Shcherbakova et al., 2003; Kunkel and Burgers, 2008). Polymerases δ and ε harbor exonuclease activity (Bebenek and Kunkel, 2004). They partake in replication and processing of Okazaki fragments during replication processes and are implicated in repair of damaged DNA. As components of recombination complexes, they are able to repair double-strand breaks and partake in HR and NHEJ.

Some members of the X-family of polymerases, such as polymerase β, are required for base excision repair. Polymerase β is not as accurate as replicative DNA polymerases because it lacks proofreading capability. Polymerase β is a key player in base excision repair, a mechanism that takes care of damaged bases and single strand breaks (Bebenek and Kunkel, 2004; Shcherbakova et al., 2003; Matsuda et al., 2003). In addition to replicative polymerases there are a number of translesion DNA polymerases, such as polymerase ι, another member of X family of polymerases. These polymerases are involved in bypassing DNA lesions that otherwise impede replication polymerases (Kunkel et al., 2003).

Detailed analysis of DNA polymerases δ, ε, β and ι demonstrated higher activity, but lower fidelity of polymerases in MCF-7/DOX resistant cells in comparison to MCF-7 cells (Figure 2.7 and Figure 2.8). Low fidelity polymerases are thought to be an evolutionary solution allowing for replication past damaged DNA and avoiding apoptosis (Beard et al., 2002; Goodman and Tippin, 2000). The ability to catalyze error-prone DNA synthesis belongs to DNA polymerase iota which was highly expressed in doxorubicin-resistant cells and was not detected in parental MCF-7 cells (Figure 2.7).

We also found higher exonuclease/proofreading activity in MCF-7 cells as compared to MCF-7/DOX cells. In the current study, we analyzed only 4 cellular DNA polymerases, therefore future analysis of other polymerases may shed more light on the mechanisms of chemo- and radiation resistance. In sum, this study revealed that drug-resistant MCF-7/DOX cells developed more rapid DNA repair, seemingly sacrificing the specificity and efficiency of this process to gain higher survival potential. In the long run this may lead to an increased probability of accumulation of mutations and further to the development of even more pronounced resistance phenotype.

Further detailed studies are needed to determine the cellular and molecular processes that are altered in resistant cells, which allow them to survive genotoxic treatments such as irradiation. This study may therefore provide a roadmap for the analysis of the roles of DNA repair, function, effectiveness and apoptosis in responses to radiation, chemotherapy and combinations of both treatment modalities.

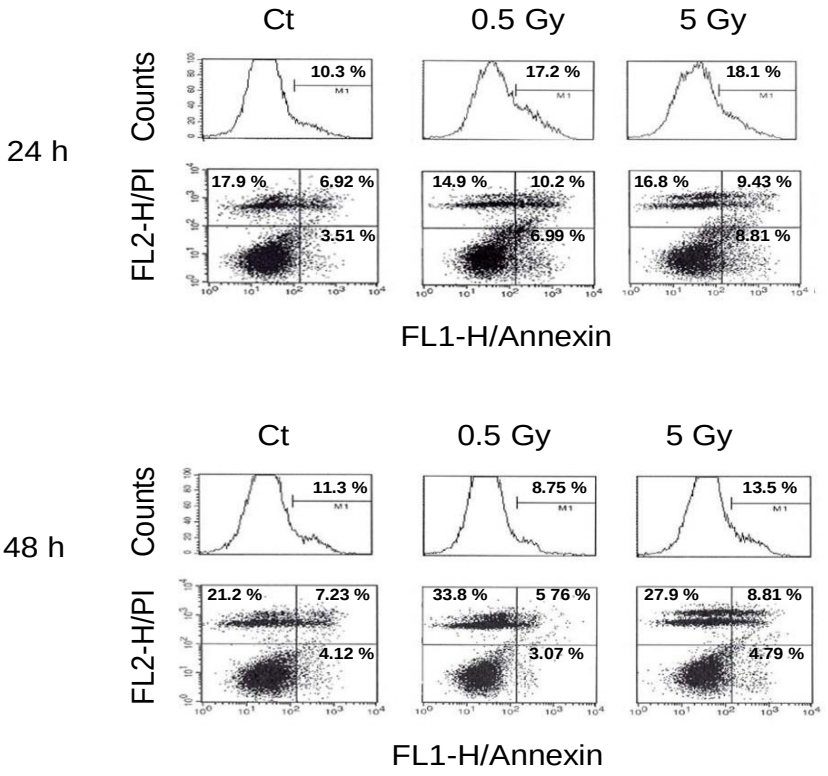

Figure 2.0. Radiation induced apoptosis in MCF-7 cells studied by Annexin V-FITC assay. M1 – average number of Annexin V positive cells. Diagrams below the M1 counts represent general cell distribution and viability. Viable cells – Annexin V and PI negative (lower left quadrants); cells in early apoptosis – Annexin V positive and PI negative (lower right quadrants); cells in late apoptosis or necrosis – Annexin V and PI positive (upper right quadrants); dead cells – (upper left quadrants). The numbers represent averages of three independent experiments.

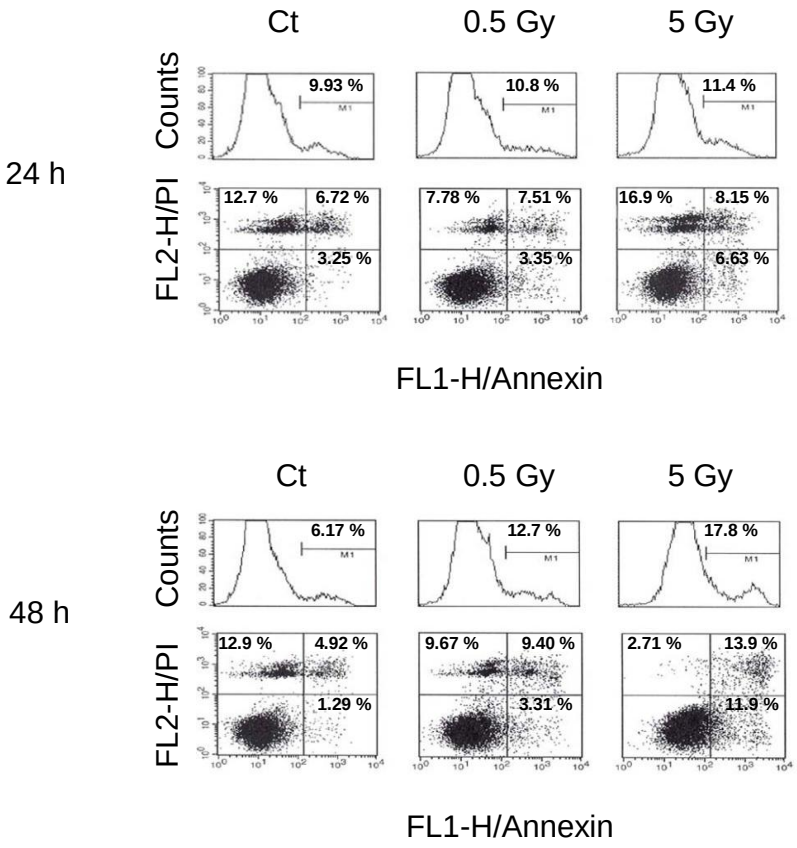

Figure 2.1. Radiation induced apoptosis in MCF-7/DOX cells studied by Annexin V-FITC assay. M1 – average number of Annexin V positive cells. Diagrams below the M1 counts represent general cell distribution and viability. Viable cells – Annexin V and PI negative (lower left quadrants); cells in early apoptosis – Annexin V positive and PI negative (lower right quadrants); cells in late apoptosis or necrosis – Annexin V and PI positive (upper right quadrants); dead cells – (upper left quadrants). The numbers represent averages of three independent experiments.

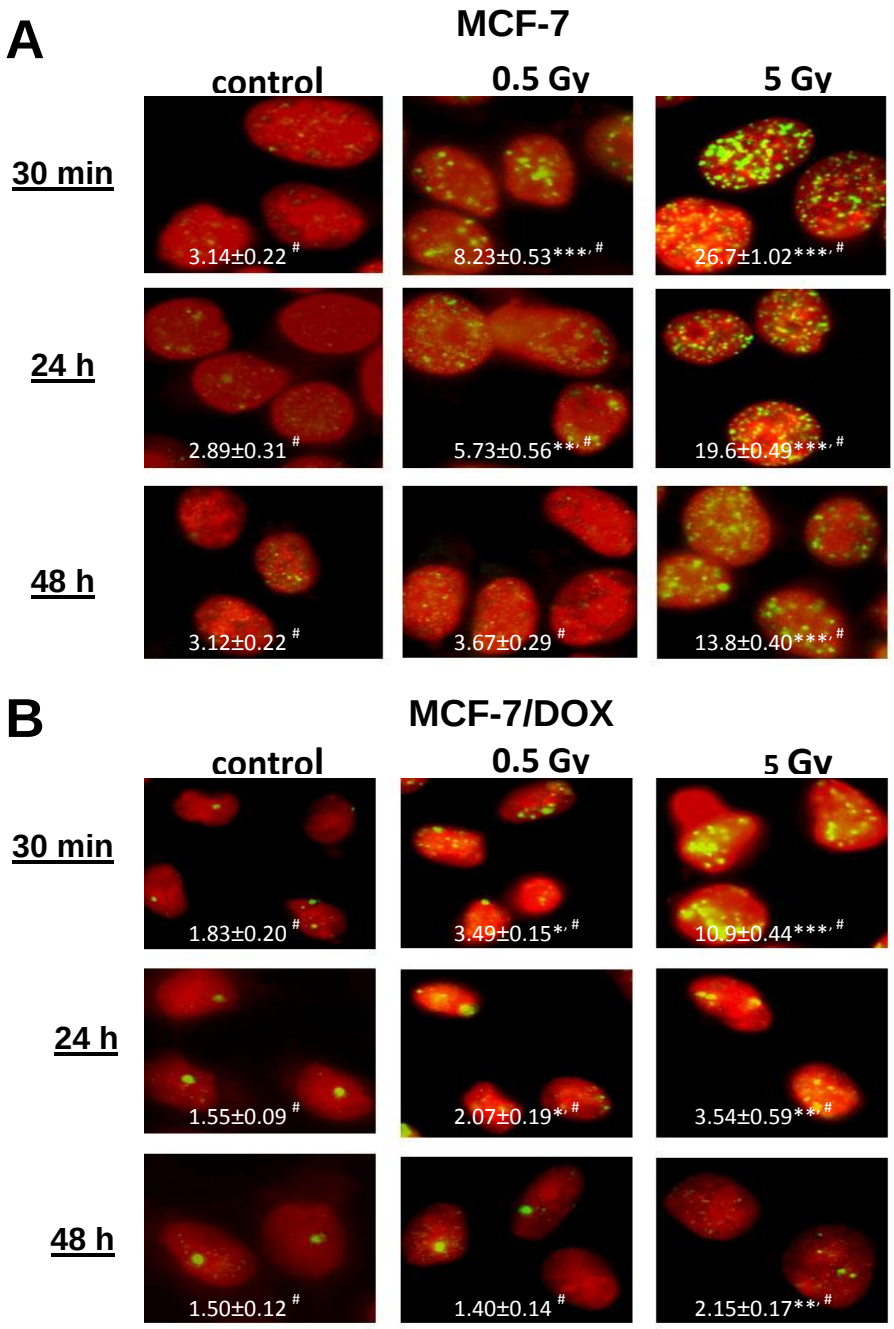

Figure 2.2. Radiation-induced H2AX phosphorylation in MCF-7 and MCF-7/DOX cells. **A** – MCF-7 cells; **B** – MCF-7/DOX cells. Results are presented as average number of γH2AX foci per cell ± SE, n = 200. ** - significantly different from the respective control; $p < 0.01$; *** - significantly different from the respective control; $p < 0.001$; Student's *t*-test. # - significantly different between the corresponding dose and time points in two cell lines- MCF-7 and MCF-7/DOX, $p<0.05$, Student's *t*-test. Magnification, × 100. Red – DAPI and Green – γH2AX.

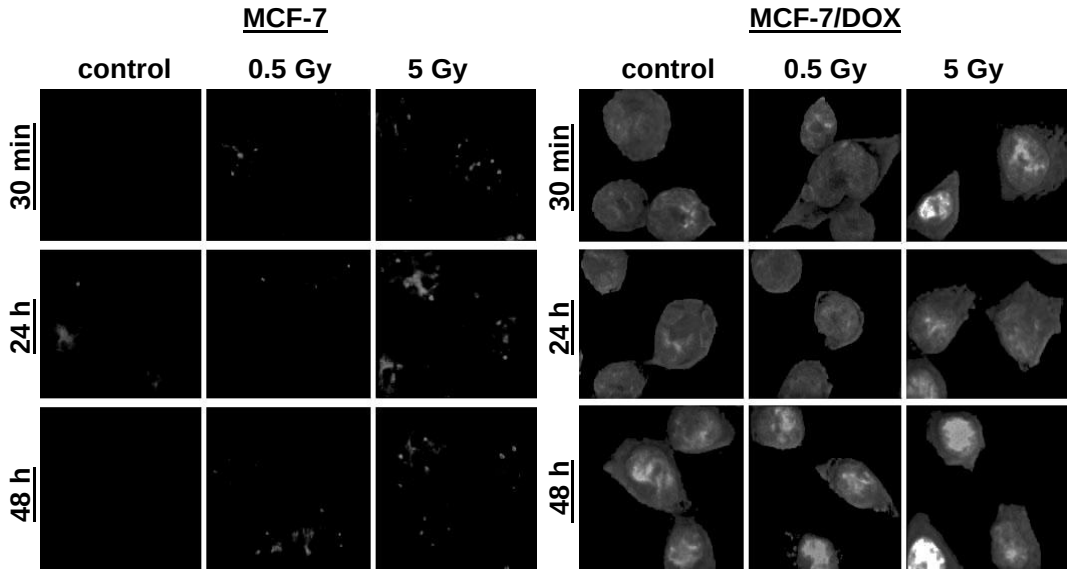

Figure 2.3. Phospho-ATM levels in MCF-7 and MCF-7/DOX cells before and after radiation exposure. Magnification, 100.

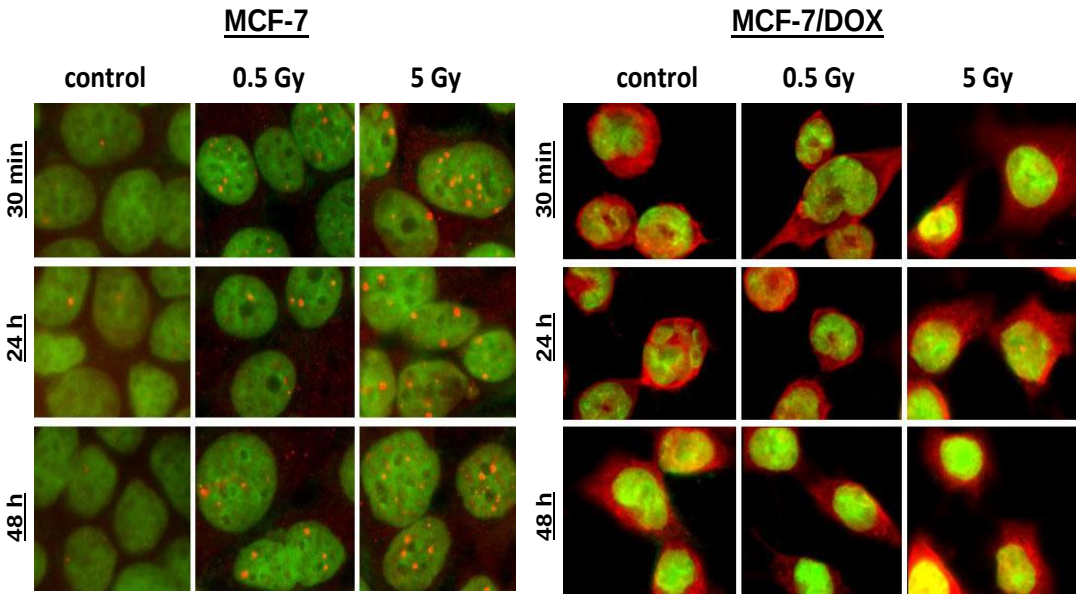

Figure 2.4. Cellular localization of phospho-ATM in MCF-7 and MCF-7/DOX cells before and after radiation exposure. Magnification, × 100. Blue – DAPI, and Green – p-ATM.

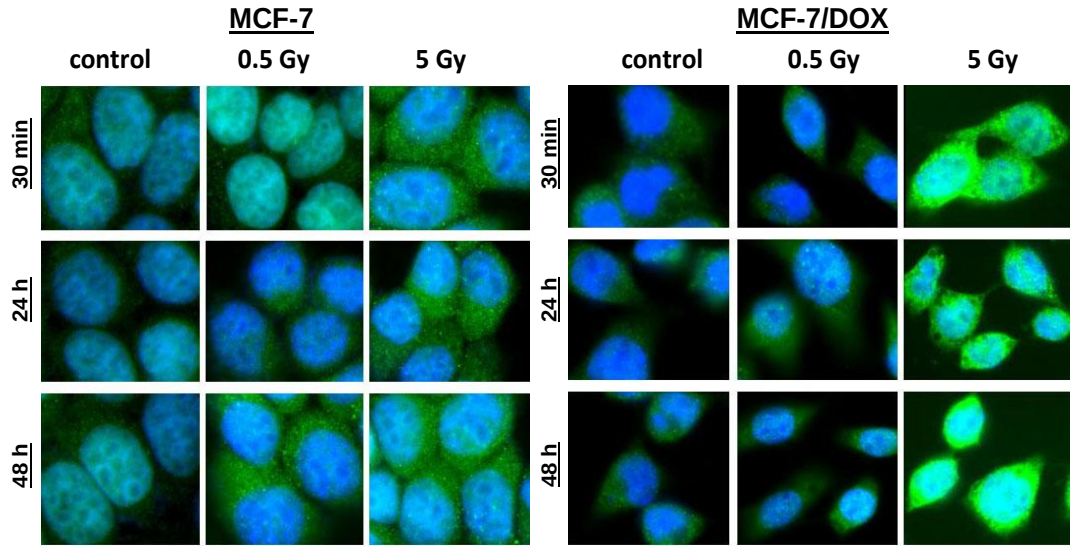

Figure 2.5. RAD51 expression in MCF-7 and MCF-7/DOX cells before and after radiation exposure. Magnification, × 100. Blue – DAPI, and Green – RAD51.

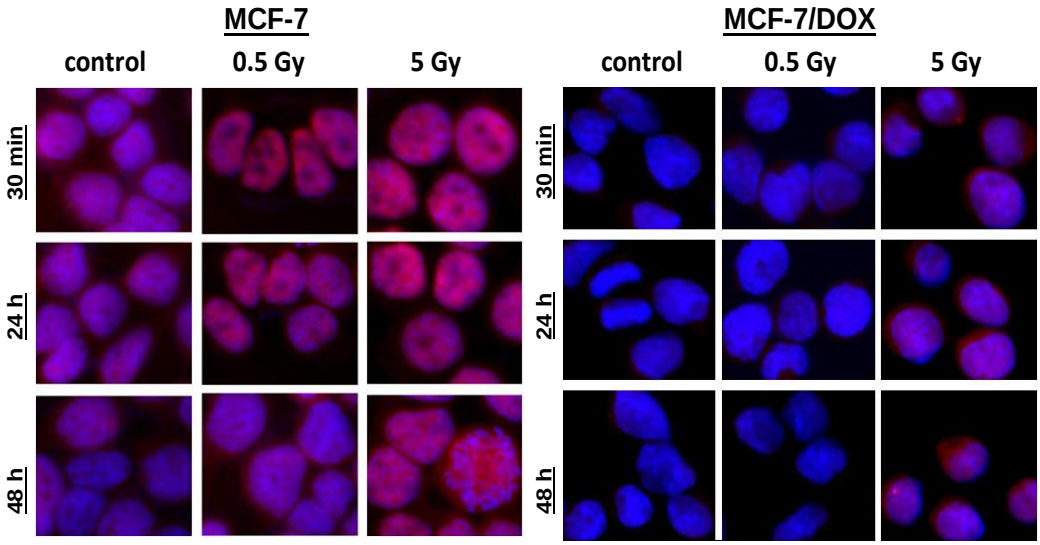

Figure 2.6. KU70 expression in MCF-7 and MCF-7/DOX cells before and after radiation exposure. Magnification, × 100. Blue – DAPI, and Red – KU70.

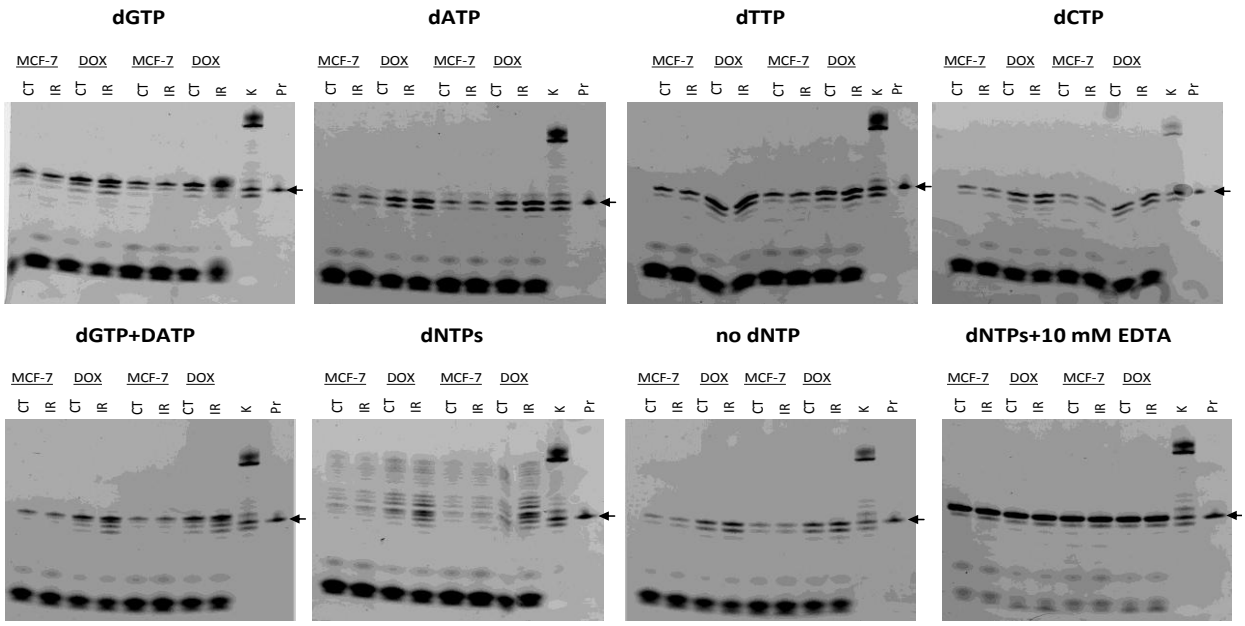

Figure 2.7. DNA Polymerase fidelity in MCF-7 and MCF-7/DOX cells. CT-control; IR-irradiated cells; K – Klenow enzyme, a positive control; Pr – primer only, 4 pM. Cell extracts were loaded in double repeats. An arrow indicates the 15bp band.

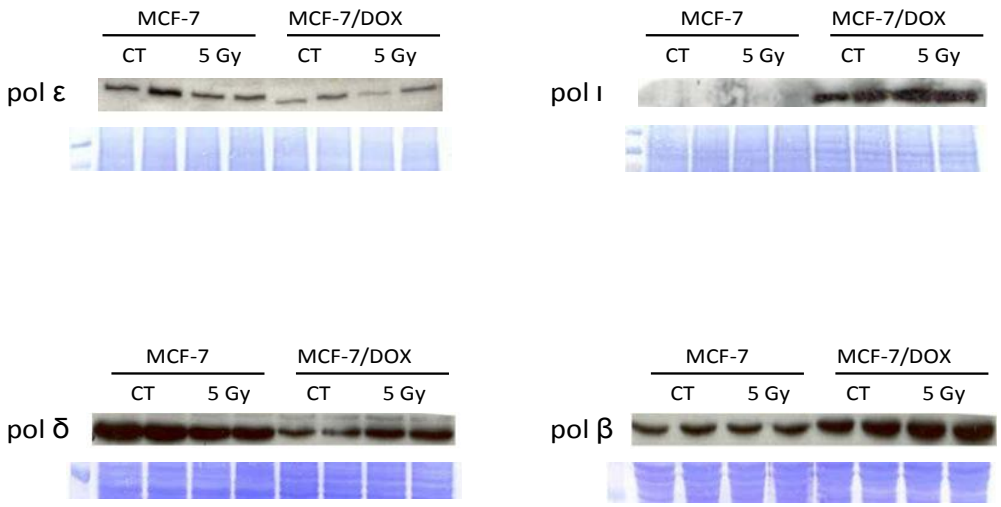

Figure 2.8. Expression of DNA Polymerases delta, beta, epsilon and iota in MCF-7 and MCF-7/DOX cells. Representative blots from 3 independent experiments are shown. PVDF membranes were stained with Coomassie Brilliant Blue G-250 to confirm an equal amount of loaded sample.

CHAPTER 3: MODULATION OF DNA METHYLATION LEVELS SENSITIZES DOXORUBICIN-RESISTANT BREAST ADENOCARCINOMA CELLS TO RADIATION-INDUCED APOPTOSIS

Chapter 3 has been submitted in its entirety in:

Luzhna L and Kovalchuk O. Modulation of DNA methylation levels sensitizes doxorubicin-resistant breast adenocarcinoma cells to radiation-induced apoptosis. Mutation Research (in review)

ABSTRACT

Chemoresistant tumors often fail to respond to other cytotoxic treatments such as radiation therapy. The mechanisms of the chemo-and radiotherapy cross resistance are not fully understood and are also believed to be epigenetic in nature.

Here we hypothesized that MCF-7 cells and their doxorubicin resistant variant MCF-7/DOX cells may exhibit different responses to ionizing radiation due to their dissimilar epigenetic status.

Similarly to the previous studies, we found that MCF-7/DOX cells harbor much lower levels of global DNA methylation than MCF-7 cells. Furthermore, we found that MCF-7/DOX cells had lower background apoptosis levels and were less responsive to radiation than the MCF-7 cells. Decreased radiation responsiveness was correlated with the significant global DNA hypomethylation of MCF-7/DOX cells.

Here we for the first time show that radiation resistance of MCF-7/DOX cells can be reversed by an epigenetic treatment – application of methyl-donor SAM. SAM-mediated reversal of DNA methylation led to elevated radiation sensitivity of MCF-7/DOX cells. Contrarily, application of SAM on the sensitive and higher methylated MCF-7cells resulted in the decrease of their radiation responsiveness. These data suggest that a fine balance of DNA methylation is needed to insure proper radiation and drug responsiveness.

INTRODUCTION

Conventional cytotoxic drugs frequently fail as a treatment modality due to the development of drug resistance that blocks their activity (Stavrovskaya, 2000). Acquired drug resistance is a multi-factorial phenomenon, involving multiple genetic and, most importantly, epigenetic mechanisms (Fojo, 2007; Gottesman, 2002; Stavrovskaya, 2000). 'Genetic' refers to heritable changes in DNA sequence, whereby the occurrence of random drug-induced mutational events leads to formation of drug-resistant cells (Fojo, 2007; Iwasa et al., 2006). 'Epigenetic' refers to information contained in chromatin rather than in the actual DNA sequence (Jaenisch and Bird, 2003), and the induction of epigenetic changes results in resistance to cytotoxic drugs (Brown and Glasspool, 2007; Chekhun et al., 2006; Glasspool et al., 2006). Epigenetic changes are heritably stable alterations and include DNA methylation, histone modifications and small RNA-mediated silencing (Jaenisch and Bird, 2003).

A number of studies have indicated substantial alterations of epigenetic profile of drug-resistant cancer cells, including changes in DNA methylation, histone modification patterns and microRNA expression (Baker and El-Osta, 2003; Brown and Glasspool, 2007; Chekhun et al., 2006; Duesberg et al., 2007; Fojo, 2007; Glasspool et al., 2006; Kovalchuk et al., 2008). DNA methylation changes in drug resistant cells comprise hypermethylation of numerous genes involved in DNA repair, cell cycle control and apoptosis (Glasspool et al., 2006; Teodoridis et al., 2004). Besides the hypermethylation-related acquired drug resistance, the hypomethylated status of some genes might cause similar chemotherapeutic responses (Chekhun et al., 2006; Chekhun et al., 2007). In addition to gene-specific changes, drug resistant cells

and tumors also exhibit global DNA hypomethylation (Chekhun et al., 2006; Chekhun et al., 2007).

Chemoresistant tumors often fail to respond to other cytotoxic treatments such as radiation therapy. The mechanisms of chemo-and radiotherapy cross resistance are not fully understood and are believed to be epigenetic in nature.

Amongst a variety of chemotherapy drugs, doxorubicin is an anthracycline drug traditionally used in curative-intent adjuvant and palliative treatment of breast cancer (Dean-Colomb and Esteva, 2008; Di Leo et al., 2002; Fornari et al., 1994; Frederick et al., 1990; T. Scandinavian Breast Group et al., 2006). While doxorubicin is one of the most active breast cancer treatment agents, many patients develop treatment resistance and experience cancer relapse (Dean-Colomb and Esteva, 2008). The data on radiation responses of doxorubicin-resistant breast tumors are contradictory and the exact nature and mechanisms of radiation responses of chemoresistant tumor cells remain obscure. The frequency of radiation resistance in doxorubicin-treated tumors also suggests an epigenetic nature to this phenomenon. Understanding the mechanisms of aberrant treatment responses is crucial for developing novel strategies for sensitizing tumors to cytotoxic regimens.

The dynamic characteristics and reversible nature of epigenetic methylation patterns suggest that this epigenetic parameter may be an attractive therapeutic target for reversing treatment resistance. Most importantly, in contrast to genetic alterations, epigenetic changes can be modified pharmacologically. Currently, DNA methylation inhibitors are being tested as potential anticancer agents. Small molecules that allow reversal of aberrant hypermethylation, such as 5′-aza-2′-deoxycytidine, are now entering clinical trials (Christman 2002).

Additionally, approaches are also available to reverse the loss of DNA methylation. S-adenosylmethionine (SAM) was proven to inhibit demethylation *in vitro* (Detich et al., 2003). SAM is a universal methyl donor which causes hypermethylation. SAM acts either by activating DNA methyltransferases or by inhibiting demethylation (Detich et al., 2003).

Here, we analyzed and compared the radiation response of MCF-7 breast adenocarcinoma cells and their doxorubicin resistant variant MCF-7/DOX cells. We noted that MCF-7/DOX cells were more resistant to radiation than sensitive cells. MCF-7/DOX cells also exhibited global DNA hypomethylation as compared to native MCF-7 cells. For the first time, we show that radiation resistance in MCF-7/DOX cells can be reversed by an epigenetic treatment – the application of methyl-donor SAM.

MATERIALS AND METHODS

Cell lines and cell culture conditions

MCF-7 and MCF-7/DOX multidrug-resistant human breast adenocarcinoma cell lines were previously developed and described (Chekhun et al., 2007; Kovalchuk et al., 2008). Cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM /F-12) with 2.5 mM L-Glutamine, without HEPES and Phenol Red (HyClone, Logan, UT), supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), in the presence of antibiotics 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO) in 5% CO_2 atmosphere at 37 ºC. Cells were treated daily either with vehicle as control or 100 mM

S-(5'-Adenosyl)-L-methionine chloride (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) by direct addition to the regular growth medium under sterile conditions for 6 days. Cells were harvested for analysis by trypsinization (Chekhun et al., 2007; Kovalchuk et al., 2008).

Irradiation conditions

Cells were irradiated at the confluency of 60% in DMEM. A radiation dose of 5 Gy (90 kV, 5 mA) was applied in order to induce cellular radiation responses. Un-irradiated cells served as control. Cells were harvested 24 hours after irradiation. All cells were tested in triplicate. Experiments were independently reproduced twice.

DNA methylation assay

Total DNA was prepared from cell suspensions using the Qiagen DNAeasy Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. A well established radiolabeled [3H] dCTP extension assay was employed to evaluate global DNA methylation levels (Pogribny et al., 1999). The assay measures the proportion of CCGG sites that lost methyl groups on both DNA strands. HpaII is a methylation-sensitive restriction enzyme that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. MspI is an isoschizomer of HpaII that is not sensitive to methylation and cleaves DNA regardless of methylation status. Both enzymes leave a 5'-guanine overhang after DNA cleavage. This overhang can be used for the subsequent single nucleotide extension with labelled [3H] dCTP. The extent of [3H] dCTP incorporation opposite to guanine in HpaII-treated DNA is directly proportional to the number of unmethylated CpG sites, while in MspI-treated DNA it is proportional to the total number of CpGs. The ratio between two indicates the level of methylation.

Briefly, 0.5 µg of genomic DNA was digested with 10 U of either methylation-sensitive HpaII or methylation-insensitive MspI enzymes (Fermentas Canada Inc., Burlington, ON, Canada) for 16-18 h at 37 ºC. A third DNA aliquot (0.5 µg) of undigested DNA served as background control. A single nucleotide extension reaction was performed in a 25 µL reaction mixture containing 0.5 µg DNA, 10 X PCR buffer, 1 mM $MgCl_2$, 0.5 U Taq DNA polymerase, and 0.1 µL [3H] dCTP (57.4 Ci/mmol) (GE Healthcare, Arlington Heights, US) and incubated at 56 ºC for 1 h. The samples were applied to Whatman DE-81 ion-exchange filters and washed three times with 0.5 M sodium phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for counting using a scintillation counter (Beckman LS 5000CE; Fullerton, CA). The [3H] dCTP incorporation into DNA was expressed as mean disintegrations per minute (dpm) per µg of DNA after subtraction of background. The percentage of methylation was calculated according to the formula:

$$\% M = 1 - ((HpaII - Ct) / (MspI - Ct))*100 \% \quad .$$

Annexin V assay

For apoptosis detection, Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) was used according to manufacturer's protocol. Cells were grown on 75 cm² cell culture flasks and irradiated as previously described. The analysis was performed 24 hours after radiation exposure. Cells were harvested, washed with PBS, resuspended in 1X binding buffer, stained with Annexin V and propidium iodide (PI) for 15 min at 25 ºC in the dark and analyzed by flow cytometry. Flow cytometry was performed at the Flow Cytometry Core Facility (University of Calgary, Calgary, AB). The results were represented as percentage of gated Annexin V positive cells.

Statistical analysis

Statistical analysis was performed using MS Excel 2007 and JMP5 software packages.

RESULTS

Different levels of radiation-induced apoptosis and DNA methylation in MCF-7 and MCF-7/DOX cells

Radiation exposure is known to induce apoptotic cell death in irradiated cells (Camphausen and Lawrence, 2008). Apoptosis is characterized by various changes in the cellular plasma membrane, the main of which is the translocation of phosphatidylserine (PS) from the inner layer to the surface of the membrane. Annexin V possesses a high affinity to PS and therefore allows early detection of apoptotic changes (Vermes et al., 1995). Here, we used the Annexin V/PI assay to study the levels of radiation-induced apoptosis in MCF-7 and MCF-7/DOX cells. Interestingly, the background apoptosis rate was much lower in MCF-7/DOX cells as compared to MCF-7 cells, which could be a characteristic feature of multidrug resistant cells (Figure 3.0). Ionizing radiation (IR) is a potent inducer of DNA damage and apoptosis. Exposure of MCF-7 cells to 5 Gy of X-rays led to a significant induction of apoptosis, while MCF-7/DOX cells were resistant to radiation-induced apoptosis (Figure 3.0)

We hypothesized that MCF-7 and MCF-7/DOX cells may be differentially sensitive to radiation due to their distinct epigenetic status, specifically – different global DNA methylation levels. To test this hypothesis, we first reconfirmed the status

of DNA methylation in MCF-7 and MCF-7/DOX cells. The cytosine extension assay revealed that MCF-7/DOX cells had significantly lower levels of methylated CCGG sites as compared to MCF-7 cells. Specifically, 73.3% of CCGGs were methylated in MCF-7 cells and 57.4% in MCF-7/DOX cells ($p<0.05$, Student *t*-test). Radiation exposure led to a slight (5.5%) increase of methylated CCGG sites in MCF-7 cells. No significant radiation-induced DNA methylation changes were seen in MCF-7/DOX cells (Figure 3.1 A)

SAM treatment reverses DNA hypomethylation and causes radiosensitivity in MCF-7/DOX cells

We hypothesized that the modification of DNA methylation status may result in the increased responsiveness of MCF-7/DOX cells to radiation. To test this prediction, we treated the MCF-7 and MCF-7/DOX cells with methyl donor SAM for 6 days. After treatment, the cells were exposed to radiation and DNA methylation and apoptosis levels were analyzed.

Treatment of cells with methyl donor SAM led to a significant increase in the level of methylation observed in both MCF-7 and MCF-7/DOX cell lines (Figure 3.1 B). Specifically, application of 100 mM SAM caused an increase in methylation level up to 70% in doxorubicin resistant cells ($p < 0.05$, Student *t*-test) which, therefore, became close to the level of methylation in untreated sensitive MCF-7 cells (Figure 3.1 A). Methylation increased up to 90% in MCF-7 cells after adding SAM (Figure 3.1 B). Importantly, upon SAM treatment the global methylation levels in both cell lines became more similar to each other. Ionizing radiation did not affect global DNA methylation in SAM-treated MCF-7 or MCF-7/DOX cells.

Next, we analyzed IR-induced apoptosis in SAM-treated MCF-7 and MCF-7/DOX cells. SAM treatment alone did not cause apoptosis in either cell line due to the non-toxic nature of SAM (Figure 3.2 and Figure 3.3). Importantly, we found SAM treatment increased radiosensitivity of previously radioresistant MCF-7/DOX cells (Figure 3.3) supposedly by increasing their methylation status. At the same time, MCF-7 cells, surprisingly, lost their sensitivity to radiation treatment after SAM treatment (Figure 3.2). Indeed, after SAM treatment, the levels of apoptosis decreased back to their background level. Such a response in drug sensitive cells could be due to the methylation and subsequent inactivation of some unknown anti-apoptotic genes, however, this requires further investigation.

DISCUSSION

In the current study, we analyzed the role of epigenetic DNA methylation status in MCF-7 and MCF-7/DOX cells and correlated this data with the radiation responsiveness of these cell lines. We found that MCF-7/DOX cells exhibited global hypomethylation as compared to sensitive MCF-7 cells (Figure 3.1 A).Our data correlates with previous findings (Chekhun et al., 2006). Importantly, drug resistant MCF-7/DOX cells were also resistant to radiation exposure. We hypothesized that if global DNA hypomethylation is responsible for the acquired treatment resistance, then inhibition of demethylation should result in a reversion to the sensitive phenotype. Because the demethylating enzymes which cause DNA hypomethylation are yet unknown, there are no specific inhibitors of hypomethylation (Szyf et al., 2004). SAM is an important cellular molecule that serves as a universal donor of methyl groups for

various processes including DNA methylation. Depletion of SAM levels in the cells can therefore result in DNA hypomethylation. After partaking in DNA methylation reactions, SAM is turned into S-adenosyl-homocysteine (SAH). Indeed, several studies have shown that the ratio of SAM to SAH in the cell is related to changes in DNA methylation (Castro et al., 2005; Yi et al., 2000). Furthermore, SAH has an inhibitory effect on DNMT activity (Hoffman et al., 1979). Since doxorubicin acquired chemoresistance is associated with increased detoxification of free radicals by the glutathione system (Stavrovskaya, 2000), which uses SAM as methyl donor for glutathione-S-transferase, the resulting depletion of SAM could cause DNA hypomethylation. Therefore, SAM treatment was chosen as a method to counteract hypomethylation in MCF-7/DOX cells (Pakneshan et al., 2004; Shukeir et al., 2006).

Previously, it has been observed that SAM treatment caused DNA methylation in tumours, with the methylation maintained for several weeks even after withdrawal of SAM (Shukeir et al., 2006). In our study, daily SAM treatment for 6 days caused a significant increase in DNA methylation levels of MCF-7 and MCF-7/DOX cells. SAM-mediated DNA methylation was correlated with an increase in radiation sensitivity of MCF-7/DOX cells. Contrarily, application of SAM to the sensitive MCF-7 cells, originally more methylated than MCF-7/DOX cells, resulted in a decrease in their radiation responsiveness. This data suggests that a fine balance of DNA methylation is needed to insure proper radiation and drug responsiveness.

Overall, our study is the first to show a correlation between radiation responsiveness and global levels of DNA methylation in cells. Future studies are needed to identify in detail the loci that change methylation status upon SAM treatment in both cell types.

Although further investigations are required to understand the mechanisms of SAM action and hypermethylation consequences, our study suggests that inhibition of hypomethylation could be a novel epigenetic approach to clinical chemo- and radiation-resistance and ultimately increase patient survival.

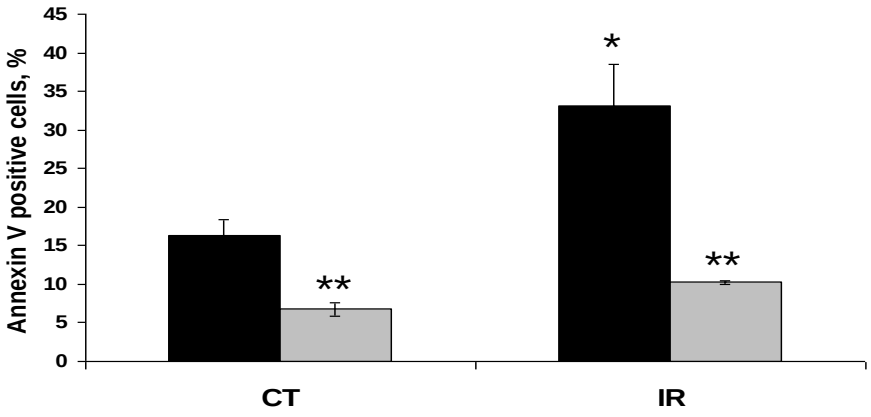

Figure 3.0. Radiation-induced apoptosis in MCF-7 and MCF-7/DOX cells. The number of cells in early apoptosis was measured using Annexin V-FITC assay for control non-irradiated (CT) and irradiated with 5 Gy of X-rays cells (IR). The results are presented as mean values ± S.E.M., n=6. * - significantly different from the respective control, $p<0.05$; ** - significantly different from respective MCF-7 cells, $p<0.05$, Student's t-test. Black bars – MCF-7 cells, and Grey bars – MCF-7/DOX cells.

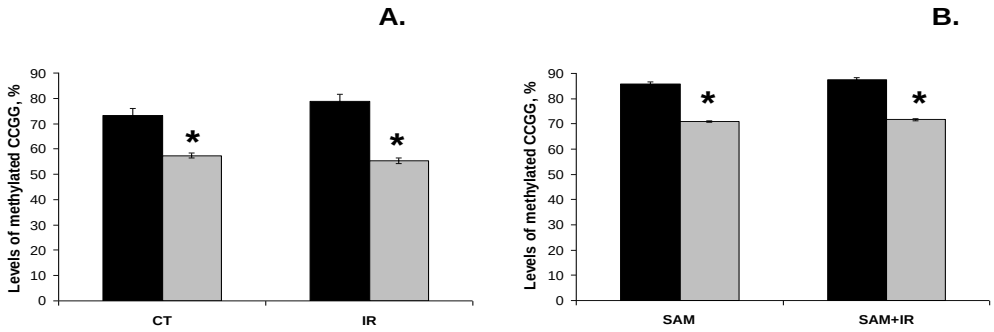

Figure 3.1 Global DNA methylation levels in MCF-7 and MCF-7/DOX cells. The levels of DNA methylation were mesured using the cytosine extension assay for control (CT), and irradiated with 5 Gy of X-rays cells (IR) (A), and for 100 mM SAM treated (SAM), and irradiated with 5 Gy of X-rays after SAM treatment cells (SAM+IR) (B). The results are presented as mean values ± S.E.M., n=4; * - significantly different from MCF-7, $p<0.05$. Black bars – MCF-7 cells, and Grey bars – MCF-7/DOX cells.

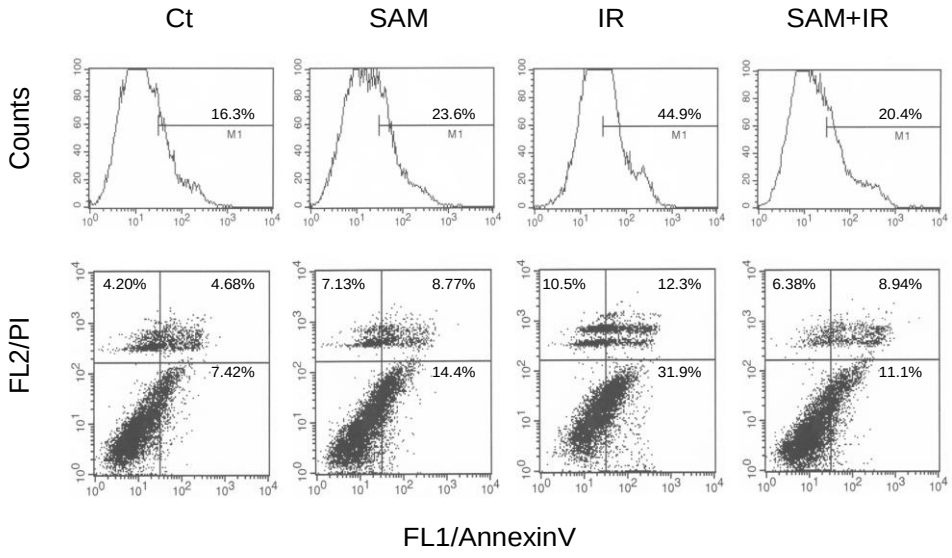

Figure 3.2 Radiation-induced apoptosis in MCF-7 cells after SAM treatment studied by Annexin V-FITC assay. M1 – average number of Annexin V positive cells. Diagrams below the M1 counts represent general cell distribution and viability. Viable cells – Annexin V and PI negative (lower left quadrants); cells in early apoptosis – Annexin V positive and PI negative (lower right quadrants); cells in late apoptosis or necrosis – Annexin V and PI positive (upper right quadrants); dead cells – (upper left quadrants). The numbers represent averages of three independent experiments.

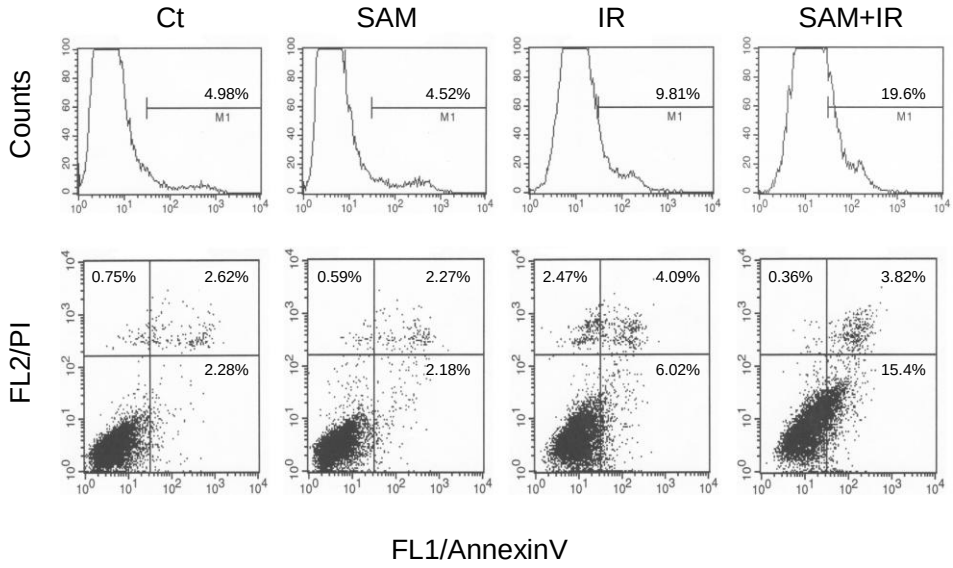

Figure 3.3 Radiation-induced apoptosis in MCF-7/DOX cells after SAM treatment studied by Annexin V-FITC assay. M1 – average number of Annexin V positive cells. Diagrams below the M1 counts represent general cell distribution and viability. Viable cells – Annexin V and PI negative (lower left quadrants); cells in early apoptosis – Annexin V positive and PI negative (lower right quadrants); cells in late apoptosis or necrosis – Annexin V and PI positive (upper right quadrants); dead cells – (upper left quadrants). The numbers represent averages of three independent experiments.

GENERAL DISCUSSION AND CONCLUSIONS

Breast cancer is the most common type of malignancy in women (Parkin, 2001; Parkin et al., 2002) with the worldwide incidence continuing to rise (Ellsworth et al., 2004).

Cytotoxic chemotherapy is extremely important for adjuvant treatment of breast cancer. Amongst a variety of cytotoxic drugs, doxorubicin is widely used in curative-intent adjuvant breast cancer therapies (Budišic et al., 1998; Verma et al., 2008). Doxorubicin is an intercalator which interferes with topoisomerase function, blocks replication and leads to induction of apoptosis (Fortune et al., 2000).

Even though cytotoxic chemotherapy is an effective treatment modality, a significant number of patients develop treatment resistance which leads to worsening of prognosis and relapse (Efferth et al., 2008; Frenkel and Caffrey, 2001; Lehnert, 1996). The development of cancer cells resistant to chemotherapeutic agents is a major clinical obstacle in the successful treatment of breast cancer (Budišic et al., 1998; Verma et al., 2008). Understanding mechanisms underlying the development of drug resistance and predisposition is critical in saving lives.

Ionizing radiation is another important treatment modality which is often combined with chemotherapy for management of breast cancer (Liang et al., 2003). However, drug-resistant breast cancer cells may fail to respond to radiation therapy (Ozols, 1988). Mechanisms of cross-resistance are not well understood.

A number of studies have indicated substantial alterations of epigenetic mechanisms in drug-resistant cancer cells, including changes in DNA methylation. DNA methylation is crucial for regulation of gene expression and chromatin state and

therefore – for normal development, cell proliferation, and proper maintenance of genome stability in an organism (Jaenisch and Bird, 2003).

The current study is aimed to analyze and compare the radiation response of MCF-7 breast adenocarcinoma cells and their counterparts resistant to doxorubicin (MCF-7/DOX).

We discovered that MCF-7/DOX cells were much more resistant to radiation than MCF-7 cells. We determined that this difference was due to differences in radiation-induced DNA damage, repair and apoptosis as well as differences in the epigenetic DNA methylation profile. We have discovered that modulation of the epigenetic DNA methylaition profle of beast adenocarcomoa cells can reverse the radiation response of cells.

The major findings of this thesis are:

1. MCF-7/DOX cells accumulate less DNA double-strand breaks following radiation treatment when compared to drug sensitive MCF-7 cells, and it is accompanied with higher but less precise DNA repair capacity.
2. Acquired doxorubicin resistance is associated with global DNA hypomethylation in MCF-7/DOX.
3. S-adenosyl-methionine (SAM) treatment causes reversal of methylation patterns and increases radiation-induced apoptosis in MCF-7/DOX.

Summary of contributions that our findings have made to already existing knowledge:

1. There has been discovered a cross-resistance between chemo- and radioresistance in breast cancer cells.

2. It has been proved that an increased DNA repair potential of drug-resistant tumor cells contributes to the development of radioresistance.
3. It has been shown that radiosensitivity of doxorubicin-resistant cells can be reverted by increasing the DNA methylation status.

Our findings are important for:

1. choosing the right modality for breast cancer treatment;
2. predicting the development of resistance to already existing treatment methods;
3. the development of novel epigenetic therapy strategies.

PROSPECT OF FUTURE RESEARCH:

The findings presented in this thesis characterize only several molecular mechanisms of resistance of breast cancer cells to chemo- and radiation therapy. The cellular response to cytotoxic agents includes a variety of defensive pathways that require further in-depth investigation. Similarly, further study is required to evaluate prospects of reversal of drug sensitivity using epigenetic therapy. Our suggestions for future studies are:

1. Further investigate the role of epigenetic changes in chemoresistance of breast cancer cells. Specifically, detailed analysis of hypomethylated DNA sequences should be performed to reveal what types of dysregulated genes are involved in the development of resistance to chemotherapy. This is especially important as it has recently been proven that alterations of DNA methylation in cancer cells occur in defined regions, suggesting that they are locus-specific and

cause non-random global DNA dysregulation (Mohn et al., 2008; Weber et al., 2005; Weber et al., 2007; Wilson et al., 2006).

2. The phenomenon of drug resistance was explained using only one out of a whole variety of therapeutic drugs as an example. There is a whole cohort of other anthracyclines and hormonal agents that might be explored in order to make a more precise conclusion about driving mechanisms of multi-drug resistance. Therefore, we assume it would be interesting to analyze and compare the radiation response of MCF-7 cells that exhibit resistance to cisplatin, tamoxifen and faslodex – some of the most common drugs used for breast cancer treatment.
3. The potential for reverting drug-resistant phenotypes by modifying the epigenetic status also needs to be studied. In the current study, we have obtained only preliminary data that allow us to propose a possible role of SAM as an epigenetic therapy agent for reversal of treatment resistance.

   Mechanisms of SAM actions are not fully understood, and it is necessary to investigate whether all genes are equally targeted by SAM, and if yes, whether it causes changes in the level of expression of tumor suppressor genes.
4. In this thesis, we studied responses of MCF-7 breast adenocarcinoma cells. They represent an ER-positive tumor phenotype with low invasive potential. Breast cancer has a wide variety of different molecular phenotypes. In the future, it would be necessary to analyze drug and radiation response of several other breast cancer cell lines.

   Molecular markers of drug resistance identified in this study can serve as a foundation for the translational approach. Emerging evidence indicates the

existence and importance of another epigenetic mechanism for regulating gene expression. This mechanism is mediated by short non-coding RNAs (Bartel, 2004; Bushati and Cohen, 2007; Sevignani et al., 2006). MicroRNAs (miRNAs) are of special interest as they inhibit the translation of a variety of proteins. MicroRNAs have only recently been identified as potent novel regulators of gene expression and as a novel class of potential tumor classifiers. Even though they are now well-established as important contributors to tumor development and chemoresistance, their functions have yet to be defined. Notwithstanding, if we define the roles of miRNAs in the regulation of anthracycline resistance, this will constitute a major breakthrough in the field of novel biomarkers for cancer prognosis and prediction of treatment responses. Therefore, miRNAs hold much promise as cancer and breast cancer drug resistance modulators and biomarkers.

Overall, this thesis may serve as a roadmap for future analysis of tumour resistance to radio- and chemotherapy and for the development of novel epigenetic strategies for reversal of breast cancer resistance to cytotoxic treatment regiments.

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