In Vitro Studies of a New Radiosensitizer for Radiotherapy of Breast Cancer

by

Yurui Wu

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Physics

Waterloo, Ontario, Canada, 2015

©Yurui Wu 2015

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Breast cancer is the most commonly diagnosed cancer among women and is the leading cause of cancer death in both developing and developed areas. Triple-negative breast cancer, one of the subtypes of breast cancer, is generally more aggressive and has fewer treatment options compared with other subtypes, due to the absence of drug-targetable receptors.

Radiotherapy delivers ionizing radiation to cancerous areas, leading to DNA damage and cell death. There has been controversy about the molecular mechanism of DNA damage induced by ionizing radiation. According to conventional notions, biological damage by ionizing radiation is primarily induced by the hydroxyl radical ($OH^{\bullet}$), but Lu et al., using femtosecond time-resolved laser spectroscopy, have discovered that the dissociative electron transfer (DET) reaction of the weakly-bound prehydrated electron (e^{-}_{pre}) plays an important role in causing chemical bond and DNA strand breaks. Building on the better understanding of DET reaction mechanisms of e^{-}_{pre}, the mechanism behind the radiosensitizing effect of cisplatin and halopyrimidines has been discovered by our group. However, cisplatin has severe toxicity, and the DET reaction efficiency of halopyrimidines is low.

A newly discovered non-platinum-based compound, FMD-Br-DAB, identified through the femtomedicine approach, was tested in this research as a potential

radiosensitizer. Our in vitro results have confirmed that FMD-Br-DAB can exert radiosensitizing effects on treated triple-negative breast cancer (MDA-MB-231) cells when combined with radiation. Our results also indicate that FMD-Br-DAB can enhance DNA damage in a dose-dependent manner when combined with radiation.

Acknowledgements

First and foremost, I would like to extend my sincerest gratitude to my supervisor, Professor Qing-Bin Lu, for his support, guidance and encouragement throughout my graduate study. I deeply appreciate Professor Lu for giving me the opportunity to conduct my undergraduate project and Master's research in his lab, and it is my great honor to be his graduate student.

I would like to express my gratitude to my advisory committee members, Dr. Bae-Yeun Ha, Dr. Russell Thompson, and Dr. Anton Burkov, for their guidance and support.

My gratitude also goes to our group members, Chunrong, Qinrong, Ting, Ning, Yang, Zhi, Jenny, and Yaowen. I appreciate the friendship and support they have given me; I could not have finished my research without their help.

I would also like to thank Mary McPherson of the Writing Centre for helping me improve my overall writing.

A special thanks to my parents and grandparents for their love and encouragement from an early age. I also like to thank my fiance, Ming, for providing me continuous encouragement throughout my study.

To my family.

Table of Contents

Abstract iii

Acknowledgements v

List of Figures ix

Chapter 1. Introduction 1

1.1 Introduction to Breast Cancer 1
1.1.1 Breast Cancer Subtypes 2
1.1.2 Breast Cancer Screening and Diagnostic Approaches 4
1.1.3 Treatments 7
1.1.4 Triple-negative breast cancer 16
1.2 Radiotherapy 17
1.2.1 Target of radiotherapy 18
1.2.2 Mechanism of radiotherapy 19
1.2.3 Radiosensitizer 21
1.2.4 FMD-Br-DAB as a Potential Radiosensitizer 23

Chapter 2. Cell Survival Study by MTT assay 25

2.1 Introduction 25
2.2 Experimental details 26
2.3 Results 29
2.4 Discussions and Conclusions 31

Chapter 3. Cell Survival Study by Clonogenic Assay 33

3.1 Introduction 33
3.2 Experimental details 34
3.3 Results 36
3.4 Discussions and Conclusions 37

Chapter 4. Gel Electrophoresis 39

4.1 Introduction 39
4.2 Experimental Details 41
4.3 Results 44
4.4 Discussions and Conclusions 50

Chapter 5. Flow Cytometry of Apoptotic Cell Death 51

5.1 Introduction 51
5.2 Experimental details 52
5.3 Results 54
5.4 Discussions and Conclusions 59

Chapter 6. Conclusions 61

References 63

List of Figures

Figure 2.1: Cell survival fraction for MDA-MB-231 cells treated with 0-60 μM of FMD-Br-DAB and 0-16 Gy of X-ray irradiation. 29

Figure 2.2: Cell survival fraction for MDA-MB-231 cells treated with 0-60 μM of FMD-Br-DAB and 0-16 Gy of X-ray irradiation. Cell survivals at different dose of X-ray without FMD-Br-DAB treatment have all been normalized to 100%. 30

Figure 3.1: Cell survival rates for MDA-MB-231 cells treated with 0-20 μM FMD-Br-DAB and 0-4 Gy X-ray 36

Figure 4.1: Gel electrophoresis of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation. 44

Figure 4.2: SSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation. 45

Figure 4.3: DSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation. 45

Figure 4.4: Gel electrophoresis of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-200 Gy of X-ray irradiation. 46

Figure 4.5: SSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-200 Gy of X-ray irradiation. 47

Figure 4.6: SSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-200 Gy of X-ray irradiation. 47

Figure 4.7: Gel electrophoresis of DNA samples treated with 0-750 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation. 48

Figure 4.8: SSBs amount (with artificial unit) of DNA samples treated with 0-750 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation. 49

Figure 4.9: SSBs amount (with artificial unit) of DNA samples treated with 0-750 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation....... 49

Figure 5.1: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 control cells without any treatment. Blue and green areas correspond to lower cell density; yellow to mid-range cell density; red and orange to high cell density. The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells. 55

Figure 5.2: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells treated with 20μM FMD-Br-DAB without any X-ray irradiation. Blue and green correspond to areas of lower cell density, yellow area represents mid-range cell density, and red and orange are areas of high cell density. The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells. 56

Figure 5.3: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells treated with 2Gy X-ray irradiation without any drug treatment. Blue and green correspond to areas of lower cell density, yellow area represents mid-range cell density, and red and orange are areas of high cell density. The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells. 57

Figure 5.4: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells treated with 20μM FMD-Br-DAB followed by 2Gy X-ray irradiation. Blue and green correspond to areas of lower cell density, yellow area represents mid-range cell density, and red and orange are areas of high cell density. The cells in the right side of the vertical line are BrdU positive cells (apoptotic cells). The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells. 58

Chapter 1. Introduction

1.1 Introduction to Breast Cancer

Breast cancer is the most common malignancy among women globally, accounting for 23% of total cancer cases in females. It is the second most common cancer in both sexes. Annually, for every ten newly diagnosed cancer cases, one is breast cancer. It is also the leading cause of cancer death among females and accounts for 14% of total cancer deaths. [1, 2, 3, 4] In Canada, one in nine women will develop breast cancer in her lifetime. It is estimated that in 2014, 13.8% of all cancer deaths in Canadian women were caused by breast cancer, making it the second leading cause in cancer deaths among Canadian women [5].

The diagnosis of breast cancer has increased in many developed countries, including the United States and Canada, since the early 1980s due to the rapid growth of the application of mammographic screening [1, 6].

1.1.1 Breast Cancer Subtypes

Breast cancer is a heterogeneous disease and can be classified as different subtypes according to phenotype and genotype or a tumor's position, stage, grade, and histology. The distinction between the subtypes of breast cancer has a great impact on prognosis and survival, and it is also critical to making appropriate treatment decisions. [7]

According to tumor histology, breast cancer can be characterized by microscopic organization and the growth pattern of cancer cells. Most breast cancers are derived from milk ducts and lobules, and these types are named ductal carcinomas and lobular carcinomas, respectively. Based on the distribution of tumor cells, breast cancer can also be classified as in situ or invasive. Ductal carcinoma in situ (DCIS) is a type of pre-cancer inside of the ductal system that has not spread to nearby tissue. Lobular carcinoma in situ (LCIS) rarely develops into invasive cancer but it can signal a higher risk of developing invasive breast cancer. [8] DCIS is the most common type of in situ breast cancer, accounting for 80-85% of all in situ tumors, while lobular carcinoma in situ (LCIS) accounts for only about 5% [9].

With respect to invasive breast cancer in developed countries, invasive ductal carcinoma (IDC), the most common subtype, accounts for about 70-73%. Invasive

lobular carcinoma (ILC), the second most common subtype, accounts for 13-16% of invasive breast cancers in developed countries [10]. Some other subtypes of breast cancer include mucinous, comedo, inflammatory, tubular, medullary, and papillary carcinomas [11].

Breast cancer can also be classified by molecular and genetic markers of tumors, including human epidermal growth factor receptor type 2 (HER2), progesterone receptor (PR) and estrogen receptor (ER). *HER2*, a growth factor receptor gene, is amplified in 25 to 30 percent of breast cancer cases, called HER2+ breast cancer [12]. ER status is also a very important factor in classifying breast cancer. Tumors that express receptors for estrogen are estrogen-receptor-positive (ER+). Estrogens can pass through cell membranes and bind the ERs, transforming them into DNA-binding transcription factors that stimulate estrogen-mediated growth pathways and impact cellular function [13]. A cancer is progesterone-receptor-positive (PR+) if it has progesterone receptors. A number of studies have confirmed that ER expression is strongly correlated with other tumor markers, for example PR. Studies have shown that more than 80% of ER+ tumors are PR+ and greater than 90% of ER- tumors are also PR- [15]. Tumors that lack expression of ER, PR, and HER2 are defined as triple-negative breast cancers. About 15 to 20% of breast cancer cases are triple-negative [14]. Therefore, with the help of

complementary DNA (cDNA) microarray technology, breast cancer is divided into five distinct subtypes: luminal A, luminal B, HER-2 overexpressed, basal-like, and normal-like [16]. Luminal A and luminal B subtypes are both ER+ and low-grade, but luminal A is HER2- and has a better prognosis, whereas luminal B is HER2+ and grows more quickly. Basal-like tumors exhibit a high expression of the characteristic genes of basal epithelial cells. Most basal-like tumors are triple-negative, meaning they are ER-, PR- and HER2-. HER2 overexpressed breast tumors have extra copies of the HER-2 gene. About six to ten percent of breast cancers are normal-like or unidentified. These tumors are usually small and tend to have a good prognosis. [18,19]

1.1.2 Breast Cancer Screening and Diagnostic Approaches

Breast cancer screening can help diagnose cancer at an early stage and avert deaths. Common screening tests include Mammography, clinical breast exam (CBE), breast self-examination (BSE), ultrasound, and magnetic resonance imaging (MRI). Mammography, a common screening method, is a type of radiography. The widespread use of screening mammography in developed countries has lead to an increased diagnosis of breast cancer and has significantly reduced breast cancer

mortality in women between 50 to 70 years of age [20, 21]. A meta-analysis has shown 14% breast cancer death reduction for women in their 50s and 32% for those in their 60s [22]. However, mammography is not very useful in finding breast tumors in younger women who tend to have dense breasts. It has been concluded by the U.S. Preventive Services Task Force (USPSTF) that the benefits of regular mammography increase, and the harm from it decreases when age increases [23]. The recommended age at which screening should begin varies across counties. For example, in Canada women age 50 to 69 are recommended to have a mammogram every two years. For many years, doing regular BSE has been suggested. It is believed that women who practice BSE regularly are more likely to find abnormalities earlier, leading to earlier treatment and therefore higher survival rates [24]. However, according to a systematic review by the USPSTF in 2009, the effectiveness of CBE and BSE in decreasing breast cancer mortality is controversial. No big differences were found in studies using mammography alone versus studies using mammography and CBE [23]. MRI, which is less commonly used, has very high negative predictive values and can be used to screen patients with radiographically dense breasts. It can also diagnose common benign conditions other than cancer. However, MRI is more expensive and may have more false positives than mammography [25,26]. Breast ultrasound, a diagnostic aid to mammography, is

frequently used as a targeted diagnostic examination focusing on a specific area. It may be used in pregnant women and women younger than 25 years old, but it has higher rates of false-positive examination results than mammography [27].

If suspicious areas are found by screening, CBE or BSE, additional imaging and biopsy may be recommended. Current biopsy techniques include fine-needle aspiration biopsy (FNAB), stereotactic core biopsy, image-guided core biopsy, non-image-guided core biopsy, and excisional biopsy [28]. The types of biopsy vary by the invasiveness level and amount of tissue acquired. FNAB, using a smaller needle, is less invasive and is less likely to cause hematoma than core biopsy. However, due to the smaller needle size of FNAB, it may obtain an insufficient sample and therefore is less reliable than core biopsy [29]. It has been shown that the use of stereotactic core needle biopsies shortens the time between abnormality detection by mammography and pathologic diagnosis [30].

1.1.3 Treatments

The treatment of most breast cancers is multidisciplinary. They can be treated by surgery, radiotherapy, and chemotherapy. Some certain cases of breast cancers can be treated by hormone therapy and targeted therapy depending on hormone receptor status, overexpression of some genes, and patient menopausal status [31].

Surgery

Surgery, a primary treatment method for breast cancer, is aim to remove the tumor and metastases in regional lymphatic tissues.

Breast-conserving surgery, including lumpectomy, quadrantectomy and partial mastectomy, is preferred when a tumor is confined to one area. Lumpectomy is the removal of a small part of the breast, including the breast tumor and some of the surrounding tissues. Quadrantectomy is the removal of around one quarter of the breast. Randomized trials have shown that radiotherapy following breast-conserving surgery decreases local recurrence and is preferred for most women with early breast cancer, but it is not recommended for women at high risk of local recurrence [32, 33].

A radical mastectomy is recommended when the tumor is too extensive or there

is a clinically positive axilla. It removes all of the breast tissue and the axillary lymph nodes [33]. Most often, breast reconstruction is performed at the time of the mastectomy.

Radiotherapy

Radiotherapy is a treatment using ionizing radiation to control or kill cancer cells. The mechanism of radiotherapy is to destroy cells in the area being treated by damaging the DNA of the cells, making them unable to grow and divide.

Typically, women with early stage breast cancer who have undergone breast-conserving surgery are treated with radiotherapy to lower their risk of local chest wall and regional lymph node recurrence. A review of ten randomized controlled trials showed that breast-conserving surgery with radiation significantly reduced the five-year local recurrence rate and appeared to decrease the 15-year mortality risk [34].

Chemotherapy

Chemotherapy can be administered prior to cancer surgery as neoadjuvant (or preoperative) chemotherapy to shrink tumors. Patients with inoperable breast cancer for surgery are considered for neoadjuvant chemotherapy because it can

shrink tumors sufficiently to make resection possible. Patients with operable breast cancer but who are poor candidates for breast-conserving surgery because of large tumor size may also be offered neoadjuvant chemotherapy to facilitate less disfiguring surgery [35]. Another benefit of neoadjuvant chemotherapy is that initial responsiveness can be assessed by measuring tumor sizes, providing prognostic information. Thus, ineffective chemotherapy can be stopped, avoiding further cytotoxicity [36]. However, there may be some unresolved concerns about neoadjuvant chemotherapy. One issue concerns the timing of sentinel lymph node biopsies (SLNBs), a preferred method for evaluating axillary lymph nodes. Chemotherapeutic drugs may induce lymphatic damage and may interfere with this procedure. It has been reported that SLNB following neoadjuvant chemotherapy may result in higher rates of false-negatives, and this may lead to the undertreatment of a subset of patients [37]. However, if SLNB is performed prior to neoadjuvant chemotherapy, patients need to undergo another operation, which would otherwise be performed during a single surgery.

Adjuvant chemotherapy is also a general treatment for breast cancer patients. It is administered by applying cytotoxic chemotherapy or ablative endocrine therapy after primary surgery. During the past few decades, adjuvant chemotherapy has undergone many significant changes. Forty years ago, only breast cancer patients

with positive nodes received adjuvant chemotherapy. It was not until the 1970s that women with node-negative breast cancer could benefit from adjuvant chemotherapy [41]. In the 1990s, anthracyclines, doxorubicin and epirubicin were first introduced. Nowadays, common chemotherapy agents used in the adjuvant setting are cyclophosphamide, methotrexate, 5-fluorouracil, doxorubicin, epirubicin, paclitaxel, and docetaxel [41]. Cyclophosphamide is an alkylating agent that adds an alkyl group to the guanine base of DNA, and therefore causes DNA cross-linking and interferes with DNA replication and cell division. Methotrexate is an antimetabolite and antifolate drug that blocks dihydrofolate reductase (DHFR), which is an enzyme participating in the conversion of folic acid to tetrahydrofolic acid, and therefore inhibits the synthesis of DNA, RNA, thymidylates, and proteins [38]. Another drug, 5-fluorouracil is a uracil analog with a hydrogen atom at the C-5 position replaced by a fluorine atom. It is converted to several active metabolites intracellularly, and these metabolites disrupt RNA synthesis and inhibit the action of thymidylate synthase, and therefore slow or stop cell growth [39]. Doxorubicin and epirubicin are both anthracyclines; epirubicin is an analogue of doxorubicin. Doxorubicin binds directly to the base pairs of the DNA or RNA and inhibits the replication of cells. Similar to Doxorubicin, epirubicin works by intercalating DNA strands, inhibiting DNA and RNA synthesis. It can also form a cleavable complex with topoisomerase II

and DNA, leading to inhibition of topoisomerase II and preventing the relaxing of supercoiled DNA, and finally blocking the DNA synthesis pathway. Paclitaxel and docetaxel are both taxanes. Paclitaxel is an antimicrotubule agent that stabilizes microtubule polymer, promoting the assembly of microtubules and protecting them from disassembly. It therefore inhibits mitotic spindle function and blocks mitosis and cell division. Docetaxel is a semisynthetic taxane and shares a similar mechanism to paclitaxel's but docetaxel is slightly more water-soluble than paclitaxel [39].

For adjuvant chemotherapy, combination therapy is more effective than single-drug therapy. The combination of cyclophosphamide, methotrexate, and fluorouracil (CMF) was the first polychemotherapy regimen, and it showed better disease-free and overall survival than single agent treatment in early breast cancer. The combination of Fluorouracil, anthracyclines, and cyclophosphamide (FA[E]C), a regimen in which the methotrexate of CMF is replaced with doxorubicin or epirubicin, has been tested in many trials and is used in routine practice [41, 42].

Hormonal Therapy

It was first recognized by Beatson in 1896 that some breast cancer patients responded favorably to oophorectomy, which is removal of ovaries. This was the first hint that some breast tumors are dependent upon hormone for growth and led to the discovery of hormonal therapy [43].

Hormonal therapy can be used to treat hormone receptor-positive tumors by lowering the amount of hormone or by blocking the stimulating action of hormone on breast cancer cells. Typical hormonal treatments include selective estrogen response modifiers (SERMs), aromatase inhibitors, gonadotropin-releasing hormone (GnRH) agonists. [44]

SERMs are compounds that can exert selective estrogen antagonistic or estrogen agonistic effects, depending on various estrogen target tissues. They can block the action of estrogen in certain tissues while mimicking the action of estrogen in other tissues. Tamoxifen was the first SERM to be investigated. It was first introduced to treat advanced breast cancer of postmenopausal women but was later approved to treat ER+ breast cancer of both premenopausal and postmenopausal women. It is still the most commonly used SERM [41]. Tamoxifen is a competitive antagonist to estrogen at the level of estrogen receptor in breast tissues while acting as an agonist in other tissues, for example bone and uterus. It

exhibits antagonistic effects by competitively binding to the ER sites on breast cell membranes and inhibiting the expression of estrogen-regulated genes that may promote cellular proliferation and tumor growth [44]. It may also decrease the production of insulin-derived growth factors in breast tissues, while increasing the production of tumor-suppressive growth factors, and as a consequence, inhibit tumor growth [46]. However, it was found that tamoxifen exerts estrogen agonistic effect on the endometrium, and it leads to increase in endometrial cancer in women taking tamoxifen for five years [47]. In addition to the therapeutic effect of tamoxifen, on existing tumors, studies have revealed that it can be used for the prevention of breast cancer as it reduces the risk of estrogen receptor-positive tumors and osteoporotic fractures in women at high risk of breast cancer [48].

Aromatase inhibitors work by blocking aromatase, an enzyme that converts the body's androgens into estrogens. Postmenopausal women get most of their estrogen from this conversion. Therefore, aromatase inhibitor treatment is considered in all postmenopausal women with hormone receptor positive breast cancer but not premenopausal women, because before reaching menopause, women produce most of their estrogen in the ovaries and aromatase inhibitors cannot stop the ovaries from making estrogen. Commonly used aromatase inhibitors are anastrozole, exemestane, and letrozole. Several studies have compared aromatase inhibitors

with tamoxifen in treating postmenopausal women with early-stage, hormone-receptor positive breast cancer [49]. It has been suggested that anastrozole is better in terms of time to progression (TTP) for patients with hormone receptor-positive tumors [50, 51]. A study comparing letrozole with tamoxifen has also shown that letrozole is significantly superior to tamoxifen in TTP, time to treatment failure (TTF), objective response rate (ORR) and clinical benefit rate [52]. Comparison between exemestane and tamoxifen has also shown a better overall response rate for exemestane than tamoxifen [53].

Targeted Therapy

Targeted therapies interfere with specific molecular targets that are involved in the growth, progression and spread of cancer. Commonly used targeted therapies include tyrosine kinase inhibitors, inhibitors of intracellular signaling pathways, angiogenesis inhibitors and agents targeting DNA repair [54].

HER-2 is a proto-oncogene that encodes a transmembrane tyrosine kinase receptor protein. *HER-2* is amplified in 20-30% of human breast cancers. Overexpression of *HER-2* is related to rapid proliferation and growth of cancer cells, and these tumors are more aggressive and resistant to chemotherapy, leading to poor prognosis, but this also makes it the most successful target [55]. A number of

drugs have been developed to target HER-2 protein, including trastuzumab, pertuzumab, trastuzumab emtansine, and lapatinib. Trastuzumab, a monoclonal antibody that blocks the effects of HER-2 protein, may be used to treat both early stage and late stage breast cancer; it has demonstrated benefit both as a single agent and when used in combination with chemotherapy in treating HER-2 positive breast cancer. Pertuzumab is also a monoclonal antibody and it can be given with trastuzumab and chemotherapy. Lapatinib, a dual tyrosine kinase inhibitor that inhibits both HER-2 and EGFR pathways, can be used to treat patients with advanced or metastatic HER-2 positive breast cancers. [53, 55]

Angiogenesis is required for invasive tumor growth and metastasis, making it a potential target to inhibit tumor growth. Vascular endothelial growth factors (VEGFs) are among the most prominent factors leading to pathological angiogenesis. Bevacizumab, the first agent targeting angiogenesis in breast cancer, is a monoclonal antibody directed against VEGF-A, one of the VEGFs [56].

Some targeted agents can be used along with hormone therapeutic drugs to improve treatment outcomes, for example palbociclib and everolimus. Palbociclib is a selective inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6. By blocking these proteins, palbociclib helps stop cell proliferation and slow cancer growth [57]. Everolimus is an inhibitor of mammalian target of rapamycin (mTOR), a protein that

promoters cell growth and division. It has been approved for postmenopausal patients with ER+ advanced or metastatic breast cancer that is resistant to aromatase inhibitors [58].

1.1.4 Triple-negative breast cancer

It has been estimated that about 15 to 20% of breast cancers are triple-negative. Unlike other subtypes of breast cancer, triple-negative breast cancer cannot be treated with hormonal therapies and therapies targeting HER2, and there is no single targeted therapy that is efficacious treating it [16, 58]. Triple-negative breast cancer tumors tend to be larger in size and higher in grade and are generally more aggressive, with a high metastatic rate. Despite higher rates of clinical response to neoadjuvant chemotherapy, no conventional therapy has been developed for triple-negative breast cancer. The lack of treatment options and the intrinsic aggressiveness of triple-negative breast cancer lead to poor prognosis and treatment outcomes [60]. Therefore, it is imperative to develop an effective therapeutic approach.

1.2 Radiotherapy

Types of radiation used for cancer treatment include photon radiation (X-rays and ϒ-rays) and particle radiation (protons, neutrons, boron, carbon and neon ions). According to the delivery method, radiotherapy can be classified as external radiotherapy, internal radiotherapy, and systemic radioisotope therapy.

External radiotherapy (or teletherapy), the most common form of radiotherapy, delivers a beam of ionizing radiation pointed at cancerous areas by a machine outside of the body. Recent advances in external radiotherapy include three-dimensional conformal radiotherapy (3D-CRT), intensity-modulated radiotherapy (IMRT), image-guided radiotherapy (IGRT), and stereotactic body radiotherapy (SBRT) [61]. 3D-CRT delivers a precisely shaped radiation beam conforming to the shape of the tumor, and therefore reduces toxicity to surrounding normal tissues [62]. IMRT is an advanced form of 3D-CRT that allows radiation doses to conform more precisely to tumor shapes while minimizing the dose to normal tissues by modulating the intensity of each segment of radiation beam during treatment sessions [63]. Studies have found that IMRT for treatment of breast cancer may be able to reduce doses delivered to the heart and lungs due to the improved target coverage [64]. IGRT incorporates contemporary imaging

techniques, such as CT and MRI, to frequently confirm the tumor and patient position during treatment, and therefore increase the accuracy of target localization and reduce damage to health tissue [61]. SBRT gives radiation from many different directions to target tumor areas and thus tumors receive a higher dose of radiation than normal tissue [65].

Internal radiotherapy (brachytherapy) delivers radiation from a radiation source placed in the body near cancerous tissue. For example, balloon catheters filled with radioisotopes can be used to limit local recurrence after the primary treatment of breast cancer [66].

In systemic radiotherapy, radioactive substances, for example iodine-131 and strontium-89, are given orally or by injection. The radioactive substance can be attached to a monoclonal antibody that targets cancer cells to improve accuracy [61].

1.2.1 Target of radiotherapy

The principle target of radiotherapy is the DNA of cancerous cells. DNA damage induced by radiation can be caused by direct interaction or indirect interaction. For the direct interaction, ionizing energy is directly deposited into DNA molecules, and

the DNA molecules are directly ionized or excited, leading to DNA damage. For the indirect interaction, ionizing radiation energy is deposited in water first, forming free radicals, and the radicals react with DNA molecules, causing damage. With high linear energy transfer (LET) radiation, for example particle radiation, the direct ionizing effect dominates, whereas under low LET radiation, for example X-ray and ϒ-ray, the indirect effect dominates.

1.2.2 Mechanism of radiotherapy

Experiments have shown that the yields of DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) are three orders of magnitude higher in the aqueous DNA samples than those in the dry condition at 25°C, indicating water plays a key role for biological effect caused by ionizing radiation [67]. Therefore, how radiolysis of water leads to DNA damage is the key to understanding the mechanism of radiation. It is well know that the major products of water radiolysis are $OH^{\bullet}$ (hydroxyl radical) and e^{-}_{hyd} (hydrated electron), but the e^{-}_{hyd} is inefficient at introducing biological damage since it is trapped in a deep potential well [68]. Some other experiments with bacterial and mammalian cell systems have also shown that one third of biological effect is induced by direct interaction between radiation and

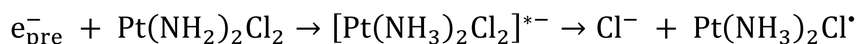
DNA, whereas two thirds of biological effect is induced by $OH^{\bullet}$. However, this conclusion is contradictory to the fact that aqueous condition enhances three orders of magnitude higher DNA damage than dry condition, and also, it has been suggested that DNA damage caused by $OH^{\bullet}$ alone can be repaired efficiently and hence the damage is inconsequential and ineffective in cell killing [69, 70].

By direct observation of the transition states of the dissociative election transfer (DET) reactions of e^{-}_{pre} (prehydrated election), the precursor to e^{-}_{hyd}, using the femtosecond time-resolved laser spectroscopy (fs-TRLS), our group has shown that the weakly-bound e^{-}_{pre} plays a key role in causing damage to aqueous DNA under ionizing radiation, and our findings unravel the longstanding mystery about how water enhances DNA damage under ionizing radiation. Indeed, it is e^{-}_{pre} not $OH^{\bullet}$ that cause biological damage to DNA under ionizing radiation [71-74]. Our results showed that cytosine and especially adenine can effectively trap an e^{-}_{pre} to form stable anions, whereas thymidine and especially guanine are vulnerable to DETs of e^{-}_{pre}, which leads to DNA bond breaks [72] Our group has also found that the reductive DNA damage induced by a e^{-}_{pre}, is twice the yield of oxidative DNA damage induced by a $OH^{\bullet}$ [74]. These findings have significance for understanding of the mechanism of radiolysis of water and the role of water in biological effect induced by ionizing radiation.

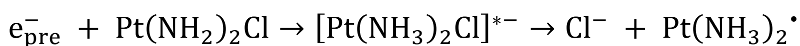
1.2.3 Radiosensitizer

The most common ionizing radiation sources used in radiotherapy are low LET sources such as X-rays and γ-rays, and there are limitations in doses and effectiveness that cause failure in treatment. A radiosensitizer is a drug that can sensitize tumor cells to radiation and therefore enhance tumor cell killing while having less effect on normal tissues. Tumor cells have different physiological characteristics as normal tissues; radiosensitizers can target these properties and therefore enhance radiosensitivity of tumor cells. [76]

Cisplatin is currently one of the most widely used chemotherapeutic drugs and has shown clinical activity against a wide variety of solid tumors [78, 79]. Also, Combination of cisplatin with radiotherapy has been shown to enhance DNA damage and is a novel cancer treatment; the radiosensitizing effect of cisplatin has also been demonstrated in many studies [80-82]. By using fs-TRLS, we found that cisplatin is very reactive for the DET reaction with e^-_{pre} produced by radiolysis of water, and this process unravels the mechanism of its radiosensitizing effect [83]. The reaction mechanism can be described as:



$$e^-_{pre} + Pt(NH_2)_2Cl_2 \rightarrow [Pt(NH_3)_2Cl_2]^{*-} \rightarrow Cl^- + Pt(NH_3)_2Cl^\bullet$$



$$e^{-}_{pre} + Pt(NH_2)_2Cl \rightarrow [Pt(NH_3)_2Cl]^{*-} \rightarrow Cl^{-} + Pt(NH_3)_2^{\bullet}$$

The $Pt(NH_2)_2Cl^{\bullet}$ and $Pt(NH_2)_2^{\bullet}$ radicals formed in this process can induce DNA strand breaks effectively.

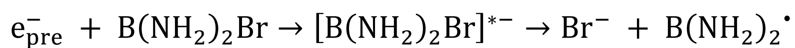
The radiosensitizing effects of halogenated pyrimidines have also been studied for many years. In the 1950's, Zamenhof, DeGiovani and Greer first demonstrated that BU replacement on the DNA of bacterial cells makes them more sensitive to ultraviolet radiation than bacterial cells without BU substitution [85, 86]. In the early 1960s, Djordjevic and Szybalski observed an increase in the sensitivity of mammalian cells to both X-ray and ultraviolet irradiation when the cells were pretreated with Bromodeoxyuridine (5- bromo-2'-deoxyuridine, BrdU, BUdR) [87]. The potential of using halogenated pyrimidines as clinical radiosensitizers was first proposed by Kaplan [88]. Thymidine in the DNA can be substituted by halogenated pyrimidines such as bromo- or ioso-deoxyuridine (BrdU or IdU), thus enhance DNA damage and cell death induced by ionizing radiation or UV phorolysis. The ultrafast DET reaction between e^{-}_{pre} and halogenated pyrimidines was first observed by our group real-timely using fs-TRLS, revealing the meachanism: $e^{-}_{pre} + BrdU \rightarrow BrdU^{*-} \rightarrow Br^{-} + dU^{\bullet}$, which is similar to the electron transfer mechanism of cisplatin[89-91]. However, the ultrafast DET reaction between halogenated pyrimidines and e^{-}_{pre} are far less efficient than that between cisplatin and e^{-}_{pre},

because $-NH_2$ groups in cisplatin act as effective promoters for electron transfer reactions [92].

1.2.4 FMD-Br-DAB as a Potential Radiosensitizer

The application of fs-TRLS to chemical and biological systems spawned the fields of femtochemistry and femtobiology, and it also led to the development of *femtomedicine* (FMD), which combines ultrafast laser spectroscopic techniques and biomedical sciences [93]. The better understanding of reductive DNA-damage mechanism and the molecular mechanism of cisplatin and halogenated pyrimidines as radiosensitizers, contributes to our discovery of a non-platinum-based radiosensitizing agent that can mimic the radiosensitizing effect of cisplatin but has less cytotoxicity than cisplatin. The new radiosensitizer contains an aromatic ring instead of platinum coordinating ion, and the aromatic ring couples two NH_2 groups as the electron transfer promoter and one bromine atom. The molecular formula of the agent is 4-bromo-1,2-diaminobenzene, which is denoted as FMD-Br-DAB or $B(NH_2)_2Br$ hereinafter. The $B(NH_2)_2Br$ is very effective in DET reactions with weakly-bound electrons (e^-_{wb}), which are rich in cancer cells [94]. Also, the e^-_{pre} produced by radiolysis of water as a result of ionizing radiation is also a

weekly-bound electron that can react with $B(NH_2)_2Br$ effectively. The mechanism can be described as:



$$e^-_{pre} + B(NH_2)_2Br \rightarrow [B(NH_2)_2Br]^{*-} \rightarrow Br^- + B(NH_2)_2^{\bullet}$$

In this reaction, $[B(NH_2)_2Br]^{*-}$ is a transient vibrationally-excited anion state, and the $B(NH_2)_2^{\bullet}$ is highly reactive that can lead to DNA strand breaks. The $B(NH_2)_2Br$ is more efficient in DET reaction than cisplatin and halogenated pyrimidines but has much less cytotoxicity than cisplatin due to the absence of platinum.

Chapter 2. Cell Survival Study by MTT assay

2.1 Introduction

In order to test drug sensitivity and cytotoxicity, it is necessary to perform cell survival assays. MTT assay is one of the most common cell survival and proliferation assays. MTT (3-(4,5-dymethylthiazolyl-2)-2,5-diphenyltetrazolium bromide), a water-soluble yellow tetrazolium salt, is reduced to a water-insoluble purple formazan in metabolically active cells by the action of mitochondrial dehydrogenase. The amount of formazan production in a given time is directly proportional to number of living cells because only living cells can actively cleave MTT. Using a solubilizing agent, for example dimethyl sulfoxide (DMSO) or sodium dodecyl sulfate (SDS), the purple formazan can be solubilized and quantified by measuring the absorbance with the help of a spectrophotometer [95-97].

2.2 Experimental details

Cell lines and culture conditions

The cell line used in this research is MDA-MB-231, a metastatic human breast cancer cell line originally isolated from a patient in 1973 [98]. It exhibits invasive properties when cultured in vitro and is widely used as a model for triple-negative breast cancer.

The MDA-MB-231 cells were cultured with L-15 Medium (Leibovitz) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100ug/mL streptomycin, and they were incubated at 37 °C in a humidified air atmosphere without CO_2. The MDA-MB-231 cells and L-15 Medium were purchased from American Type Culture Collection. The FBS was purchased from Hyclone Laboratories.

The cells were sub-cultured every 4 days or when they were 80-90% confluent. To subculture the cells, they were washed with phosphate buffered saline (PBS) twice after removing the original culture medium, then an appropriate volume of 0.25% trypsin-0.53mM EDTA solution was added to the flask. After the cells had de-attached and rounded up, a proper amount of complete growth medium was added to neutralize the trypsin. Then the cells were harvested by gently washing

with the medium added. The cell-medium solution was removed and centrifuged at 700 rpm. After centrifugation, the old medium was gently aspirated, and the completed medium was re-added; the pellet of cells was re-suspended by gentle pipetting. A proper amount of cell-medium solution was transferred to a new T-75 flask to reach a sub-cultivation ratio of 1:2 to 1:4, and complete growth medium was added to a total of 14mL. Then cell cultures were incubated at 37 ^{o}C without CO_2.

MTT assay

A cell suspension was prepared and seeded in 96-well plates at 5000 cells/well and incubated at 37°C without CO_2 for 24 hours. Various concentrations of FMD-Br-DAB were added to each well. After 24 hours of drug treatment, the cells were irradiated with different doses of X-ray using X-ray irradiator IR225. The duration of irradiation was adjusted to reach the desired dose. The cells were then incubated for 6 days. After incubation, the cells were replaced with 100ul serum-free medium containing 1mg/ml MTT and were incubated for a further 4 hours in the dark at 37°C without CO_2. Next, 100ul of 10% SDS/0.01M HCl solution was added to each well as a solubilizing agent. After 2-4 hours of incubation, the fraction of live cells was determined by measuring the absorbance at 570nm by a spectrophotometer. The cell survival rate of untreated control cells is taken as 100%,

and since the number of live cells has a linear relationship with the absorbance value, the cell survival rate of the experimental group is calculated as the absorbance value of the experimental group divided by the absorbance of the untreated control group.

2.3 Results

MDA-MB-231 triple-negative breast cancer cells were treated with 0 µM, 10 µM, 20 µM and 60 µM FMD-Br-DAB for 24 hours, followed by X-ray irradiation at 0 Gy, 4 Gy, 8 Gy and 16 Gy. The cell survival fraction 6 days post-irradiation was measured by MTT assay, and results are shown in Figure 2.1. Normalized results are shown in Figure 2.2.

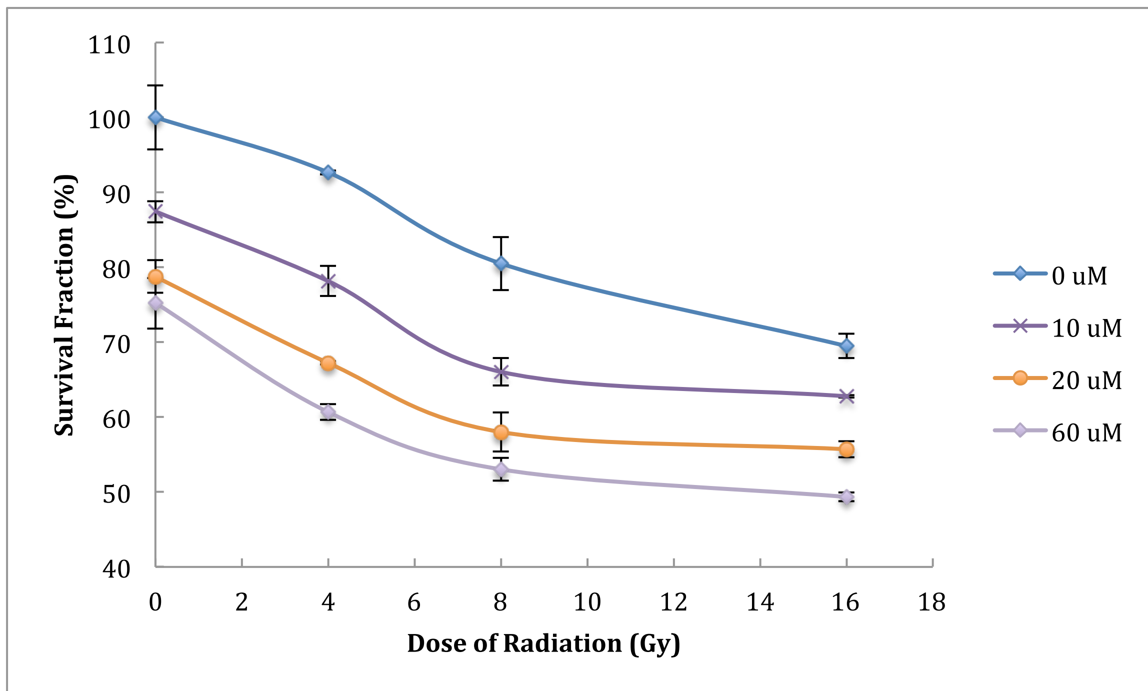


Figure 2.1: Cell survival fraction for MDA-MB-231 cells treated with 0-60 µM of FMD-Br-DAB and 0-16 Gy of X-ray irradiation.

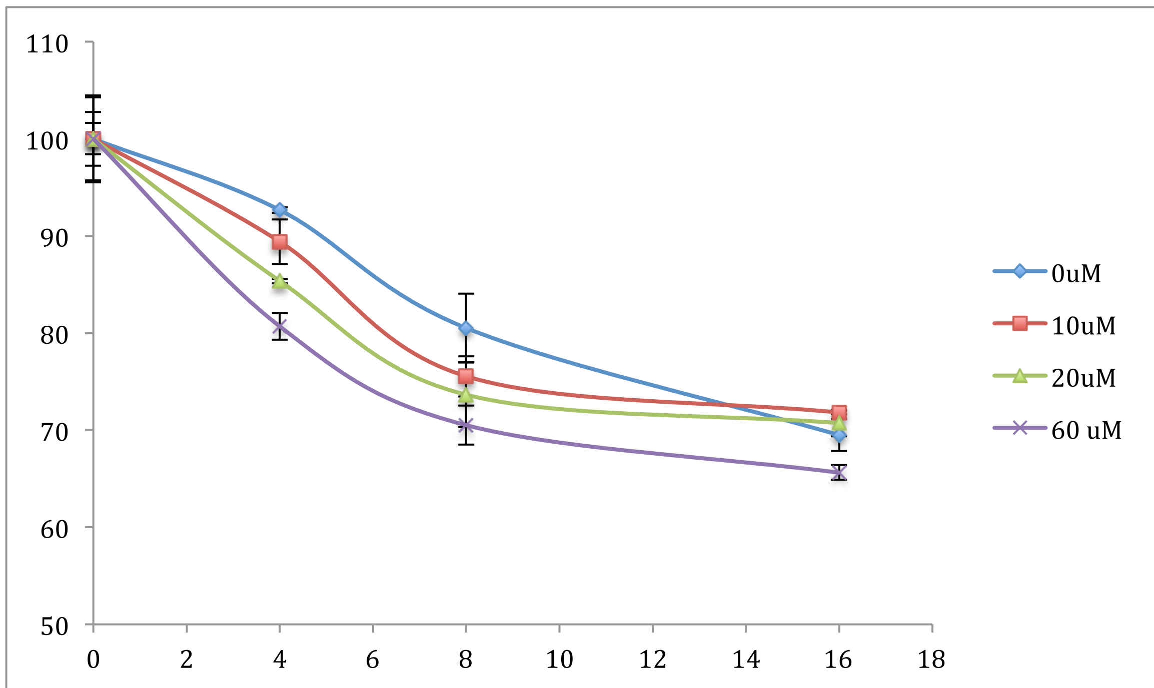


Figure 2.2: Cell survival fraction for MDA-MB-231 cells treated with 0-60 μM of FMD-Br-DAB and 0-16 Gy of X-ray irradiation. Cell survivals at different dose of X-ray without FMD-Br-DAB treatment have all been normalized to 100%.

2.4 Discussions and Conclusions

The data plotted in Figure 2.1 show that when cells were treated with FMD-Br-DAB alone (no X-ray irradiation), the cell survival fraction is around 80%. Even at the highest concentration of FMD-Br-DAB, which is 60 μM, the cell survival fraction is 75%, which indicates that FMD-Br-DAB only has little cytotoxicity when used alone. When cells were treated with 4 Gy of X-ray irradiation and various concentrations of FMD-Br-DAB, the cell survival fraction reduces from 93% to 61% as the concentration of FMD-Br-DAB increases from 0 μM to 60 μM with a concentration dependent manner. When cells were treated with 8 Gy of X-ray irradiation and various concentrations of FMD-Br-DAB, the cell survival fraction reduces from 80% to 53% in a concentration dependent manner. Similarly, when cells were treated with 16 Gy of X-ray irradiation and various concentrations of FMD-Br-DAB, the cell survival fraction reduces from 70% to 45% as the concentration of FMD-Br-DAB increases from 0 μM to 60 μM.

The cell survival fractions treated with different concentration of FMD-Br-DAB alone are normalized to 100% and plotted in Figure 2.2. This figure provides evidence of the synergistic effect between FMD-Br-DAB and radiation, which is induced by the radiosensitizing effect of FMD-Br-DAB.

These MTT results reveal that FMD-Br-DAB treatment alone has limited cytotoxicity effects on MDA-MB-231 cells up to the highest concentration of 60 μM, but cytotoxic effect increases, when FMD-Br-DAB is used in combination with radiation. The results also suggest the radiosensitizing effect of FMD-Br-DAB, which enhances cell killing synergistically with radiation.

The promising MTT results opens up the possibility of using FMD-Br-DAB as a radiosensitizer to treat breast cancer and especially triple-negative breast cancer, which cannot be treated with hormonal therapy and is generally more aggressive.

Chapter 3. Cell Survival Study by Clonogenic Assay

3.1 Introduction

Clonogenic assay measures cell survival and proliferation based on the ability of a single cell to reproduce into a colony consisting of at least 50 cells. These colonies can be visualized with naked eye and are countable. The ratio of colonies to the number of seeded cells indicates the capacity of cells to reproduce. A cell survival curve describes the relationship between the dose of the agent and the fraction of the cells that retain the capability to produce colonies. Unlike MTT assay, which distinguishes live and dead cells by metabolic function, clonogenic assay only takes into account cells that can divide "unlimitedly". A cell that retains the ability to go through only one or two mitoses but cannot form a colony is considered dead in clonogenic assays [99, 100]. Although they are more time consuming than MTT assays and usually takes about 2-3 weeks incubation time to form colonies, clonogenic assays are considered to be the optimal method to determine cell radiosensitivity and reproductive death after treatment with ionizing radiation [101].

3.2 Experimental details

Cells were obtained from sub-cultured MDA-MB-231 cell line by trypsinization, and the process is similar to the sub-culture process described in the last chapter. When the cells started to detach, complete growth medium was added to neutralize trypsin. The cells were harvested by gently pipetting the cell-medium solution. Then the solution was centrifuged at 700 rpm, and the supernatant, which contained medium and trypsin, was removed. A proper amount of complete growth medium was re-added to the sediment, which contained the cells, and a single cell suspension was obtained by pipetting the solution. Then, cell concentration was counted using a Moxi Z automated cell counter. Desired seeding concentration can be obtained by diluting the cell suspension with a proper amount of complete growth medium, and the dilution can be used to seed cells in culture dishes. After seeding, the cells were incubated at 37° C without CO_2 until they had attached to the dishes. Various concentrations of compound D were added to each dish, and the cells were incubated for a further 24 hours at the same condition. After the drug treatment, the cells were irradiated with desired doses of X-ray using X-ray irradiator IR225. The dishes were then incubated until the cells in control group have formed sufficient large clones. It usually took about more than 14 days.

The colonies were dyed with glutaraldehyde crystal violet mixture, which contains 6% glutaraldehyde and 0.5% crystal violet. The medium was removed by gentle aspiration, and the dishes were rinsed twice with PBS. After removing the PBS, an amount of 3ml glutaraldehyde crystal violet was added to each dish. After 30 min, the glutaraldehyde crystal violet was removed, and the dishes were rinsed gently with tap water. When the dishes became dry, the number of colonies was counted by naked eye or microscope. The survival fraction of the untreated control group was set to 100%, and the survival fraction of other groups was calculated by the number of colonies of the experimental group divided by the number of colonies of the untreated control group.

3.3 Results

MDA-MB-231 triple-negative breast cancer cells were treated with 0 μM, 10 μM and 20 μM FMD-Br-DAB for 24 hours, followed by X-ray irradiation at 0 Gy, 2 Gy, and 4 Gy. The cell survival rates 20 days post-irradiation were measured by clonogenic assay, and results are shown in Figure 3.1.

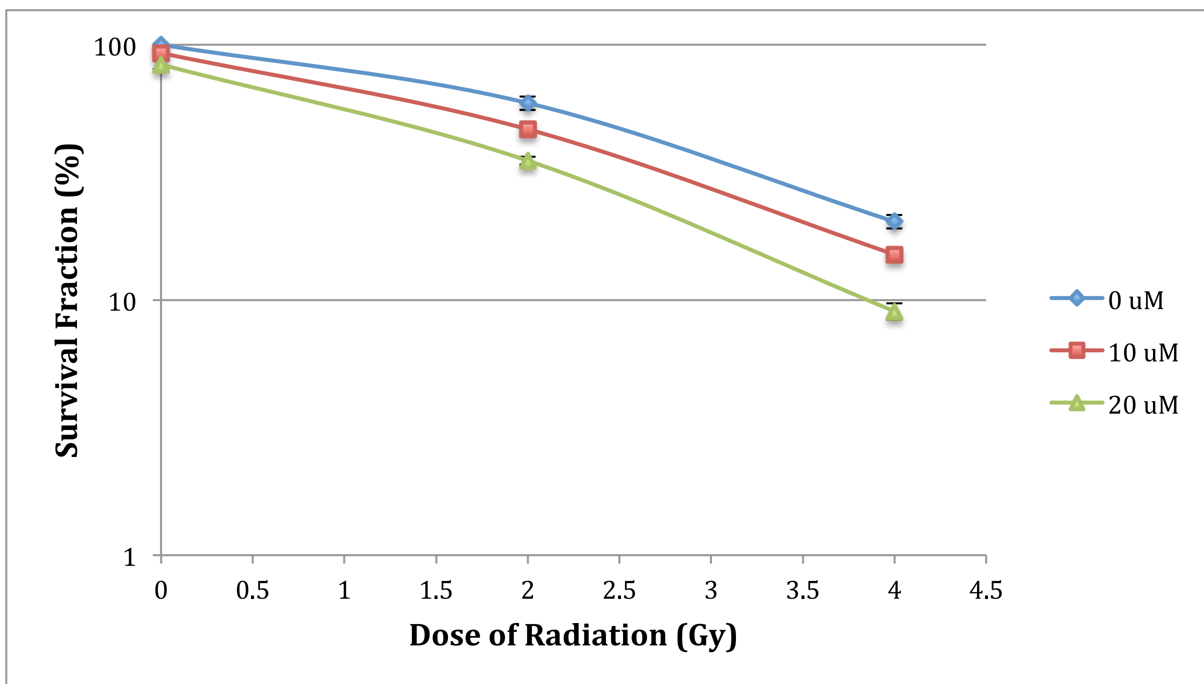


Figure 3.1: Cell survival rates for MDA-MB-231 cells treated with 0-20 μM FMD-Br-DAB and 0-4 Gy X-ray. The sensitizer enhancement ratio (SER) at 20% survival fraction in MDA-MB-231 cells treated with 20 μM of FMD-Br-DAB is 1.36.

3.4 Discussions and Conclusions

As shown in Figure 3.1, the survival fractions at 0 Gy, 2 Gy, and 4 Gy without FMD-Br-DAB treatment are 100% $\pm$ 5.3%, 59% $\pm$ 3.5% and 20% $\pm$ 1.2%, respectively. For cells pretreated with 10 μM FMD-Br-DAB, the survival fractions at 0 Gy, 2 Gy, and 4 Gy are 93% $\pm$ 6.3%, 47% $\pm$ 1.0% and 15% $\pm$ 1.2%, respectively. For cells pretreated with 20 μM FMD-Br-DAB, the survival fraction at 0 Gy, 2 Gy, and 4 Gy are 84% $\pm$ 3.2%, 35% $\pm$ 1.3% and 9% $\pm$ 0.7%, respectively.

As seen in Figure 3.1, the X-ray dose required to produce a 50% cell killing effect (IC_{50}) is between 2 Gy and 4 Gy, whereas when combined with 24 h pre-incubation of 20 μM FMD-Br-DAB, the IC_{50} of X-ray is less than 2 Gy. Even at lower concentration (10 μM) of FMD-Br-DAB, the IC_{50} of X-ray is less than 2 Gy. These results show that the IC_{50} of X-ray is significantly reduced when cells are pretreated with FMD-Br-DAB, and the higher the concentration of FMD-Br-DAB, the lower the IC_{50} at specific dose of X-ray.

In the absence of X-ray, the survival fraction is 93% $\pm$ 6.3% at 10 μM concentration of FMD-Br-DAB, and the survival fraction is 84% $\pm$ 3.2% at the highest FMD-Br-DAB concentration of 20 μM. Our data indicates that FMD-Br-DAB alone has only minimal effects on cytotoxicity and clonogenic survival (long-term

cell survival). However, radiation-induced cytotoxicity is significantly enhanced as the dose of FMD-Br-DAB increases. This enhancement provides evidence of the radiosensitizing effect of FMD-Br-DAB, and is also consistent with the results of the MTT assay. The clonogenic survival at 20 μM FMD-Br-DAB treatment combined with 4 Gy X-ray irradiation is even less than 10%, suggesting that FMD-Br-DAB has the potential to be an effective radiosensitizer.

Also, the sensitizer enhancement ratio (SER) at 20% cell survival is 1.36, calculated by dividing the magnitude of X-ray dose leading to a 20% cell survival at 20μM of FMD-Br-DAB with the magnitude of X-ray dose leading to a 20% cell survival without FMD-Br-DAB treatment.

Chapter 4. Gel Electrophoresis

4.1 Introduction

Agarose gel electrophoresis is the most effective way of separating DNA fragments based on their size. DNA samples are loaded into pre-cast wells in the gel, and the gel is placed in an electrophoresis chamber, which is connected to an electric power source. The electric field consists of a negative charge at one end and a positive charge at the other. Since DNA is negatively charged due to the phosphate groups in the backbone of DNA, when the electric current is applied, DNA fragments will migrate from the negatively charged electrode to the positively charged one. The gel prepared by agarose can form a solid but porous matrix, and the pore size of 1% agarose gel ranges from 200-500nm [102]. As DNA fragments move through the pores of the gel, they meet with resistance. Therefore, the movement of the DNA is affected by the conformation of the DNA; the larger pieces move more slowly through the gel, while the smaller pieces move faster. Several samples can be loaded into individual wells in the same gel, and DNA molecules of a similar size will pass through the gel at the similar speed and end up in the same band. The moving rate is

related to the conformation and size of the DNA molecule. The larger the DNA molecule, the faster and the further it will migrate.

The DNA sample used in this experiment was plasmid, purified from *E. coli* cultures using the GeneJET™ Plasmid Miniprep Kit purchased from Thermo Scientific. A plasmid DNA purified from a bacterial cell will exist a naturally occurring supercoiled shape, which has extra twists between double helix strands, thus forming a compact conformation, and this compact conformation makes the plasmid run faster than damaged DNA [103]. If supercoiled circular DNA is SSB (single strand break) damaged, its conformation changes to circular relaxed conformation nicked circular. The mass does not change, but the volume becomes larger, thus the moving rate and migration length of SSB DNA is smaller than undamaged supercoiled form. If supercoiled circular DNA is DSB (double strand break) damaged, its conformation changes to linear. Typically, the rate of linear DNA migration is slower than that of its supercoiled form, but faster than its circular form. Therefore, the moving rate and migration length of different conformations of the same DNA are in the order of: supersoiled (undamaged) > linear (DSB) > circular (SSB).

4.2 Experimental Details

Preparing Plasmid DNA

DNA samples used in this experiment were prepared by a GeneJET™ Plasmid Miniprep Kit purchased from Thermo Scientific. The kit contains Resuspension Solution, Lysis Solution, Neutralization Solution, Wash Solution, RNase A, Elution Buffer, GeneJET Spin Columns, and Collection Tubes.

A single colony of *E. coli* cultures was picked and incubated for 14 hours at 37° C while being shaken at 200-250 rpm. After incubation, the *E. coli* cells were harvested by centrifugation at 8000 rpm for 2 minutes. All remaining medium was removed, and the pelleted cells were resuspended in 250 μL of Resuspension Solution. The cell suspension was transferred to a microcentrifuge tube, and 250 μL of the Lysis Solution was added and mixed well. Then, 350 μL of the Neutralization Solution was added and mixed immediately and thoroughly. The solution was centrifuged for 5 minutes to pellet cell debris and chromosomal DNA, and the supernatant was transferred to the GeneJET spin column by pipetting. The tube together with the spin column was centrifuged for a further 1 minute, and the flow-through was discarded. Next, 500 μL of the Wash Solution, prepared by adding 35 mL of Ethanol to 20 mL of concentrated Wash Solution, was added to the GeneJET spin column.

Then, the wash procedure was repeated and the column was transferred to a collection tube, which was then centrifuged for 1 minute to segregate and remove the remaining Wash Solution. The GeneJET spin column was then transferred to a 1.5 mL microcentrifuge tube, and 50 μL of the Elution Buffer was added to the center of the column membrane to wash the plasmid. Finally, the column was discarded and the purified plasmid DNA was stored at -20° C.

Preparing Agarose Gel

The buffer solution used in this experiment was 1×TAE diluted by adding 40 mL of 10×TAE to 360mL of pure water. Then, 0.4 g of agarose powder was added to 40 mL 1×TAE, and the mixture was heated in a microwave until clear and uniform. When the flask was cooled to 60° C, 4 μL of Ethidium Bromide (EtBr) was added to the agarose-TAE solution, which was then poured into an apparatus for solidificantion. Next, a comb was inserted to generate 12 wells. After 50 minutes, the solution was solidified to gel and the comb removed. The remaining 1×TAE was added to the apparatus to cover the gel.

Loading DNA Treatment Samples

The concentration of purified plasmid DNA was 100 ug/mL, and this solution was used as DNA stock solution to prepare DNA tratmenet samples. Each DNA sample was prepared by mixing 6 μL various concentrations of FMD-Br-DAB to 2 μL of DNA stock solution, which contained 0.2 μg DNA, to a total volume of 8 μL. The DNA samples were then irradiated with different doses of X-ray. Then, 8 μL of each sample was added to 1.6 μL of 6×loading dye and mixed well to obtain a total volume of 9.6 μL of DNA-dye solution. Then, 8 μL of each solution from last step was added to each well on the gel carefully. Power was turned on and running time was set as 2 hours.

At the end of gel electrophoresis, power was turned off and gel was taken out carefully. The gel was placed on a UV light box and photos of the fluorescent EtBr-stained DNA bands were taken.

4.3 Results

Electrophoresis of DNA samples treated with 0 μM, 50 μM, 100 μM, and 200 μM of FMD-Br-DAB followed by X-ray irradiation at 0 Gy, 50 Gy and 100 Gy is shown in Figure 4.1. The quantified amounts of SSBs and DSBs are given in Figure 4.2 and 4.3, respectively.

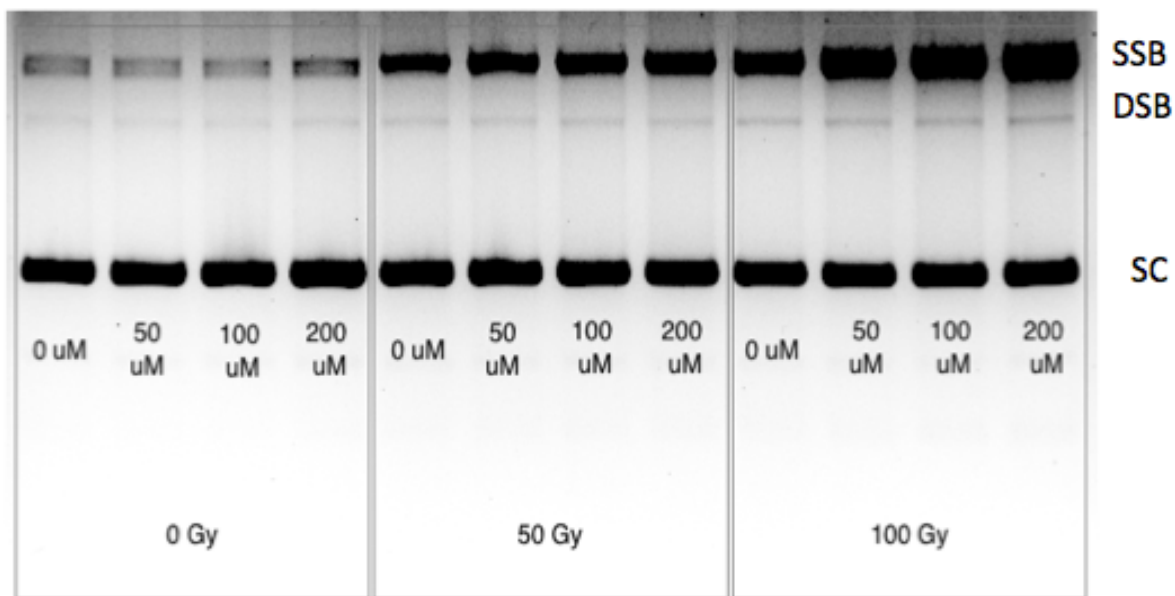


Figure 4.1: Gel electrophoresis of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation.

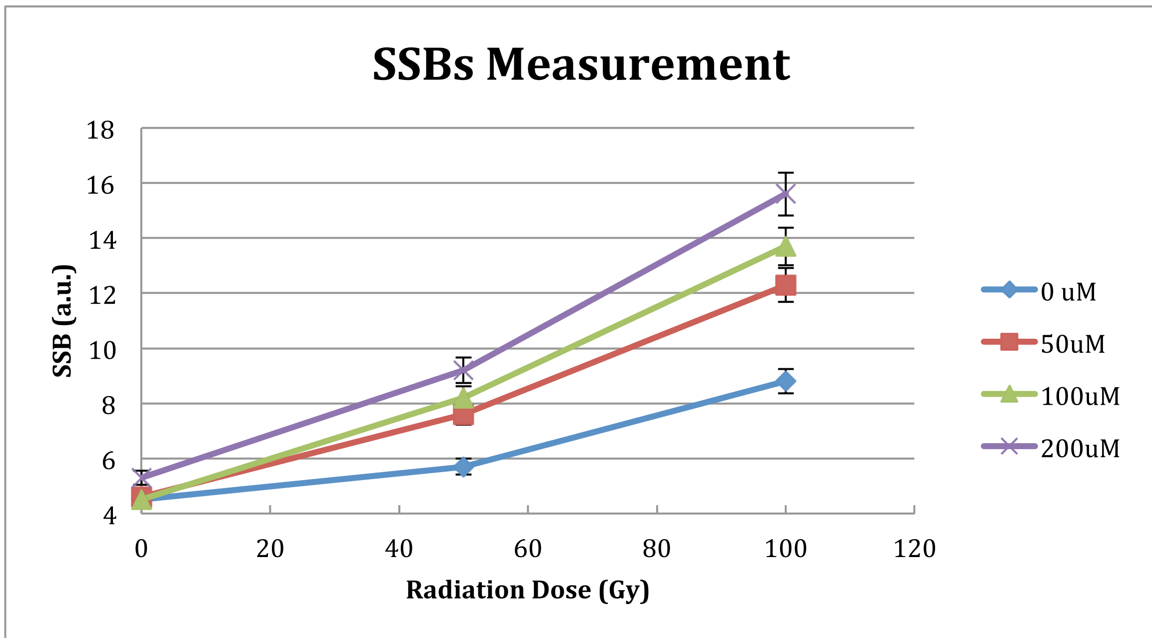


Figure 4.2: SSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation.

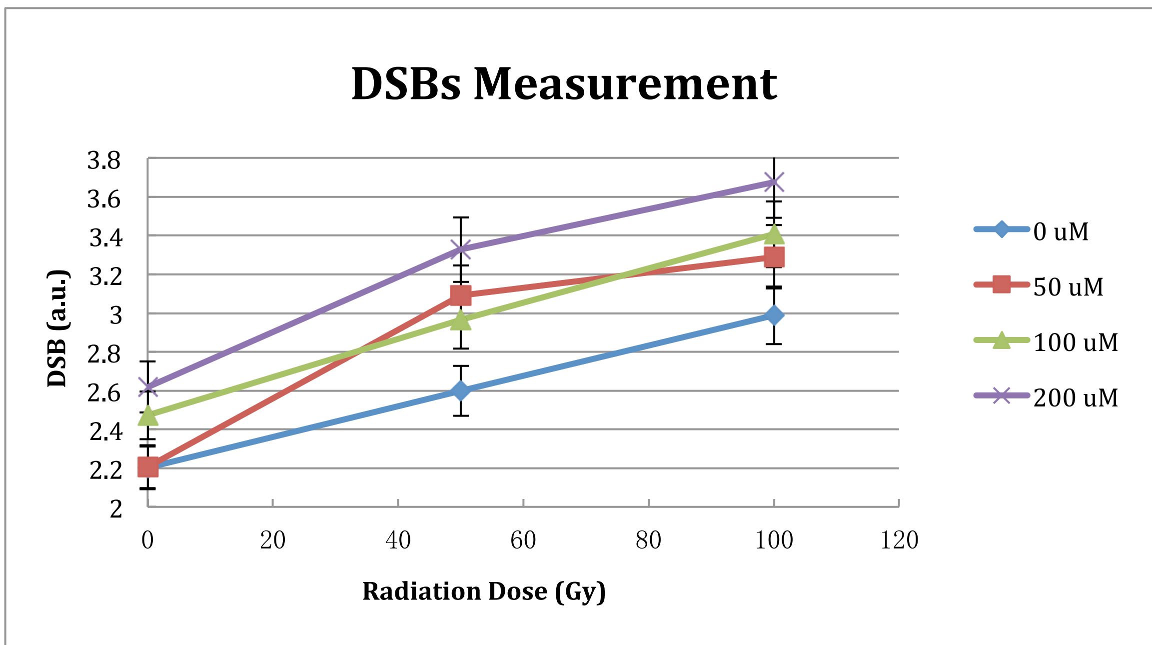


Figure 4.3: DSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation.

The electrophoresis of DNA samples treated with 0 μM, 50 μM, 100 μM, and 200 μM of FMD-Br-DAB followed by X-ray irradiation at 0 Gy, 100 Gy and 200 Gy is shown in Figure 4.4. Quantified amounts of SSBs and DSBs are given in Figure 4.5 and 4.6, respectively.

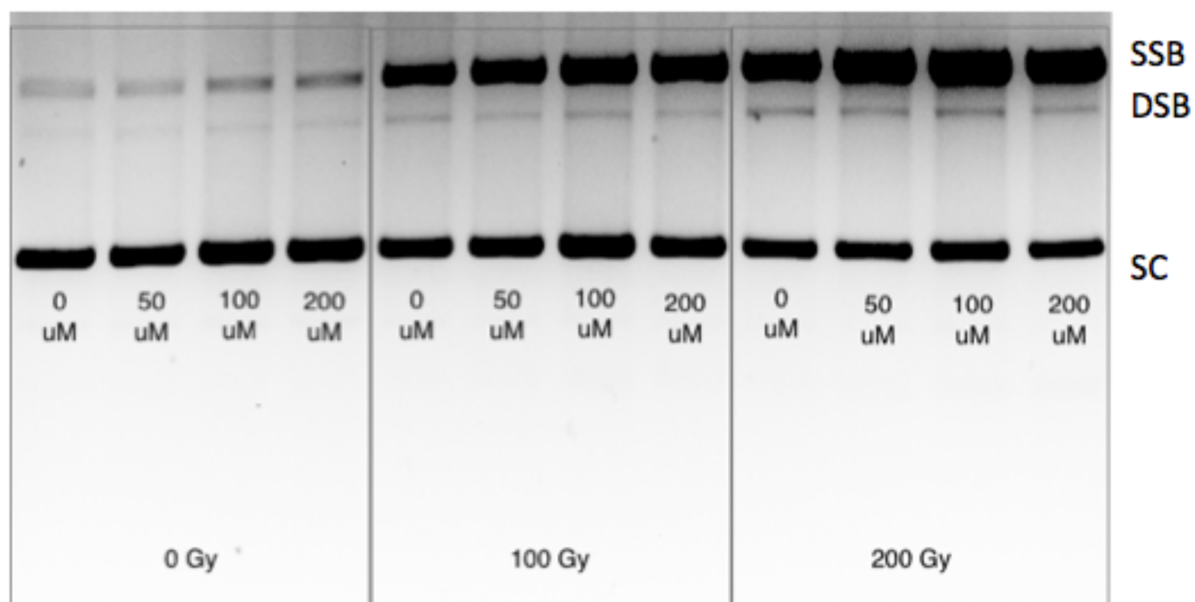


Figure 4.4: Gel electrophoresis of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-200 Gy of X-ray irradiation.

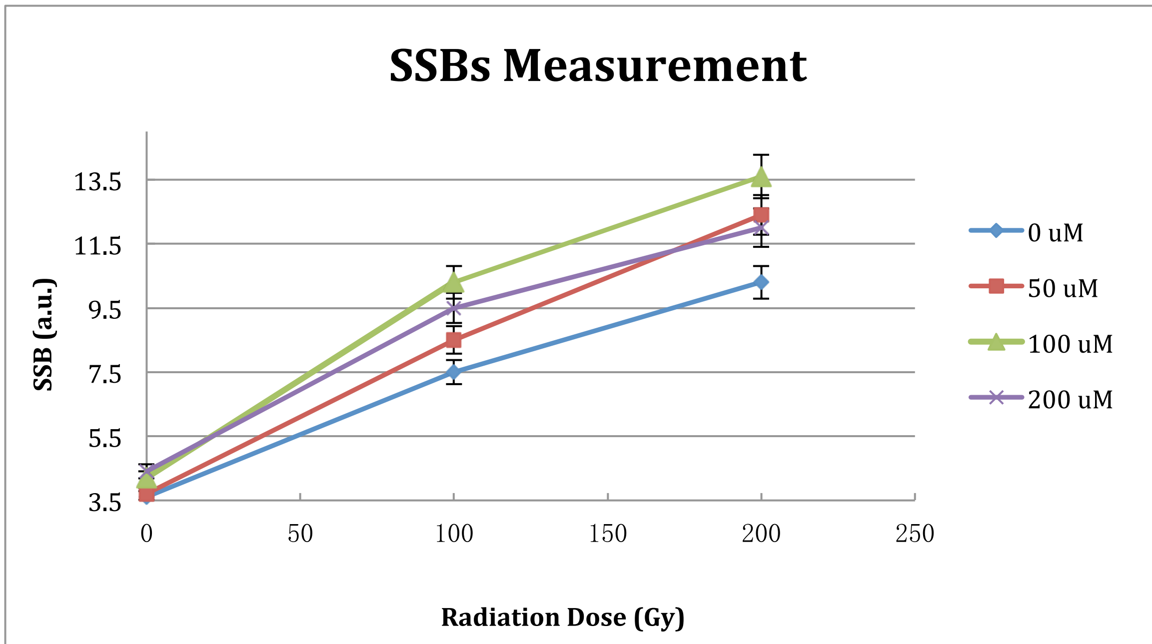


Figure 4.5: SSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-200 Gy of X-ray irradiation.

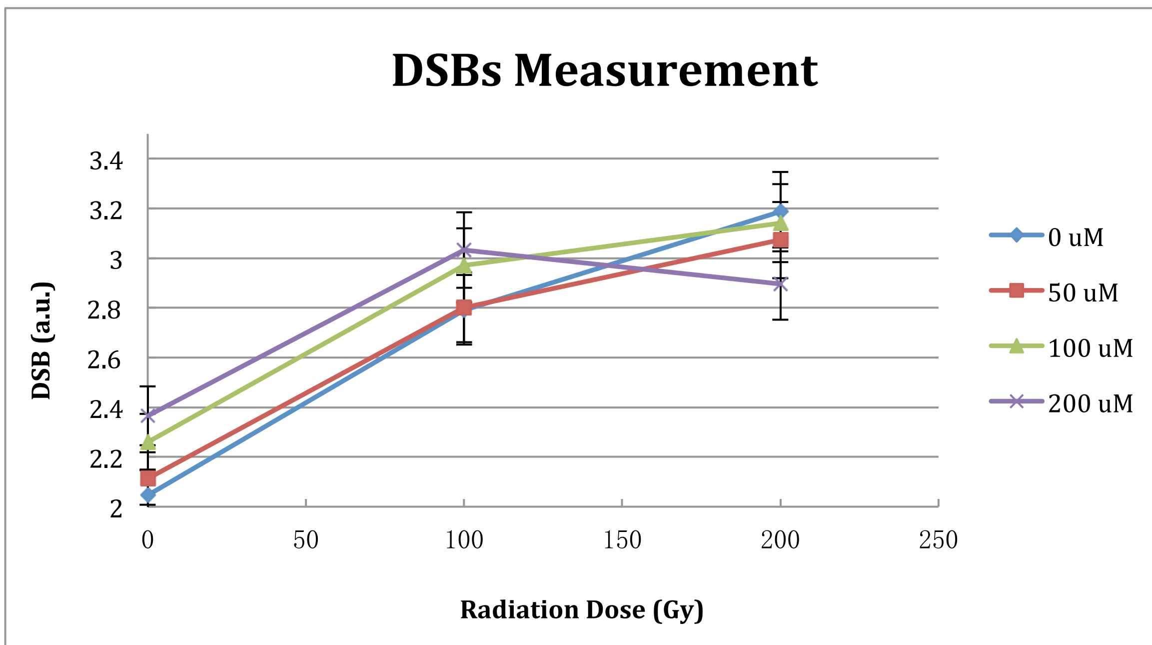


Figure 4.6: SSBs amount (with artificial unit) of DNA samples treated with 0-200 μM FMD-Br-DAB combined with 0-200 Gy of X-ray irradiation.

The electrophoresis of DNA samples treated with 0 μM, 250 μM, 500 μM, and 750 μM of FMD-Br-DAB followed by X-ray irradiation at 0 Gy, 50 Gy and 100 Gy is shown as Figure 4.7. Quantified amount of SSBs and DSBs are given in Figure 4.8 and 4.9, respectively.

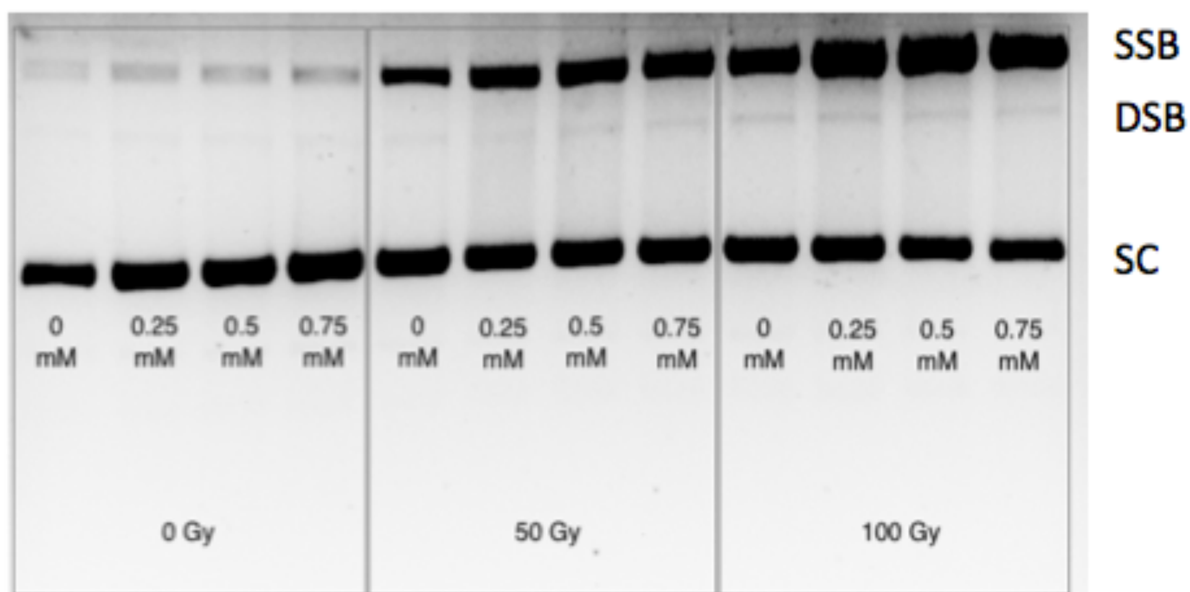


Figure 4.7: Gel electrophoresis of DNA samples treated with 0-750 μM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation.

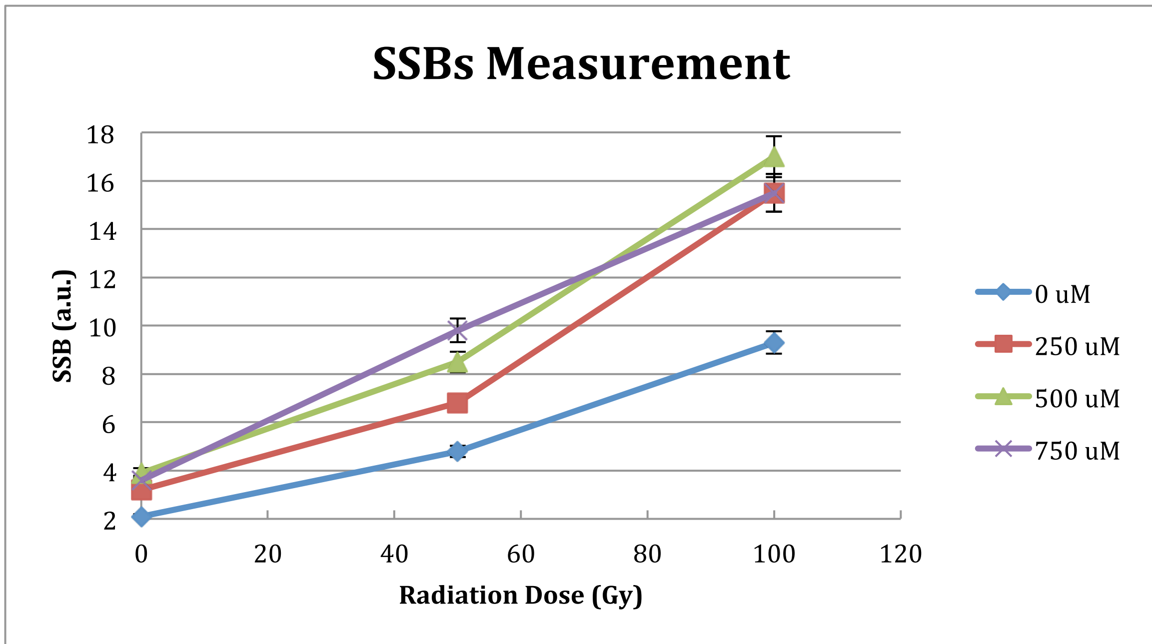


Figure 4.8: SSBs amount (with artificial unit) of DNA samples treated with 0-750 µM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation.

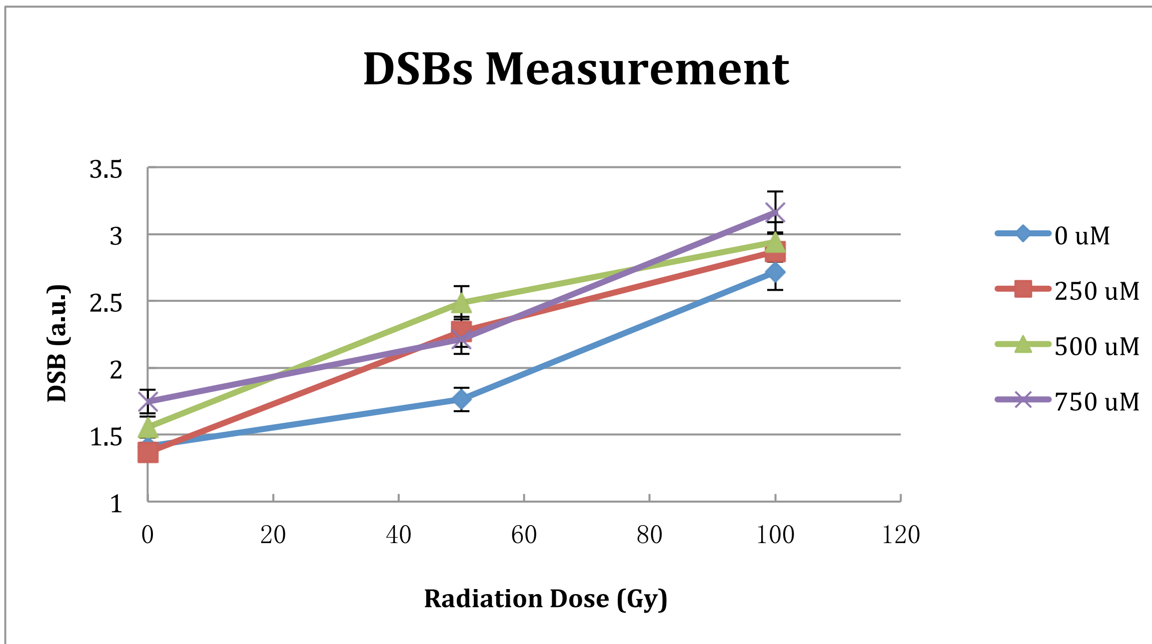


Figure 4.9: SSBs amount (with artificial unit) of DNA samples treated with 0-750 µM FMD-Br-DAB combined with 0-100 Gy of X-ray irradiation.

4.4 Discussions and Conclusions

In Figure 4.1, it can be noticed that when the concentration of FMD-Br-DAB increases, the amount of SSBs increases significantly, especially when X-ray irradiation is given together with FMD-Br-DAB. This finding can also be confirmed from Figure 4.2. As Figure 4.3 indicates, DSBs tend to increase when the concentration of FMD-Br-DAB increases.

When X-ray doses increase to 100 Gy and 200 Gy, SSBs still increases as the concentration of FMD-Br-DAB increases, as is shown in both Figure 4.4 and 4.5. DSBs also show an upward tendency when the concentration of FMD-Br-DAB increases, even though one inflection point is shown at 200 μM 100 Gy in Figure 4.6.

When the concentration of FMD-Br-DAB increases to 250 μM, 500 μM and 750 μM, as shown in Figure 4.7, the amount of SSBs increases accordingly, as shown in Figure 4.8. Enhancement of DSBs as a result of concentration increase is also observed in Figure 4.9.

Therefore, it can be concluded that FMD-Br-DAB can enhance SSB DNA damages, especially when it is combine with X-ray irradiation. The amount of DSBs also has an upward tendency when the concentration of FMD-Br-DAB increases, but this trend is not as significant as that of SSBs.

Chapter 5. Flow Cytometry of Apoptotic Cell Death

5.1 Introduction

Apoptotic cells are morphologically and biochemically different from live cells or necrotic cells. For apoptotic cells, nucleases are activated and the nuclear DNA is degraded into fragments with lengths of approximately 200 base pairs, and the DNA break sites expose a large number of 3'-hydroxyl ends. The hydroxyl groups can be used as starting points for terminal deoxynucleotidyl transferase to add nucleotides (or analogs of nucleotides). For example, the 5-bromo-2'-deoxyuridine 5'-tri-phosphate (BrdUTP), a deoxythymidine analog can be added to label the break sites. The incorporated BrdU can be detected by a fluorescent dye conjugated with anti-BrdU antibody. [107-110]

The APO-BrdU™ TUNEL Assay Kit, purchased from Invitrogen, was used to detect the DNA fragmentation of apoptotic cells. An Alexa Fluor® 488 dye-labeled anti-BrdU antibody was used to detect the BrdU incorporation at DNA break sites. Propidium iodide was also included to determine the total cellular DNA content.

5.2 Experimental details

Cell Preparation and Fixation

Cells were seeded in fourteen 100mm dishes, with 10^6 cells per dish, and incubated at 37°C without CO_2 for 12 hours. Nine of the dishes were used as the experimental group, two were used as the negative controls, and three were used as positive controls. After incubation, various concentrations of FMD-Br-DAB were added to the corresponding dishes from the experimental group. After 12 hours of incubation, the samples from the experimental group were exposed to X-ray irradiation at appropriate doses. For the negative control group, no drug treatment or X-ray irradiation was applied. For the positive control group, cells were treated with 200 μM H_2O_2 for 18 hours before harvesting. After another 12 hours of incubation, the cells were harvested by the procedure described in the previous chapter, and the cells from each sample were suspended in a separate 0.5 mL phosphate-buffered saline (PBS) solution. Then, 4mL of 1% paraformaldehyde was added to fix the cells, and the cells were centrifuged at 300×g and the supernatant was discarded. Next, the cells were washed twice by adding 4.5 mL of PBS, then centrifuged. After that, 0.5 mL of PBS was added and the cells were transferred to 4 mL of 70% ice-cold ethanol. Then the cells were stored at -20°C. For the positive

control group, no drug treatment or X-ray irradiation was applied but cells were treated with 200 μM H_2O_2 for 18 hours before harvesting.

Detection of apoptosis

The cells from each tube were re-suspended in 1 mL of wash buffer provided in the kit and then centrifuged for 5 minutes at 300×g, and the supernatants were removed by aspiration. The above operation was repeated once, and 50 μL of DNA-labeling solution was added to each tube. The DNA-labeling solution was prepared by mixing 150 μL of reaction buffer, 11.25 μL of TdT enzyme, and 120 μL of BrdUTP and 468.75 μL of deionized water. The cells were then incubated for 60 minutes at 37° C and were shaken every 15 minutes to keep them in suspension. After incubation, 1.0 mL of rinse buffer was added to each tube and the cells were pelleted by centrifugation. Then each cell pellet was re-suspended with 100 μL of antibody solution, which was prepared by mixing 75 μL of the Alexa Fluor® 488 dye-labeled anti-BrdU antibody with 1.43 mL of rinse buffer provided from the kit, and the cells were incubated for 30 minutes at room temperature without light. Then, 0.5 mL of the propidium iodide/RNase A staining buffer was added to each sample, and the cells were incubated for an additional 30 minutes without light. After incubation, the samples were analyzed by flow cytometry.

5.3 Results

The results of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells, without any treatment, are shown as pseudocolor plots in Figure 5.1. A pseudocolor plot is a type of bivariate density plot that displays the number of events by colored pixels. Blue and green areas represent low population density; yellow areas correspond to mid-range cell density; red and orange areas represent high cell density. The cells to the right of the vertical line represent BrdU positive cells, which are cells undergoing apoptosis. The cells to the left of the vertical line represent BrdU negative cells, which are non-apoptosis cells. Therefore, the amount of Q_1 represents the percentage of apoptotic cells.

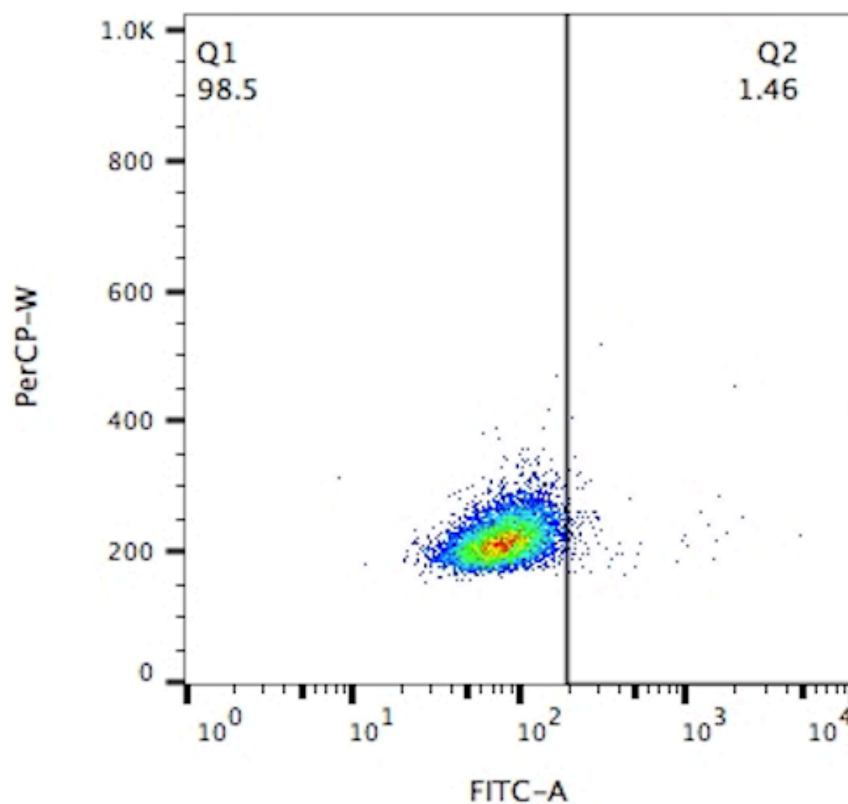


Figure 5.1: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 control cells without any treatment. Blue and green areas correspond to lower cell density; yellow to mid-range cell density; red and orange to high cell density. The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells.

Results of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells, treated with 20µM FMD-Br-DAB for 24 hours without any X-ray irradiation, are shown as pseudocolor plots in Figure 5.2.

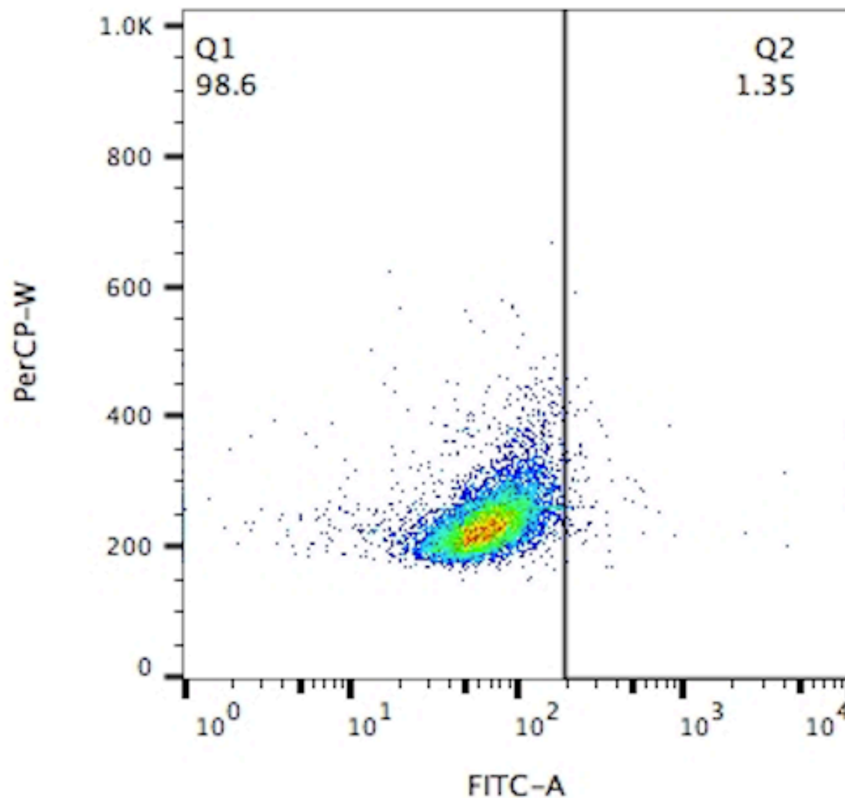


Figure 5.2: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells treated with 20µM FMD-Br-DAB without any X-ray irradiation. Blue and green correspond to areas of lower cell density, yellow area represents mid-range cell density, and red and orange are areas of high cell density. The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells.

Results of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells, treated with 2Gy X-ray irradiation without any drug treatment, are shown as pseudocolor plots in Figure 5.3.

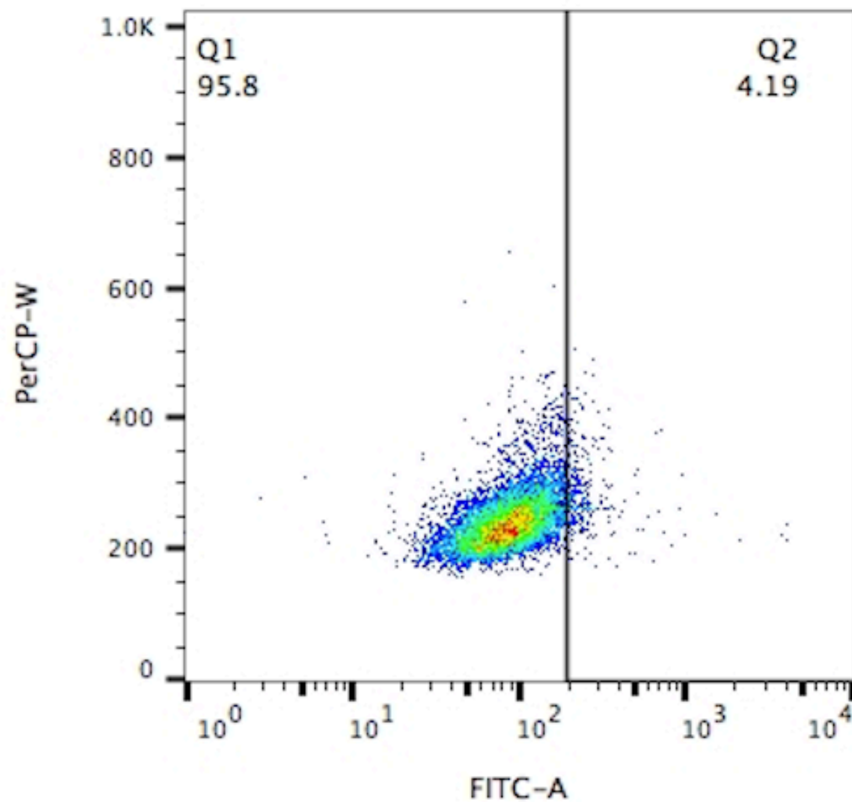


Figure 5.3: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells treated with 2Gy X-ray irradiation without any drug treatment. Blue and green correspond to areas of lower cell density, yellow area represents mid-range cell density, and red and orange are areas of high cell density. The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells.

Results of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells, treated with 20μM FMD-Br-DAB followed by 2Gy X-ray irradiation, are shown as pseudocolor plots in Figure 5.4.

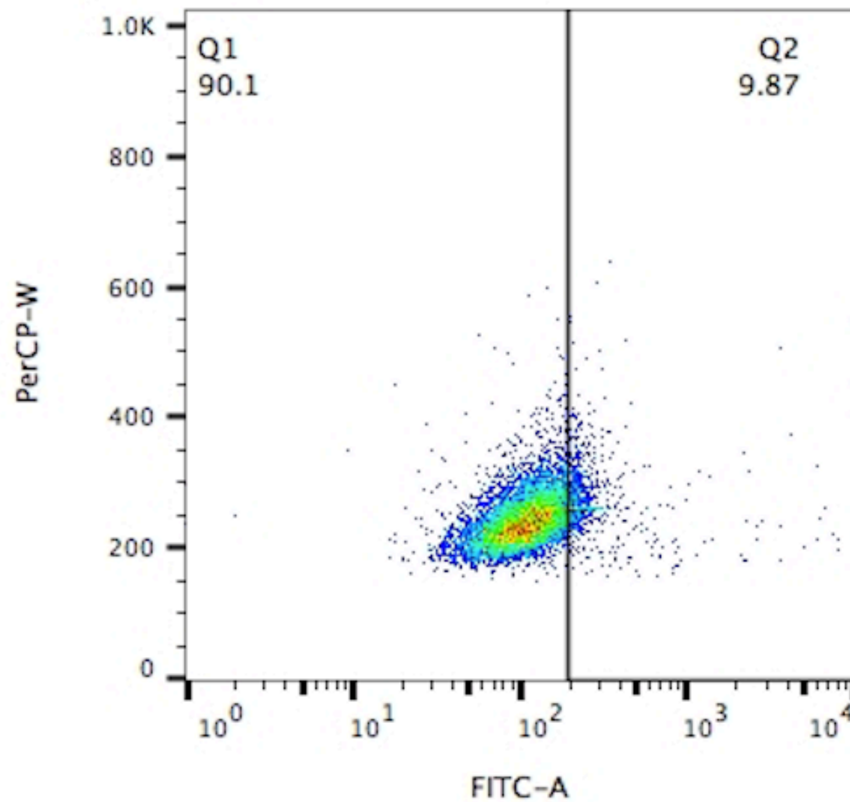


Figure 5.4: Bivariate density plot of APO-BrdU™ TUNEL Assay for MDA-MB-231 cells treated with 20μM FMD-Br-DAB followed by 2Gy X-ray irradiation. Blue and green correspond to areas of lower cell density, yellow area represents mid-range cell density, and red and orange are areas of high cell density. The cells in the right side of the vertical line are BrdU positive cells (apoptotic cells). The cells to the right of the vertical line are BrdU positive (apoptotic). Value of Q_2 represents percentage of apoptotic cells.

5.4 Discussions and Conclusions

As shown in Figure 5.1, 1.46% of untreated MDA-MB-231 cells undergo apoptosis and exhibit DNA fragmentation. Figure 5.2 shows that when MDA-MB-231 cells are treated with 20 μM FMD-Br-DAB alone, only 1.35% of the cells undergo early apoptosis and exhibit DNA fragmentation. Remarkably, the percentage of apoptotic cells from the FMD-Br-DAB treatment group is close to the percentage of apoptotic cells from the control group, and the percentage is even slightly lower that that of control group. These results reveal that FMD-Br-DAB treatment alone at the concentration of 20μM does not induce apoptosis.

In Figure 5.3, when MDA-MB-231 cells are treated with 2 Gy X-ray irradiation alone, 4.19% cells are apoptotic and exhibit DNA fragmentation. As shown in Figure 5.4, when MDA-MB-231 cells are treated with 20 μM FMD-Br-DAB followed by 2 Gy X-ray irradiation, 9.87% cells are apoptotic and exhibit DNA fragmentation. It can be seen from these results that when there is 2 Gy of X-ray irradiation, the percentage of apoptotic cells is significantly enhanced (from 4.19% to 9.87%) when cells are pretreated with FMD-Br-DAB.

In contrast to the results in Figure 5.1 and Figure 5.2, in which the amount of apoptotic cells does not increase when cells were pretreated with FMD-Br-DAB in

the absence of X-ray, the results in Figure 5.3 and Figure 5.4 show significant enhancement of the amount of apoptotic cells. These findings provide evidence of the synergistic effect between FMD-Br-DAB and radiation, attributed to the radiosensitizing effect of FMD-Br-DAB.

Chapter 6. Conclusions

This study shows the radiosensitizing effect of a newly discovered non-platinum-based regimen, FMD-Br-DAB, used as a radiosensitizer for radiotherapy of breast cancer.

In Chapters 2 and 3, MTT assays and clonogenic assays have been conducted to evaluate the cytotoxicity and radiosensitizing effects of FMD-Br-DAB. Both results have shown that FMD-Br-DAB treatment alone has certain cytotoxicity effects on MDA-MB-231 triple-negative breast cancer cells. When FMD-Br-DAB is used in combination with radiation, cytotoxic effect appears and increases as concentration of FMD-Br-DAB increases. Both MTT assays and clonogenic assays open up the possibility of using FMD-Br-DAB as a radiosensitizer.

In Chapters 4, gel electrophoresis has been done to confirm whether the radiosensitizing effect of FMD-Br-DAB is due to the enhancement of DNA damage. Plasmid DNA has been treated with FMD-Br-DAB and radiation, and the results have shown that DNA damage is significantly increased when DNA is treated with both FMD-Br-DAB and X-ray irradiation. These results are consistent with results obtained in previous chapters and directly prove that FMD-Br-DAB can enhance DNA damage when combined with radiation.

In Chapters 5, apoptotic cell death is detected and the percentage of apoptotic cells is measured by flow cytometry. The results are consistent with previous chapters, in which synergistic effects are observed between FMD-Br-DAB and X-ray, and no apoptosis is induced by FMD-Br-DAB alone.

In summary, this work confirms the radiosensitizing effect of a newly discovered non-platinum-based agent that can effectively enhance radiosensitivity of MDA-MB-231 cells and enhance DNA damage in a dose-dependent manner when combined with radiation.

References

1. Ferlay, J., Héry, C., Autier, P., & Sankaranarayanan, R. (2010). Global burden of breast cancer. In *Breast Cancer Epidemiology* (pp. 1-19). Springer New York.

2. Bray, F., McCarron, P., & Parkin, D. M. (2004). The changing global patterns of female breast cancer incidence and mortality. *childhood*, *4*(5).

3. *Siegel, R., Ma, J., Zou, Z., & Jemal, A. (2014). Cancer statistics, 2014. CA: a cancer journal for clinicians, 64(1), 9-29.*

4. Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: a cancer journal for clinicians*, *61*(2), 69-90.

5. *Canadian Cancer Society's Advisory Committee on Cancer Statistics. Canadian Cancer Statistics 2014. Toronto, ON: Canadian Cancer Society; 2014.*

6. Wun, L. M., Feuer, E. J., & Miller, B. A. (1995). Are increases in mammographic screening still a valid explanation for trends in breast cancer incidence in the United States?. *Cancer Causes & Control*, *6*(2), 135-144.

7. Phipps, A. I., & Li, C. I. (2010). Breast cancer biology and clinical characteristics. In *Breast Cancer Epidemiology* (pp. 21-46). Springer New York.

8. Franceschi, S., Levi, F., La Vecchia, C., Randimbison, L., & Te, V. C. (1998). Second cancers following in situ carcinoma of the breast. *International journal of cancer*, *77*(3), 392-395.

9. Li, C. I., & Daling, J. R. (2007). Changes in breast cancer incidence rates in the United States by histologic subtype and race/ethnicity, 1995 to 2004. *Cancer Epidemiology Biomarkers & Prevention*, *16*(12), 2773-2780.

10. Levi, F., Te, V. C., Randimbison, L., & La Vecchia, C. (2003). Increase in lobular breast cancer incidence in Switzerland. *International journal of cancer*,*107*(1), 164-165.

11. Li, C. I., Daling, J. R., & Malone, K. E. (2005). Age-specific incidence rates of in situ breast carcinomas by histologic type, 1980 to 2001. *Cancer Epidemiology Biomarkers & Prevention*, *14*(4), 1008-1011.

12. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., ... & Norton, L. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England Journal of Medicine*, *344*(11), 783-792.

13. Levin, E. R. (2005). Integration of the extranuclear and nuclear actions of estrogen. *Molecular Endocrinology*, *19*(8), 1951-1959.

14. Mohamed, A., Krajewski, K., Cakar, B., & Ma, C. X. (2013). Targeted therapy for breast cancer. *The American journal of pathology*, *183*(4), 1096-1112.

15. Lacroix, M., Toillon, R. A., & Leclercq, G. (2004). Stable 'portrait'of breast tumors during progression: data from biology, pathology and genetics.*Endocrine-related cancer*, *11*(3), 497-522.

16. Gelmon, K., Dent, R., Mackey, J. R., Laing, K., McLeod, D., & Verma, S. (2012). Targeting triple-negative breast cancer: optimising therapeutic outcomes. *Annals of oncology*, *23*(9), 2223-2234.

17. Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., ... & Børresen-Dale, A. L. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences*, *98*(19), 10869-10874.

18. Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., ... & Botstein, D. (2000). Molecular portraits of human breast tumours.*Nature*, *406*(6797), 747-752.

19. Carey, L. A., Perou, C. M., Livasy, C. A., Dressler, L. G., Cowan, D., Conway, K., ... & Millikan, R. C. (2006). Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *Jama*, *295*(21), 2492-2502.

20. Kerlikowske, K., Grady, D., Rubin, S. M., Sandrock, C., & Ernster, V. L. (1995). Efficacy of screening mammography: a meta-analysis. *Jama*, *273*(2), 149-154.

21. Gartlehner, G., Thaler, K., Chapman, A., Kaminski‐Hartenthaler, A., Berzaczy, D., Van Noord, M. G., & Helbich, T. H. (2013). Mammography in combination with breast ultrasonography versus mammography for breast cancer screening in women at average risk. The Cochrane Library.

22. SEER stat fact sheets: breast. (http://seer.cancer.gob/statfacts/html/breast.html.)

23. US Preventive Services Task Force. (2009). Screening for breast cancer: US Preventive Services Task Force recommendation statement. *Annals of internal medicine*, *151*(10), 716.

24. Hackshaw, A. K., & Paul, E. A. (2003). Breast self-examination and death from breast cancer: a meta-analysis. *British journal of cancer*, *88*(7), 1047-1053.

25. Saslow, D., Boetes, C., Burke, W., Harms, S., Leach, M. O., Lehman, C. D., ... & Russell, C. A. (2007). American Cancer Society guidelines for breast screening with MRI as an adjunct to mammography. *CA: a cancer journal for clinicians*, *57*(2), 75-89.

26. Hrung, J. M., Sonnad, S. S., Schwartz, J. S., & Langlotz, C. P. (1999). Accuracy of MR imaging in the work-up of suspicious breast lesions: a diagnostic meta-analysis. *Academic radiology*, *6*(7), 387-397.

27. Elmore, J. G., Armstrong, K., Lehman, C. D., & Fletcher, S. W. (2005). Screening for breast cancer. *Jama*, *293*(10), 1245-1256.

28. Fajardo, L. L., Pisano, E. D., Caudry, D. J., Gatsonis, C. A., Berg, W. A., Connolly, J., ... & Radiologist Investigators of the Radiologic Diagnostic Oncology Group V. (2004). Stereotactic and sonographic large-core biopsy of nonpalpable breast lesions: Results of the Radiologic Diagnostic Oncology group V study1. *Academic radiology*, *11*(3), 293-308.

29. Pisano, E. D., Fajardo, L. L., Caudry, D. J., Sneige, N., Frable, W. J., Berg, W. A., ... & McNeil, B. J. (2001). Fine-Needle Aspiration Biopsy of Nonpalpable Breast Lesions in a Multicenter Clinical Trial: Results from the Radiologic Diagnostic Oncology Group V 1. *Radiology*, *219*(3), 785-792.

30. Kerlikowske, K., Grady, D., Rubin, S. M., Sandrock, C., & Ernster, V. L. (1995). Efficacy of screening mammography: a meta-analysis. *Jama*, *273*(2), 149-154.

31. Kurian, A. W., & Carlson, R. W. (2010). Principles of breast cancer therapy. In*Breast Cancer Epidemiology* (pp. 371-388). Springer New York.

32. Morris, A. D., Morris, R. D., Wilson, J. F., White, J., Steinberg, S., Okunieff, P., ... & Van Dongen, J. A. (1996). Breast-conserving therapy vs mastectomy in early-stage breast cancer: a meta-analysis of 10-year survival. *The cancer journal from Scientific American*, *3*(1), 6-12.

33. Kurtz, J. M., Jacquemier, J., Amalric, R., Brandone, H., Ayme, Y., Hans, D., ... & Spitalier, J. M. (1990). Risk factors for breast recurrence in premenopausal and postmenopausal patients with ductal cancers treated by conservation therapy. *Cancer*, *65*(8), 1867-1878.

34. Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, E., ... & Wang, Y. (2005). Early Breast Cancer Trialists Collaborative Group (EBCTCG). Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet*, *366*(9503), 2087-2106.

35. Hudis, C., & Modi, S. (2007). Preoperative chemotherapy for breast cancer: miracle or mirage?. *JAMA*, *298*(22), 2665-2667.

36. Charfare, H., Limongelli, S., & Purushotham, A. D. (2005). Neoadjuvant chemotherapy in breast cancer. *British journal of surgery*, *92*(1), 14-23.

37. Jones, J. L., Zabicki, K., Christian, R. L., Gadd, M. A., Hughes, K. S., Lesnikoski, B. A., ... & Smith, B. L. (2005). A comparison of sentinel node biopsy before and after neoadjuvant chemotherapy: timing is important. *The American journal of surgery*, *190*(4), 517-520.

38. Rajagopalan, P. R., Zhang, Z., McCourt, L., Dwyer, M., Benkovic, S. J., & Hammes, G. G. (2002). Interaction of dihydrofolate reductase with methotrexate: ensemble and single-molecule kinetics. *Proceedings of the National Academy of Sciences*, *99*(21), 13481-13486.

39. Pazdur, R., Kudelka, A. P., Kavanagh, J. J., Cohen, P. R., & Raber, M. N. (1993). The taxoids: paclitaxel (Taxol®) and docetaxel (Taxotere®). *Cancer treatment reviews*, *19*(4), 351-386.

40. Longley, D. B., Harkin, D. P., & Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nature Reviews Cancer*, *3*(5), 330-338.

41. Dow, K. H. (2004). *Contemporary issues in breast cancer: a nursing perspective*. Jones & Bartlett Learning.

42. von Minckwitz, G., & Loibl, S. (2015). Evolution of adjuvant chemotherapy for breast cancer. *The Lancet*.

43. Beatson, G. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *The Lancet*, *148*(3803), 162-165.

44. VASSILOPOULOU‐SELLIN, R. E. N. A. (2003). Breast cancer and hormonal replacement therapy. *Annals of the New York Academy of Sciences*, *997*(1), 341-350.

45. Arteaga, C. L., & Osborne, C. K. (1991). Growth factors as mediators of estrogen/antiestrogen action in human breast cancer cells. In *Regulatory mechanisms in breast cancer* (pp. 289-304). Springer US.

46. Vogel, C. L. (1996, August). Hormonal approaches to breast cancer treatment and prevention: an overview. In *Seminars in oncology* (Vol. 23, No. 4 Suppl 9, pp. 2-9).

47. Fisher, B., Costantino, J. P., Redmond, C. K., Fisher, E. R., Wickerham, D. L., & Cronin, W. M. (1994). Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *Journal of the National Cancer Institute*, *86*(7), 527-537.

48. Fisher, B., Costantino, J. P., Wickerham, D. L., Cecchini, R. S., Cronin, W. M., Robidoux, A., ... & Wolmark, N. (2005). Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *Journal of the National Cancer Institute*, *97*(22), 1652-1662.

49. Wong, Z. W., & Ellis, M. J. (2004). First-line endocrine treatment of breast cancer: aromatase inhibitor or antioestrogen&quest. *British journal of cancer*,*90*(1), 20-25.

50. Bonneterre, J., Thürlimann, B., Robertson, J. F. R., Krzakowski, M., Mauriac, L., Koralewski, P., ... & Arimidex Study Group. (2000). Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex

Randomized Group Efficacy and Tolerability study. *Journal of Clinical Oncology*, *18*(22), 3748-3757.

51. Nabholtz, J. M., Buzdar, A., Pollak, M., Harwin, W., Burton, G., Mangalik, A., ... & Arimidex Study Group. (2000). Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. *Journal of Clinical Oncology*,*18*(22), 3758-3767.

52. Mouridsen, H., Gershanovich, M., Sun, Y., Pérez-Carrión, R., Boni, C., Monnier, A., ... & Dugan, M. (2001). Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group.*Journal of Clinical Oncology*, *19*(10), 2596-2606.

53. Dirix, L., Piccart, M. J., Lohrisch, C., Beex, L., Nooij, M., Cameron, D., ... & Paridaens, R. (2001). Efficacy of and tolerance to exemestane (E) versus tamoxifen (T) in 1st line hormone therapy (HT) of postmenopausal metastatic breast cancer (MBC) patients (pts): a European Organisation for the Research and Treatment of Cancer (EORTC Breast Group) Phase II trial with Pharmacia and Upjohn. In *Proc Am Soc Clin Oncol* (Vol. 20, p. 29a).

54. Higgins, M. J., & Baselga, J. (2011). Targeted therapies for breast cancer. *The Journal of clinical investigation*, *121*(10), 3797.

55. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., & McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*, *235*(4785), 177-182.

56. Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E. A., ... & Davidson, N. E. (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *New England Journal of Medicine*, *357*(26), 2666-2676.

57. Rocca, A., Farolfi, A., Bravaccini, S., Schirone, A., & Amadori, D. (2014). Palbociclib (PD 0332991): targeting the cell cycle machinery in breast cancer.*Expert opinion on pharmacotherapy*, *15*(3), 407-420.

58. Mohamed, A., Krajewski, K., Cakar, B., & Ma, C. X. (2013). Targeted therapy for breast cancer. *The American journal of pathology*, *183*(4), 1096-1112.

59. Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of clinical investigation*, *121*(7), 2750.

60. Duffy, M. J., McGowan, P. M., & Crown, J. (2012). Targeted therapy for triple‐negative breast cancer: Where are we?. *International Journal of Cancer*,*131*(11), 2471-2477.

61. Gerber, D. E., & Chan, T. A. (2008). Recent advances in radiation therapy.*American family physician*, *78*(11), 1254-1262.

62. Bucci, M. K., Bevan, A., & Roach, M. (2005). Advances in radiation therapy: conventional to 3D, to IMRT, to 4D, and beyond. *CA: a cancer journal for clinicians*, *55*(2), 117-134.

63. Gaspar, L. E., & Ding, M. (2008). A review of intensity-modulated radiation therapy. *Current oncology reports*, *10*(4), 294-299.

64. Krueger, E. A., Fraass, B. A., & Pierce, L. J. (2002, July). Clinical aspects of intensity-modulated radiotherapy in the treatment of breast cancer. In *Seminars in radiation oncology* (Vol. 12, No. 3, pp. 250-259). WB Saunders.

65. Teh, B. S., Lu, J. J., & Schefter, T. E. (2012). *Stereotactic body radiation therapy*. Springer.

66. Vicini, F., Beitsch, P. D., Quiet, C. A., Keleher, A. J., Garcia, D., Snider, H. C., ... & Lyden, M. (2008). Three‐year analysis of treatment efficacy, cosmesis, and toxicity by the American Society of Breast Surgeons MammoSite Breast Brachytherapy Registry Trial in patients treated with accelerated partial breast irradiation (APBI). *Cancer*, *112*(4), 758-766.

67. Ito, T., Baker, S. C., Stickley, C. D., Peak, J. G., & Peak, M. J. (1993). Dependence of the yield of strand breaks induced by γ-rays in DNA on the physical conditions of exposure: Water content and temperature. *International journal of radiation biology*, *63*(3), 289-296.

68. Michael, B. D., & O'Neill, P. (2000). A sting in the tail of electron tracks.*Science*, *287*(5458), 1603-1604.

69. NIKJOO, P. O'NEILL, DT GOODHEAD and M. TERRISSOL, H. (1997). Computational modelling of low-energy electron-induced DNA damage by early physical and chemical events. *International journal of radiation biology*, *71*(5), 467-483.

70. Chapman, J. D., & Gillespie, C. J. (1981). Radiation-induced events and their time scale in mammalian cells. *Adv. Radiat. Biol*, *9*(143-198), 54.

71. Lu, Q. B., Baskin, J. S., & Zewail, A. H. (2004). The presolvated electron in water: Can it be scavenged at long range?. *The Journal of Physical Chemistry B*, *108*(29), 10509-10514.

72. Wang, C. R., Nguyen, J., & Lu, Q. B. (2009). Bond breaks of nucleotides by dissociative electron transfer of nonequilibrium prehydrated electrons: a new molecular mechanism for reductive DNA damage. *Journal of the American Chemical Society*, *131*(32), 11320-11322.

73. Wang, C. R., Luo, T., & Lu, Q. B. (2008). On the lifetimes and physical nature of incompletely relaxed electrons in liquid water. *Physical Chemistry Chemical Physics*, *10*(30), 4463-4470.

74. Nguyen, J., Ma, Y., Luo, T., Bristow, R. G., Jaffray, D. A., & Lu, Q. B. (2011). Direct observation of ultrafast-electron-transfer reactions unravels high effectiveness of reductive DNA damage. *Proceedings of the National Academy of Sciences*, *108*(29), 11778-11783.

75. Lu, Q. B. (2007). Molecular reaction mechanisms of combination treatments of low-dose cisplatin with radiotherapy and photodynamic therapy. *Journal of medicinal chemistry*, *50*(11), 2601-2604.

76. Wardman, P. (2007). Chemical radiosensitizers for use in radiotherapy. Clinical Oncology, 19(6), 397-417.

77. Lu, Q. B., Kalantari, S., & Wang, C. R. (2007). Electron transfer reaction mechanism of cisplatin with DNA at the molecular level. *Molecular pharmaceutics*, *4*(4), 624-628.

78. Siddik, Z. H. (2003). Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*, *22*(47), 7265-7279.

79. Wang, D., & Lippard, S. J. (2005). Cellular processing of platinum anticancer drugs. *Nature reviews Drug discovery*, *4*(4), 307-320.

80. Geldof, A. A., & Slotman, B. J. (1996). Radiosensitizing effect of cisplatin in prostate cancer cell lines. *Cancer letters*, *101*(2), 233-239.

81. Candelaria, M., Garcia-Arias, A., Cetina, L., & Dueñas-Gonzalez, A. (2006). Radiosensitizers in cervical cancer. Cisplatin and beyond. *Radiation Oncology*,*1*(1), 15.

82. Begg, A. C., Van der Kolk, P. J., Dewit, L., & Bartelink, H. (1986). Radiosensitization by cisplatin of RIF1 tumour cells in vitro. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine*, *50*(5), 871-884.

83. Lu, Q. B., Kalantari, S., & Wang, C. R. (2007). Electron transfer reaction mechanism of cisplatin with DNA at the molecular level. *Molecular pharmaceutics*, *4*(4), 624-628.

84. Lu, Q. B. (2007). Molecular reaction mechanisms of combination treatments of low-dose cisplatin with radiotherapy and photodynamic therapy. *Journal of medicinal chemistry*, *50*(11), 2601-2604.

85. Greer, S., & Zamenhof, S. (1957, April). Effect of 5-bromouracil in deoxyribonucleic acid of E. coli on sensitivity to ultraviolet radiation. In *Abstr. Am. Chem. Soc. 131st Meeting p. C* (Vol. 3).

86. Perez, L. M., & Greer, S. (1986). Sensitization to X ray by 5-chloro-2′-deoxycytidine co-administered with tetrahydrouridine in several mammalian cell lines and studies of 2′-chloro derivatives. *International Journal of Radiation Oncology* Biology* Physics*, *12*(8), 1523-1527.

87. Djordjevic, B., & Szybalski, W. (1960). GENETICS OF HUMAN CELL LINES III. INCORPORATION OF 5-BROMO-AND 5-IODODEOXYHRIDINE INTO THE DEOXYRIBONUCLEIC ACID OF HUMAN CELLS AND ITS EFFECT ON RADIATION SENSITIVITY. *The Journal of experimental medicine*, *112*(3), 509-531.

88. Kaplan, H. S., Smith, K. C., & Tomlin, P. (1961). Radiosensitization of E. coli by purine and pyrimidine analogues incorporated in deoxyribonucleic acid. *Nature*,*190*, 794-796.

89. Wang, C. R., & Lu, Q. B. (2007). Real‐Time Observation of a Molecular Reaction Mechanism of Aqueous 5‐Halo‐2′‐deoxyuridines under UV/Ionizing Radiation. *Angewandte Chemie International Edition*, *46*(33), 6316-6320.

90. Wang, C. R., Hu, A., & Lu, Q. B. (2006). Direct observation of the transition state of ultrafast electron transfer reaction of a radiosensitizing drug bromodeoxyuridine. *The Journal of chemical physics*, *124*(24), 241102.

91. Wang, C. R., & Lu, Q. B. (2010). Molecular mechanism of the DNA sequence selectivity of 5-halo-2′-deoxyuridines as potential radiosensitizers. *Journal of the American Chemical Society*, *132*(42), 14710-14713.

92. Lu, Q. B., & Madey, T. E. (1999). Giant enhancement of electron-induced dissociation of chlorofluorocarbons coadsorbed with water or ammonia ices: Implications for atmospheric ozone depletion. *Journal of Chemical Physics*,*111*(7).

93. Lu, Q. B. (2010). Effects and applications of ultrashort-lived prehydrated electrons in radiation biology and radiotherapy of cancer. *Mutation Research/Reviews in Mutation Research*, *704*(1), 190-199.

94. Lu, Q. B., Zhang, Q. R., Ou, N., Wang, C. R., & Warrington, J. (2015). In Vitro and In Vivo Studies of Non-Platinum-Based Halogenated Compounds as Potent Antitumor Agents for Natural Targeted Chemotherapy of Cancers.*EBioMedicine*.

95. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1), 55-63.

96. van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. In *Cancer Cell Culture* (pp. 237-245). Humana Press.

97. Morgan, D. M. (1998). Tetrazolium (MTT) assay for cellular viability and activity. In *Polyamine protocols* (pp. 179-184). Humana Press.

98. Cailleau, R., Young, R., Olive, M., & Reeves, W. J. (1974). Breast tumor cell lines from pleural effusions. Journal of the National Cancer Institute, 53(3), 661-674.

99. Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., & Van Bree, C. (2006). Clonogenic assay of cells in vitro. *Nature protocols*, *1*(5), 2315-2319.

100. Munshi, A., Hobbs, M., & Meyn, R. E. (2005). Clonogenic cell survival assay. In*Chemosensitivity* (pp. 21-28). Humana Press.

101. Banasiak, D., Barnetson, A. R., Odell, R. A., Mameghan, H., & Russell, P. J. (1999). Comparison between the clonogenic, MTT, and SRB assays for determining radiosensitivity in a panel of human bladder cancer cell lines and a ureteral cell line. *Radiation oncology investigations*, *7*(2), 77-85.

102. Viovy, J. L. (2000). Electrophoresis of DNA and other polyelectrolytes: physical mechanisms. Reviews of Modern Physics, 72(3), 813.

103. Sinden, R. R. (2012). *DNA structure and function*. Elsevier.

104. Evan, G. I., & Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature*, *411*(6835), 342-348.

105. Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: enemies within. *Science*,*281*(5381), 1312-1316.

106. Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature*, *407*(6805), 770-776

107. Arends, M. J., Morris, R. G., & Wyllie, A. H. (1990). Apoptosis. The role of the

108. endonuclease. The American journal of pathology, 136(3), 593.

109. Vermes, I., Haanen, C., & Reutelingsperger, C. (2000). Flow cytometry of apoptotic cell death. Journal of immunological methods, 243(1), 167-190.

110. Arends, M. J., Morris, R. G., & Wyllie, A. H. (1990). Apoptosis. The role of the endonuclease. The American journal of pathology, 136(3), 593.

111. Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: enemies within. Science,281(5381), 1312-1316.

112. Hengartner, M. O. (2000). The biochemistry of apoptosis. Nature, 407(6805), 770-776.

113. Begg, A. C. (1990). Cisplatin and radiation: interaction probabilities and therapeutic possibilities. *International Journal of Radiation Oncology* Biology* Physics*, *19*(5), 1183-1189.

114. Al-Sarraf, M., LeBlanc, M., Giri, P. G., Fu, K. K., Cooper, J., Vuong, T., ... & Ensley, J. F. (1998). Chemoradiotherapy versus radiotherapy in patients with advanced nasopharyngeal cancer: phase III randomized Intergroup study 0099.*Journal of Clinical Oncology*, *16*(4), 1310-1317.