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RADIO-SENSITIZING PROSTATE CANCER CELLS: A POSSIBLE ROLE FOR ZOLEDRONIC ACID AND PRAVASTATIN (ZOPRA)

by
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"If you have knowledge, let others light their candles in it." – Margaret Fuller.

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ABSTRACT

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Title: Radio-Sensitizing Prostate Cancer Cells: A Possible Role For Zoledronic Acid And Pravastatin (ZoPra)

Prostate cancer is highly prevalent in men worldwide and in Lebanon specifically. Radiotherapy is one of the first-line treatments aimed at reducing tumor size and further disease progression. Its efficacy relies on the radio sensitivity of tumor cells and the patient. However, it's significantly reduced by the radioresistance of tumor cells and deleterious effects on surrounding normal cells. Radioresistance of tumor cells is brought about by the hyperactivation of non-homologous end-joining DNA repair proteins, pATM and H2AX, that increase repair signaling and recognition respectively. Therefore, targeting these proteins in tumor cells to impede resistance is of high importance.

Bisphosphonates such as Zoledronic acid are widely used in the treatment of bone loss-related diseases. Statins, such as Pravastatin, are used in the management of lipid levels. Recently, a combination of both ZoPra was shown to radioprotect normal tissues and radiosensitize cancer cells.

Therefore, the aim of this study is to assess the effect of Zoledronic acid and Pravastatin, alone and in combination on the radio-response of human prostate cancer cell lines, DU-145 and PC-3, *in vitro,* on a cellular and molecular level.

The cytotoxic effect of different concentrations of Zoledronic acid and Pravastatin were tested using MTT assay. The optimum concentration of both was determined to be 1 μM and was therefore used in further experiments. Cells were treated with 1 μM Zoledronic acid and Pravastatin, alone and in combination, prior to a 2 Gy irradiation. Clonogenic assay was performed to assess cell survival and colony forming ability in both cell lines with treatment. Immunofluorescence analysis of pATM and γH2AX was performed to study DNA DSB repair kinetics.

Pre-treatment with 1 μM ZoPra prior to a 2 Gy irradiation was shown to radiosensitize DU-145 and PC3 cell lines. The treatment was shown to increase the residual number of γH2AX foci. A significant decrease in cell survival in both cell lines was observed. This study presents novel findings on the potential use of ZoPra as a radio-sensitizing agent for radio-resistant prostate cancer cells.

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ABBREVIATIONS

ACS: American Cancer Society

ADT: Androgen deprivation therapy

ASR: Age-standardized rate

ATM: Ataxia telangiectasia mutated

ATR: ATM and Rad3 related

AUA: American Urology Association

BPH: Benign prostatic hyperplasia

CZ: Central Zone

DDR: DNA damage repair

DRE: Digital rectal exam

DNA: Deoxyribonucleic acid

DNA-PK: DNA-dependent protein kinase.

DSB: Double-strand Break

EBRT: External beam radiation therapy

EMT: Epithelial to Mesenchymal Transition

Gy: Grey

H2AX: Variant histone H2A

γH2AX: Phosphorylated H2AX

HDR: High-dose-rate

HIFU: High-intensity focused ultrasound

HMG Co-A: Hydroxymethylglutaryl coenzyme-A

HR: Homologous recombination

HSP90: Heat Shock Protein 90

IR: Ionizing Radiation

LET: Linear energy transfer

MN: Micronuclei

MRN: MRE11-Rad50-Nbs1

MTT: 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NF1: Neurofibromatosis type 1

NHEJ: Non-homologous end joining

OS: Osteosarcoma

PCa: Prostate cancer

PE: Plating efficiency

PIKK: Phosphatidylinositol-3-kinase-like kinase

Pra: Pravastatin

PSA: Prostate-specific antigen

PZ: Peripheral Zone

Qol: Quality of Life

RIANS: Radiation-induced nucleo-shuttling of the ATM kinase

ROS: Reactive oxygen species

RR: Radiation resistance

RT: Radiotherapy

SEM: Standard error mean

SF: Surviving fraction

SSB: Single strand breaks

ssDNA: Single strand DNA

TGF-β: Transforming Growth Factor beta

TRUS: Transrectal ultrasound

TZ: Transitional zone

Zo: Zoledronic acid

ZoPra: Combination of Zoledronic acid and Pravastatin

CHAPTER I

INTRODUCTION

A. Overview of Prostate Cancer: Epidemiology

Cancer is a complex pathophysiological disease. It is multifactorial and the dysregulation of cell proliferation is due to the accumulation of several gene mutations (1). It is a worldwide burden, having the second highest mortality rate worldwide after cardiovascular disease (2). The COVID-19 pandemic overwhelmed health care systems worldwide, and cancer screening, diagnosis, and treatment became less accessible. This may momentarily improve the numbers in regards to diagnosis but may increase them in regards to higher staged cancer cases in the future (3). In 2020, according to the GLOBOCAN estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer (IARC), the total incidence of cancer worldwide is an estimated 19.3 million with about 10 million death cases (4). According to the World Health Organization (WHO) in 2020, prostate cancer (PCa) was the second most prevalent type in males with 1.41 million cases and was the fifth leading cause of male cancer deaths. Low- and middle-income countries, such as Lebanon, suffer the most deaths from this disease (4, 5). The most frequent types of cancer in Lebanon are breast, lung, and prostate, with their prevalence in that order. In 2020, there were 11,589 recorded cases, of which 1,027 new cases and 360 deaths were of PCa. PCa had the highest Age-standardized (World) rate (ASR) of incidence (28.5) in males, and the third highest mortality rate (9.7)(6).

B. Prostate

1. Gland anatomy and physiology

The prostate is a gland located in the retroperitoneal space in the male anatomy (7). It is about the size of a walnut and has an inverse conical shape. Its base faces upward and is continuous with the inferior surface of the bladder and its apex faces downward in contact with the superior fascia of the external urethral sphincter (8, 9). According to McNeal, the prostate's parenchyma is anatomically divided into 4 zones with the relationship of each to the urethra providing a central anatomic reference (figure 5) (10):

- Preprostatic region – constituting the transition zone (TZ), that surrounds the proximal urethra, and periurethral ducts glands, that extend from the bladder base to the verumontanum, where the ejaculatory ducts empty into the urethra. Hyperplastic proliferative lesions usually emanate in this zone.
- The central zone (CZ) – forms the base of the gland that surrounds the ejaculator ducts and constitutes 25% of the glandular prostate.
- The peripheral zone – surrounds most of both the TZ and the CZ and surrounds the distal urethra. It constitutes 70% of the glandular prostate. Most carcinomas emanate from this zone.
- The anterior fibromuscular stroma – forms the anterior surface of the prostate and is devoid of any glandular tissue.

The most prevalent prostate pathology include prostatitis, benign prostatic hyperplasia (BPH), and malignant prostate cancer (7). The growth of the cells in the prostate is androgen-dependent and the lack of androgens leads to atrophy. Cell proliferation and atrophy must be controlled to maintain prostate homeostasis. The etiology of prostate cancer remains elusive; however, several risk factors contribute to an

imbalance between proliferation and cell death resulting in pathology (11, 12). These risk factors include age, genetic family history, ethnicity, bladder cancer history, and increased circulating sex steroid hormones (13, 14).

2. Screening

The American Urological Association (AUA) has specific guidelines regarding the importance of PCa screening in those that are at higher risk than others (15).

Screening aims to find evidence of cancer in patients, especially in those with symptoms. Alarming signs include a weak or interrupted flow of urine, sudden urge to urinate frequently, straining to empty the bladder, blood in urine or semen, and pain caused while sitting from an enlarged prostate (16). PCa screening, according to the American Cancer Society (ACS) recommendation has risks and benefits that should be weighed (17). Detection of asymptomatic PCa may expose patients to unnecessary treatment side effects, ultimately causing more harm than benefit. Screening, when appropriate, usually takes place biannually to decrease the risk of overdiagnosis, according to the AUA. The two most common screening modalities are (18):

- Prostate Specific Antigen (PSA) – produced by malignant and non-malignant epithelial cells. Elevated PSA may be due to several factors such as prostatitis, BPH, or recent genitourinary tract manipulation, according to the AUA.
- Digital Rectal Exam (DRE) – the PZ can be palpated when a gloved finger is inserted into the rectum to track prostate size and consistency.

These are the usual first-line screening tests. PSA is a poor predictor of disease on its own and should be interpreted in the context of the patient; it is therefore supported by a DRE. There are adjunct PCa screening tests that include PSA kinetics, free/total

PSA, prostate health index, magnetic resonance imaging, and urinary markers that may be used by the physician (e.g. PCA3) (18).

3. Diagnosis

PCa diagnosis is facilitated with a transrectal ultrasound (TRUS)-guided needle biopsy and an MRI in the case screening results are abnormal. 12 samples are usually taken from representative areas of the prostate and examined and graded by pathologists and graded. Men with an abnormal PSA and negative biopsy will usually undergo additional testing from those mentioned above (19). If cancer is detected, it is given a grade to describe the cell's morphology compared to normal cells and it is given a stage, which describes if cancer has spread or remains localized in the prostate.

4. Staging, grading, and types of PCa

The stage is determined by classifying the cells histologically, and by using the TNM staging system. TNM stands for T (tumor), N (nodes), and M (metastasis); a system developed by the American Joint Committee on Cancer. The stages range from 1 through 4 and include substages to precisely specify the extent to which the tumor has grown and spread. In stages I and II the tumor has not spread beyond the prostate, stage III is the locally advanced stage where the tumor has spread to nearby tissues in a process known as extracapsular extension, and stage IV describes a tumor that has undergone metastasis to lymph nodes and/or bones (figure 6) (20-22). Cancer metastasis can occur through the tissue, lymph system, and blood. Some tests can be used to detect metastasis and its degree. These include a bone scan to check for rapidly dividing cells in the bone, MRI, CT scan, pelvic lymphadenectomy where lymph nodes in the pelvis are removed and

examined by a pathologist, seminal vesicle biopsy, and/or a ProstaScint scan where radioactive material that attaches to prostate cancer cells is injected into the vein and is detected by a scanner (16).

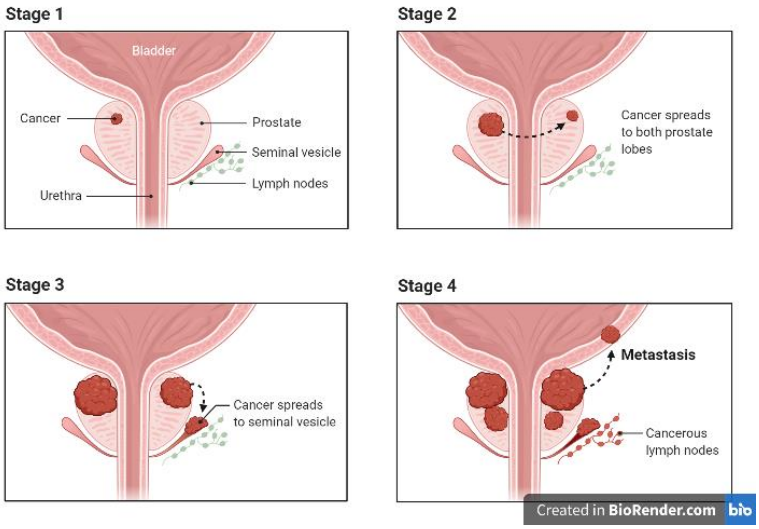

Figure 1. Stages of prostate cancer showing metastatic extent.

The grade is determined using the Gleason Score. Pathologists describe the tumor cells by comparing them to normal prostate tissue and assigning them a grade; a grade of 3 means that they look most like normal prostate cells and a grade of 5 looks most abnormal. Adding two scores from the biopsy will give a Gleason score ranging from 6-10. Table 1 summarizes this score and compares it to the cell's Grade Group which is also used to grade PCa tumors (16).

Table 1. Gleason score of prostate cancer, its equivalent Grade group, and the meaning assigned to each.

Gleason score	Grade Group	Meaning
Gleason score 6	1	Cells are very similar to normal prostate cells They are not expected to grow/ expected to grow slowly
Gleason score 7	2	Most cells are similar to normal prostate cells They are expected to grow slowly
Gleason score 8	3	Cells look less like normal prostate cells They are expected to grow moderately
Gleason score 9	4	Some cells look abnormal They are expected to grow moderately or quickly
Gleason score 10	5	Most cells look abnormal They are expected to grow quickly

There are two types of staging prostate cancer, and these are:

- Clinical staging – this is by taking into consideration the DRE result, PSA testing and Gleason score.
- Pathological staging – this is based on the pathology of the prostate gland and/or the lymph nodes affected after surgery.

The importance of both staging and grading is to identify the patient's prognosis and also help determine the most effective therapeutic modality (21). Another factor that aids in this process is identifying its cellular origin. The most common types of cancers develop in the epithelium. Of those, acinar adenocarcinomas are the most common and others include ductal adenocarcinomas, urothelial carcinoma, squamous cell neoplasms, basal cell carcinoma, as well as neuroendocrine carcinomas. Tumors of other origins include prostatic mesenchymal tumors and hematological neoplasia which have a rarer occurrence compared to adenocarcinomas (23).

5. Treatment

As previously mentioned, using the information gathered from the grade, stage, origin of cancer, and the overall medical status of the patient, physicians can now make a constructive decision regarding which mode(s) of therapy will yield the best results while taking the side effects of each into consideration. According to the AUA, therapeutic modalities fall within two categories: localized prostate cancer management and advanced prostate cancer management. The most common therapeutic modalities are briefly explained in the section below.

a. Localized prostate cancer management

i. Active surveillance

This is where a patient's condition is monitored carefully in order to track any changes. This is beneficial when a patient is of old age or has a condition that would be exacerbated by other treatments and maintaining his quality of life (QoL) is more beneficial. PCa is a heterogeneous disease and its aggressiveness is relative, therefore with people who have more favorable symptoms or none at all, treatment is unsubstantiated (24). Frequent DREs, PSA tests, transrectal ultrasounds, and biopsies are used to track any changes (25).

ii. Radiotherapy

According to the AUA, the two most common forms of radiation therapy used are external beam radiation (EBRT) and brachytherapy. High energy rays, specifically X-rays, are targeted at tumor cells to kill them or halt their growth in EBRT, with minimal damage to normal tissue. This can be combined with different types of treatments, such

as hormonal therapy also known as androgen deprivation therapy (ADT) in high-risk patients, chemotherapy, and surgery.

Brachytherapy is when radioactive seeds or particles are placed in or nearby a tumor. It allows the delivery of a very high dose of radiation directly to the tumor while reducing its effect on surrounding cells. (26)

iii. Focal therapy

A novel type of treatment aimed at bridging between active surveillance and invasive treatment for prostate cancer. A percentage of prostate cancers in patients put under active surveillance have a chance of becoming aggressive. In order to delay invasive manipulation, focal therapy aims at keeping the patients' QoL adequately under control. This involves cryotherapy, high-intensity focused ultrasound (HIFU), both high-dose-rate (HDR) brachytherapy and low-dose-rate (LDR) brachytherapy, radiotherapy, and thermotherapy (27).

iv. Hormone therapy

Hormone therapy is a modality usually chosen in the case of stage III, stage IV, and/or relapsed PCa. Chemical castration is achieved through anti-androgen treatment, as androgen receptors (AR) found in epithelial and stromal tissue are blocked. This is more effective than orchidectomy as it ensures a lack of androgens even from the adrenal glands (28). However, PCa cells develop androgen-independent growth. This is possibly achieved by the activation of AR by other ligands, increased AR expression, and sensitivity to minute amounts of androgens. This type of PCa is known as castration-resistant PCa and metastatic castration-resistant PCa (28).

b. Advanced prostate cancer management

v. Surgery

Radical prostatectomy is one of the first steps in the multitherapeutic approach in patients with tumors in Groups 2-5 and with a Gleason score of 7-9. This opposes earlier practices of this procedure when it was done only on low-grade prostatic tumors. This is due to the increased use of active surveillance programs and molecular insights that identify phenotypically indolent tumors. Surgery is opted for in order to prevent the beginning or further metastasis of PCa cells (29). According to the AUA, adjuvant radiotherapy may be given after surgery.

vi. Chemotherapy

According to the AUA, chemotherapy is generally used in metastatic PCa to stop the growth of cancer cells. For those who have not undergone ADT, androgen antagonists are used accompanied by several agents. These agents may be chemotherapeutic such as Docetaxel and Cabazitaxel. Docetaxel is one of the first chemotherapeutic agents to result in the overall improvement in survival rate in metastatic castration-resistant PCa (30).

vii. Immunotherapy

This uses the patient's immune system to fight cancer. This is done either by boosting, restoring, or directing the body's immune response against tumor cells by injecting material made by the body or in the laboratory. It is most usually used in metastasized PCa (16).

C. Radiotherapy

Radiation therapy (RT) is a clinical modality that is used to treat over 50% of patients with malignancy (31). It aids in inhibiting cell growth, metastasis, and proliferation of tumor cells. Unfortunately, the therapeutic window of RT is delimited by the resistance of tumor cells and the deleterious effect on patient quality of life (QoL) by targeting healthy surrounding tissue (32). Adverse effects include erectile dysfunction in 36-59%(33), urinary dysfunction in 45%, bowel dysfunction such as rectal bleeding in 5% increasing to 25% 2 years post-EBRT, diarrhea and fecal incontinence in 10-20% (34), and incompetence in 36-68% of patients that undergo EBRT. Other side effects coupled with EBRT include damaged pelvic nerves, induced fibrosis, loss of functional urethral length, climacturia, and ejaculatory dysfunction. Some of the inauspicious effects of EBRT are acute and resolve within 90 days, however, several others that last longer than 2 years, such as proctitis, cystitis, urinary/rectal bleeding, narrowing of the rectum/urethra, chronic diarrhea/frequency/ incontinence, and ulcer development in the rectum (35, 36).

1. Production of X-rays: bremsstrahlung effect

X-rays are produced through the process known as Bremsstrahlung radiation (in English: braking radiation). In an x-ray tube, free electrons are created at a cathode filament, accelerated through the tube using a voltage to the anode where they interact with tungsten atoms (or other material). Some of these electrons are attracted to the positive nucleus of the atoms. When the electrons reach the orbit of the atom, the electrons decelerate or 'break' and deflect, changing their path direction. The lost energy in this process is released as X-ray photons (figure7). These electrons cause ionization and

excitation of atoms in other mediums. Deep-seated tumors are targeted by high-energy beams, as low-energy beams are hindered by obstacles. Radiation is quantified by the radiation absorbed dose. This is the energy deposited by secondary charged particles in the medium. The unit of absorbed energy is the gray (Gy) (37). X-ray radiation is considered low linear energy transfer (LET), which is defined as the energy lost per unit path length by the particle (38). According to the AUA guidelines, EBRT is usually given in the highest number of sessions (fraction of radiation) with a relatively low dose of 1.8-2 Gy, resulting in 37-45 sessions, 5 sessions a week, depending on the patient's case. Hypofraction, lower fractions of radiation, with increased doses of radiation of 2.5-3 Gy are being used more frequently as it has proved to be more favorable.

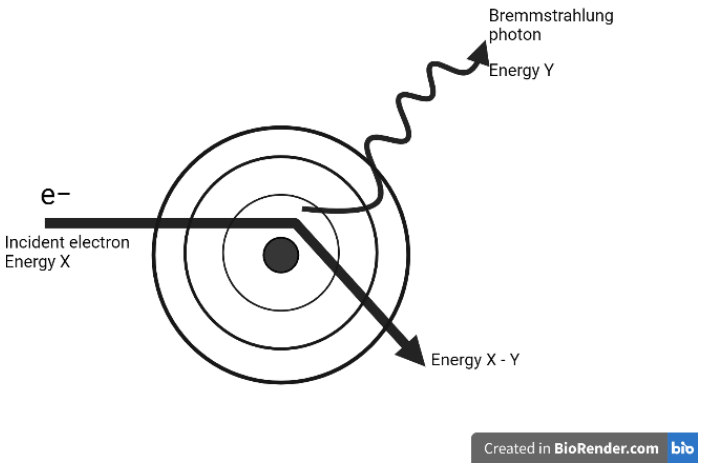

Figure 2. The bremsstrahlung effect. High energy electron deflected in the electric field of an atomic nucleus produces bremsstrahlung radiation.

2. Interaction between X-ray and biological matter

There is a direct and indirect (bystander) effect of radiation on cells and their DNA (figure 8).

- Direct effect – Electrons resulting from the radiation act directly on cellular molecules and cause DNA damage.

- Indirect effect – Radiation also leads to the ionization or excitation of water components and produces free radicals. These free radicals, such as hydrogen (H+) and hydroxyl (OH-) ions, hit DNA causing damage. This mechanism is responsible for 70% of radio-induced damage (39, 40).

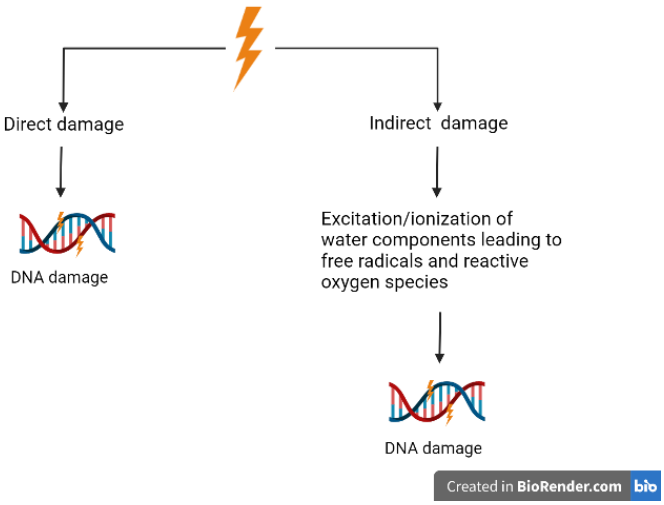

Figure 3. Illustration of the direct and indirect effects of ionizing radiation. Radiation energy may be deposited directly in the DNA or surrounding species ultimately leading to DNA damage.

3. Types of DNA damage

There are several types of DNA lesions induced by low-LET IR interactions. The most prominent damages include chemical lesions, single strand break (SSB), and double-strand break (DSB) (41).

- Chemical lesions -This is the most common type of DNA lesions and is also known as base damage.
- Single strand break (SSB) – IR may induce cleavage in one strand of the DNA double helix resulting in the loss of a single nucleotide and damaged 5’ and/or 3’ termini (42).

- Double strand break (DSB) – They are formed when two SSBs occur on opposite strands of the DNA double helix resulting in 3’ and 5’ overhangs. It is considered the most lethal of all three DNA damage and is the main lesion responsible for IR-induced cell death (43-45).

In comparison to one another, per cell per Gy, IR induces about 450 purine and 850 pyrimidine lesions, 1000 SSB, and 20-40 DSB with low LET radiation (46).

4. DNA repair after IR

Cellular homeostasis is dependent on DNA damage response (DDR) machinery to rectify lesions, mainly DSB, induced by IR. If unrepaired they may lead to cell death, and if misrepaired they may lead to chromosomal translocation and genomic instability. Tumor resistance and sensitivity to IR are reflected by the cells’ ability to repair the damage induced or not (47). In general, a cell’s resistance to IR is controlled by DNA repair, recombination, and replication. However, IR also affects normal, healthy surrounding cells contributing to treatment-related toxicity (48).

The two principal ways in which IR-induced DSBs are repaired are non-homologous end joining (NHEJ) and conservative homologous recombination (HR) (figure 9). HR takes place only in the S/G2 phase and is slow. The homolog of the damaged DNA guides the repair and therefore there is no lost genetic information. NHEJ, on the other hand, may occur in any phase of the cell cycle but is dominant in G0/G1, and is generally error-prone. This is because the DNA 3’ and 5’ overhangs at the DSB are cut or modified, and then ligated regardless of the homology. This results in deletions, insertions, mutations or genomic rearrangements (49). Of these, NHEJ is the major repair pathway as solid tumor cells are predominantly in the G0/G1 phase (44, 50).

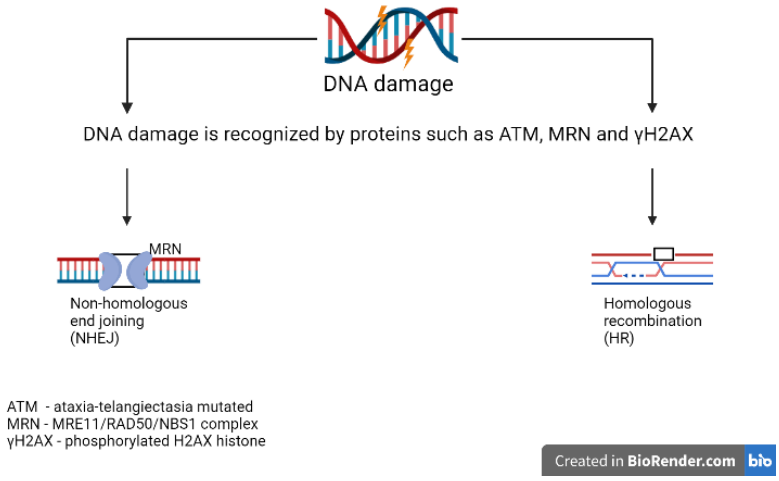

Figure 4. Illustration of the two main repair mechanisms after IR-induced DNA damage. NHEJ is dominant in G0/G1 of the cycle while HR is dominant in the S/G2 phase of the cycle.

c. NHEJ and the role of ATM and γH2AX

Double-strand breaks in DNA result in the activation of signal transduction pathways to ensure repair. This includes phosphatidylinositol-3-kinase-like kinase (PIKK)-dependent signaling pathways involving the proteins ataxia-telangiectasia mutated (ATM), ATM and Rad3 related (ATR), and DNA-dependent protein kinase (DNA-PK). These transducer proteins relay and amplify the damage signal, and are crucial in checkpoint signaling and DNA repair. In NHEJ, ATM of the PIKK family of proteins is involved and has several downstream targets that mediate DNA repair, senescence, and apoptosis. In response to IR-induced DNA damage, the cytoplasmic inactive ATM dimer monomerizes, auto-phosphorylates on Ser1981, and translocates into the nucleus by a process known as nucleo-shuttling (51, 52). ATM is recruited by an evolutionarily conserved protein complex that binds to the DSB ends, Mre11-Rad50-Nbs1 (MRN), and in turn recruits enzymes and exonucleases to cleave the overhangs and facilitate end-joining, forming single-stranded DNA (ssDNA) in a process called resection (53, 54). pATM then phosphorylates the histone variant H2AX on Ser139,

forming γH2AX (figure 10). γH2AX is responsible for the recruitment of several DNA repair proteins and chromatin-remodeling complexes resulting in the repair of the DSB (41, 51, 55). From this, it can be concluded that the localization of pATM and γH2AX is associated with IR-induced DDR, and the lack of it is associated with radiosensitivity (56).

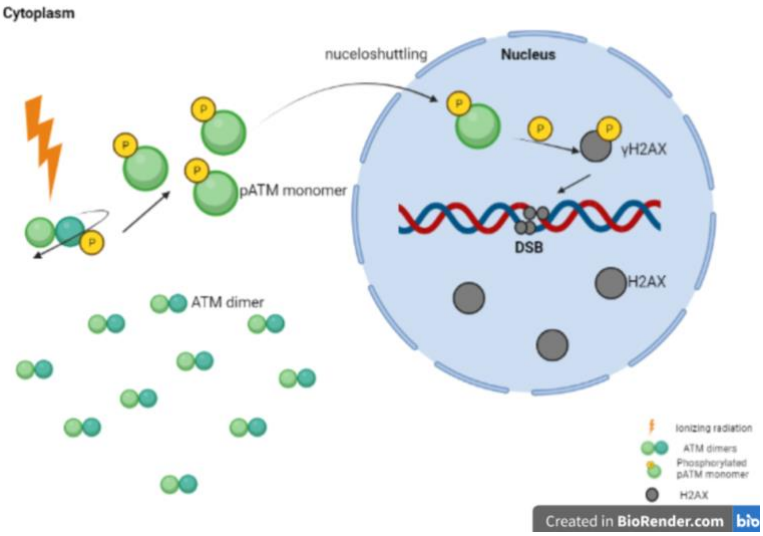

Figure 5. A simplified representation of the role of ATM and H2AX in the NHEJ repair. Upon IR, dimeric ATM autophosphorylates and monomerizes forming pATM. pATM relocates into the nucleus and phosphorylates histone variant H2AX into γH2AX.

d. Radio-induced cell death

Another role of ATM is the activation and accumulation of p53 protein due to IR-induced DNA damage. P53 is a tumor suppressor that is known to be a key player in inhibiting cell cycle progress, Induce apoptosis, and senescence (57). In a case in which DNA damage repair (DDR) systems fail, apoptosis, senescence, or mitotic catastrophe are initiated.

- Apoptosis – a common IR-induced cell death mechanism. It is a process of programmed cell death initiated by DNA damage. It is characterized by pyknosis

(condensation of chromatin), cell shrinkage, and inter-nucleosomal breakage of chromatin.

- Senescence – a state of permanent cycle arrest. According to studies, PCa cells entered a state of cytostatic arrest which caused tumor regression following RT (58-60).
- Mitotic catastrophe – the main IR-induced cell death in solid tumors. A result of the delayed or aberrant entry of the cell into mitosis, usually due to the inactivation or mutation of p53 (61).

These three modes of IR-induced cell deaths all fall below one main type of cell death, which is clonogenic cell death; this prevents the cells' replicative ability (62).

D. Radiosensitivity and radioprotection

There are several mechanisms by which tumor cells adapt to radiation therapy, hence leading to clinical treatment failure. One way in which this is achieved is by alterations in the expression of oncogenes, tumor suppressor genes, and tumor microenvironment ultimately favoring the resistance of cancer cells to therapy (63, 64). Another notable mechanism is the hyperactivation of DNA repair pathways. In PCa radiation-resistant cell lines (when compared to normal prostate cancer cell lines PrEC and PrSC) such as PC-3, DU-145, and LNCaP, radioresistance (RR) is mainly mediated through the NHEJ DNA repair mechanism, as these cells show cell cycle arrest in G0/G1 phase (65-67).

Therefore, it can be deduced that one way to increase the radiosensitivity of cancer cells would be by reducing the efficiency of targeted DNA repair, mainly those involved in the NHEJ pathway. The nucleoshuttling of ATM can predict and describe

radiosensitivity (51, 52). Thus, targeting and/or inhibiting the activity of ATM and γH2AX in cancer cells may make the cells more sensitive to radiation therapy. The importance of pATM is reflected in a quantitative study showing that decreased pATM activity and delayed nucleoshuttling are correlated with radiosensitivity (52). ATM and H2AX in IR-induced DDR are quantifiable by immunofluorescence. It is an immunohistochemistry technique that relies on fluorophores to visualize antigens, such as the proteins of interest (68). Cells that are defective in G1/S checkpoint arrest, especially in the signaling pathway involving the ATM protein, may have enhanced sensitivity (41). There are several diseases, such as Progeroid syndromes, Bruton's disease, neurofibromatosis, Huntington's disease, or Usher's syndrome, associated with radiosensitivity without hard evidence that there are mutations in their DSB repair and signaling proteins. However, a mutated cytoplasmic protein is found in these diseases which sequesters ATM and thereby inhibits its activity by delaying its nucleoshuttling (51). According to another study, there are three groups of human radiosensitivity. The scale depends on the availability of ATM. The highest sensitivity is when ATM is mutated, followed by when it is sequestered in the cytoplasm. This reinforces the idea that when ATM nucleoshuttling is inhibited or delayed, the more radiosensitized the cells will be (55).

There have been several agents aimed at radiosensitizing cancer cells, their effectiveness was limited as they also radiosensitized normal cells and caused more pathologies induced by RT (69). Clinical and molecular studies show that zoledronic acid and pravastatin individually radioprotect normal tissues and are suggested to radiosensitize cancer cells (70-72). There is recent evidence suggesting the combination of two drugs, Zoledronic acid (Zo) and pravastatin (Pra) or ZoPra, is involved in the

radioprotection of normal cells found in diseases such as Huntington's disease, progeria, neurofibromatosis 1 and tuberous sclerosis (73-76). Thus, these drugs have been implicated in both radioprotection and radiosensitization. These drugs in combination have been previously reported to have synergistic effects without any lapping toxicities, as well as being tolerated without any major side effects *in vitro* and *in vivo* assays (77, 78).

5. Zoledronic acid

Zoledronic acid (ZO) is a third-generation nitrogen-containing bisphosphonate. It targets osteoclasts in the bone to inhibit bone resorption in patients with osteoporosis, Paget disease of bone, metastatic disease to the bone, and multiple myeloma and is also used in the treatment of hypercalcemia of malignancy (79, 80). Bisphosphonates are prescribed in clinical practice to patients with malignancy as 90% of patients with metastatic disease develop bone lesions. Bone cancer metastasis is accompanied by hypercalcemia, severe bone pain, and skeletal destruction (81). Several studies support the anti-tumor and synergistic effects of Zo. It has been shown to reduce skeletal bone-related risks by 11% in 2 years, when compared with placebo, in men with hormone-refractory PCa (82, 83). In addition, in a randomized controlled trial of Zo, it was shown to be useful in reducing gonadotropin-releasing hormone agonist-induced bone loss in PCa, such as in those undergoing ADT (84). Its anti-tumor effect is evident in increasing the effectiveness of chemotherapeutic drugs, paclitaxel or tamoxifen in breast cancer when used in conjunction (85).

In a study assessing the effects of Zo on radioresistant osteosarcoma (OS) cells undergoing RT, it was concluded that Zo radiosensitized the cells. This claim was

supported by the decrease in radiation dosage required to kill 90% of the cells. This was facilitated by the increase in DNA damage which was achieved by counting the γH2AX foci, decreased DNA damage repair protein expression of both HR and NHEJ, and increased level of ROS when compared to the cells that underwent RT alone. The DNA repair proteins that were significantly down-regulated in this study were Rad52, DNA-PKs and specifically ATR and ATM were moderately down-regulated. Zo was also noted to affect the EMT markers and thereby affect migration and metastasis of the OS cells (72). Zo was shown to have synergistic cytotoxic events when coupled with RT in a study where two cell lines of PCa and myeloma cancer were treated with RT and Zo (86).

6. Pravastatin

Pravastatin (Pra; a statin) is a competitive hydroxymethylglutaryl coenzyme-A (HMG Co-A) reductase inhibitor. It is used to treat and manage high lipid levels such as in primary hypercholesterolemia, hyperlipidemia, and mixed dyslipidemia (87). Statins also show anti-tumor features. In retrospective studies with patients undergoing chemoradiotherapy and taking statins, patients with bladder cancer showed improved local control (88) and patients with rectal cancer showed improved pathologic complete response (89).

There are also several preclinical and clinical studies that show the radioprotective effect statins have on normal cells and their anti-inflammatory effect post-RT (90-100). Haydont *et al* reported that pravastatin reduced late side effects, specifically intestinal fibrosis when administered post-RT. And they also reported that the drug did not reduce the effect of RT on the tumor cells (93, 94). However, Mahmoudi *et al* and Nübel *et al* reported a statin, specifically Lovastatin, accelerated DNA repair by accelerating ATM

phosphorylation in atherosclerotic rabbits and radioprotected endothelial cells by inhibiting IR-induced DNA damage-dependent stress responses, respectively (96, 97). In a study by Hiroshi *et al,* in which pravastatin was administered to mice prior to receiving RT, the drug was shown to reduce IR-induced cell death, the induction of DSB, and the expression of both ATM and γH2AX in RT-treated intestine cells (101). Therefore, pravastatin was shown to provide radioprotection to normal cells while having no negative effects on the response of RT on tumor cells. Aside from having the radioprotective and accelerating ATM nucleoshuttling in normal cell properties, statins are shown to decrease the radioresistance of ej*ras*-transformed human osteosarcoma cells (102). According to Fritz *et al*, HeLa cells treated with lovastatin prior to IR therapy were radiosensitized by increasing IR-induced death (103).

7. Effect of ZoPra on IR-induced damage repair: ATM and H2AX

a. Radioprotection of non-cancerous cells

The studies on ZO and PRA individually and in combination suggest they might have a radiosensitizing role in cancer cells while having a radioprotecting role on normal cells. Combemale *et al* and Ferlazzo *et al* have conducted studies on the effect of a combination of those drugs on radiation-induced ATM nucleoshuttling (RIANS) in Neurofibromatosis type 1 (NF1), Tuberous sclerosis, and Huntington's Disease (75, 76, 104). In NF1, a mutated cytoplasmic neurofibromin protein leads to the formation of benign and malignant tumors. IR treated quiescent NF1 fibroblast cell lines showed a decrease in γH2AX foci meaning a decrease in DSB recognition and a decrease in the nucleoshuttling of pATM. It is associated with radiosensitivity and radio-susceptibility as neurofibromin mutated protein might bind to and sequester ATM proteins delaying

RIANS (figure 11). However, the additional treatment of the cells using ZoPra prior to IR showed that RIANS was accelerated, thereby increasing the recognized DSB repair. This shows the radioprotective effect of ZoPra (75). Another study achieved the same outcome with quiescent tuberous sclerosis fibroblast cell lines. In this case, radiosensitivity is due to mutated hamartin or tuberin proteins and sequestration of ATM due to TS2. And the treatment using ZoPra leads to the increased RIANS (76). This was also achieved in Huntington's Disease, whose radiosensitivity is due to mutated huntingtin protein sequestering pATM in the nucleus (104). This means that ZoPra led to the radioprotection of these fibroblasts. The suggested action of this drug combination is through the inhibition of farnelysation and geranylgeranylation thereby leading to an increased ATM

nucleoshuttling (73).

b. Radiosensitization of cancer cells

In an *in vitro* study by our group on two breast cancer cell lines, it was presented that pretreatment using ZoPra radiosensitized the cell lines. This was supported as there was a decrease in breast cancer cell survival and an increase in the residual number of γH2AX foci. In one of the cell lines in particular, the activity of pATM was shown to be reduced as well (105).

Table 2. A summary of the results of studies done on the radio-response of ZoPra.

Effect of ZoPra on:	Neurofibromatosis 1	Tuberous sclerosis	Huntington's disease	Breast cancer
pATM nucleoshuttling	Accelerated	Accelerated	Accelerated	Delayed
γH2AX foci	Increased	Increased	Not tested	Decreased
Radioprotecting/ Radiosensitizing?	Radioprotecting	Radioprotecting	Radioprotecting	Radiosensitizing
Suggested mechanism	Nuclear membrane farnelysation	Nuclear membrane farnelysation	Nuclear membrane farnelysation	Unknown

E. Aims of the study

Using Zo and Pra can be an example of repurposing FDA-approved drugs in order to widen the therapeutic window of RT.

Henceforth, the aim of this thesis project is to assess the effect of Zoledronic acid and Pravastatin, alone and in combination, with a 2 Gy irradiation, on the radio-response of human prostate cancer cell lines DU-145 and PC3 *in vitro*.

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

1. DU-145 and PC-3: Human Prostate Cancer Cell Lines

Two prostate cancer cell lines, DU-145 and PC-3, were used and purchased from the American Tissue Culture (ATCC, USA). They are both epithelial cell lines and are extracted from metastatic sites of the brain and bone respectively *(ATCC)*.

2. Cell Growth

DU-145 and PC-3 cells were cultured and maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS-Sigma-Aldrich), 1% Penicillin- Streptomycin (Sigma-Aldrich), 0.2% Plasmocin-Prophylactic (InvivoGen), 1% non-essential amino acids (Sigma, USA), and 1% sodium pyruvate (Sigma, USA). Cells were incubated at 37°C, 5% CO_2 humidified incubator.

3. Treatment with Zoledronic acid, Pravastatin, and ZoPra

Zoledronic Acid (ZO) (Sigma-Aldrich SML 0223 cat.no 0000020794) and Pravastatin (PRA) (Sigma-Aldrich P4498 cat.no 0000027976) were solubilized with phosphate-buffered saline (PBS) at a concentration of 10mM and 8.96mM for ZO and PRA, respectively, and were stored at -20°C for long-term storage. In each experiment, cells were incubated with PRA for 24 hours, and or ZO for 12 hours, followed or not by a 2 Gy irradiation.

B. Ionizing radiation

Cells were irradiated with or without prior treatment with ZO and PRA, alone or in combination. A 225KV Precision X-Ray (PXi) irradiator model No. X-RAD 225 was used at 2Gy.min-1 with a 1.5 mm Aluminium filter.

C. MTT Cell Proliferation Assay

The effect of Zo and Pra, alone and in combination, with and without a 2 Gy irradiation, on the viability of both DU-145 and PC3, was investigated using the MTT ([3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay. Both cell lines were plated in triplicates in 100 µl complete media in 96-well culture plates, at a density of $3x10^3$ cells per well. Cells were incubated overnight, then treated with PBS (as the vehicle) and various indicated concentrations of Zo alone, Pra alone, or the combination of both drugs diluted in complete media for 24, 48, and 72 hours. For each time point, the media containing treatment was removed, fresh complete media was added, and 10µL of 5mg/mL MTT reagent (dissolved in 1X PBS) was added to each well and incubated at 37°C for 3 hours. In this step, metabolically active/viable cells have the ability to convert the yellow tetrazolium salt (MTT) into insoluble purple formazan crystals due to the high levels of NADH and NADPH, which is a measure of mitochondrial metabolic activity. Afterward, the reagent was removed and 100µL of solubilizing solution (Isopropanol) was added to solubilize the formed crystals. The plate was covered by foil and incubated for 1 hour at room temperature. Finally, the reduced MTT optical density was measured at a wavelength of 595nm using an ELISA reader (Multiskan EX). The percentage of cell proliferation is expressed as percentage growth relative to control wells. The blank well

was used for the baseline zero. The data are derived from the mean of triplicate wells of three independent experiments.

D. Clonogenic Cell Survival Assay

DU-145 and PC-3 cells' capacity to form colonies after treatment with Zo and Pra, alone and in combination, with or without a 2 Gy irradiation was assessed using clonogenic assay. Cells were seeded in 12 well plates and treated with 1 μM Pra for 24 hours and/or 1 μM Zo for 12 hours when they reach 70% confluency. Cells were then subjected to a 2 Gy IR and then plated after 24 hours. A plating efficiency (PE) experiment, describing the surviving fraction of cells without prior treatment, was carried out to determine the optimal seeding density of each cell line, in 6 well plates.

$$P.E. = \frac{Number\ of\ colonies\ formed}{Number\ of\ cells\ plated}$$

PE was used to determine the optimum density of cells to use in the clonogenic assay. A delayed plating technique was implemented, where 24 hrs post-radiation, cells were trypsinized and counted using a hemocytometer. DU-145 cells were seeded at a density of 4000 cells per well while PC-3 cells were seeded at 3000 cells per well. After incubation for 6-8 days, cells were fixed with 95% ethanol, washed with PBS, and stained with cresyl violet (KODAK) for about 5 minutes then washed with distilled water. Stained colonies were counted (colony ≥ 50 cells) and the surviving fraction was calculated using the following formula:

$$SF = \frac{number\ of\ colonies\ counted}{number\ of\ cells\ seeded * (\frac{PE}{100})}$$

SF: surviving fraction

PE: plating efficiency

Each experiment was repeated at least 3 times.

E. Immunofluorescence

Anti-γH2AX and anti-pATM IF were performed to assess the effect of a 2 Gy irradiation with and without ZoPra on the DSB signaling and repair kinetics in both DU-145 and PC3 cell lines. Cells were seeded on 12 mm coverslips at the bottom of 24 well-plates. When reaching 70% confluency, cells were either left untreated (0 Gy or control), treated with PBS, 1 μM Pra for 24 hours, 1 μM Zo for 12 hours, or 1 μM ZoPra, followed by a 2 Gy irradiation. Treated cells were fixed with 4% Paraformaldehyde (PFA) 0 min, 10 min, 1 hr, 4 hrs, and 24 hrs post-irradiation. Post-fixation, cells were permeabilized with a mixture of 0.1% Triton-x 100 (Bio-Rad), 10% normal goat serum (NGS-Gibco), and 3% bovine serum albumin (BSA-Sigma-Aldrich) for 1 hr at room temperature. Cells were then incubated with anti-γH2AX (ser139) anti-mouse antibody (dilution 1:350, Millipore; cat # 05636), and with anti-pATM (ser1981) monoclonal anti-mouse antibody (dilution 1:80, Abcam cat #05740), for 1 hr at 37°C in an incubator, then washed twice with PBS. Next, cells were incubated with the secondary antibody Alexa Fluor 488 goat anti-mouse IgG (dilution 1:100, ab150113) for 30 min at 37°C and washed twice with PBS. Coverslips were then mounted using Fluoroshield Mounting Medium with 4',6'Diamidino-2- Phenyl-indole (DAPI) (Abcam; cat #ab104139).

DAPI counterstaining permitted the indirect evaluation of the yield of G1 cells (nuclei with homogeneous DAPI staining), G2 cells (nuclei with heterogeneous DAPI staining), and metaphase (visible chromosomes): nuclear foci were scored in G0/G1 phase cells only. Briefly, more than 50 nuclei were analyzed per experiment per post-

irradiation time and three independent replicates were performed. Images were taken with an Upright fluorescent microscope DM6 B (Wetzlar, Germany).

F. Micronuclei Assay

The DAPI counterstaining performed during immunofluorescence experiments permitted us to quantify the micronuclei caused by unrepaired chromosomal breaks *(36)*. For each condition, the percentage of cells with micronuclei was counted. Experiments we performed three times.

G. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 analysis software. The significance of the data was analyzed using one-way and two-way analysis of variance (ANOVA) and non-parametric t-tests where appropriate. P values of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) were considered significant.

CHAPTER III

RESULTS

A. The effect of Zoledronic acid and Pravastatin, alone and in combination, with and without a 2 Gy irradiation, on the cell proliferation of DU-145 and PC-3 cell lines using MTT assay.

The cytotoxic study was carried out to determine the optimal dose of Zo and Pra, on DU-145 and PC-3 cell lines, which will exert no significant cytotoxic effect. Cells were treated with increasing doses (1, 3, 9, and 18 μM) of each drug, separately. The results suggest that Zo exerts no significant cytotoxicity on either cell line (**Figure 6)**.

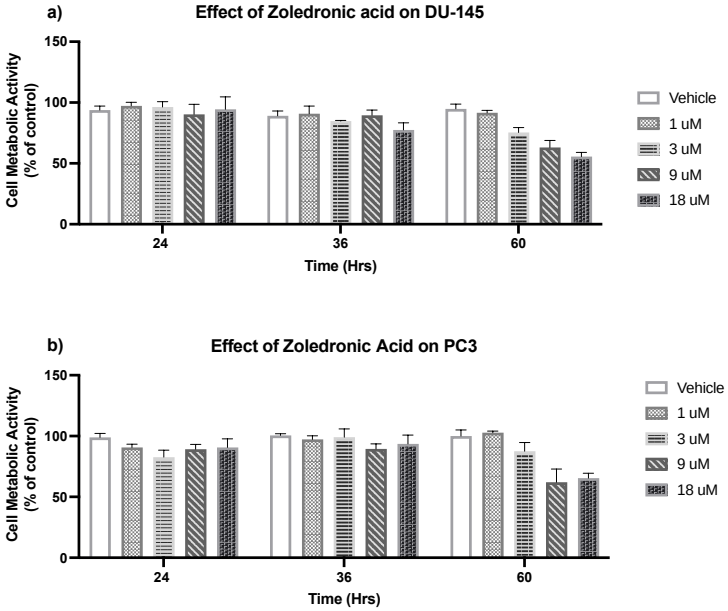

Figure 6. Effect of varying concentrations of Zoledronic acid, on DU-145 and PC3 cell proliferation. After incubation of DU-145 and PC-3 cells for 24, 48, and 72 h with or without treatment (Zo), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM).

Increasing doses of Pra had no significant impact on the cell proliferation of DU-145 and PC-3 cells. (**Figure 7**). Hence, the minimal effective dose of 1 μM Zo and 1 μM Pra was used to carry out further optimization experiments.

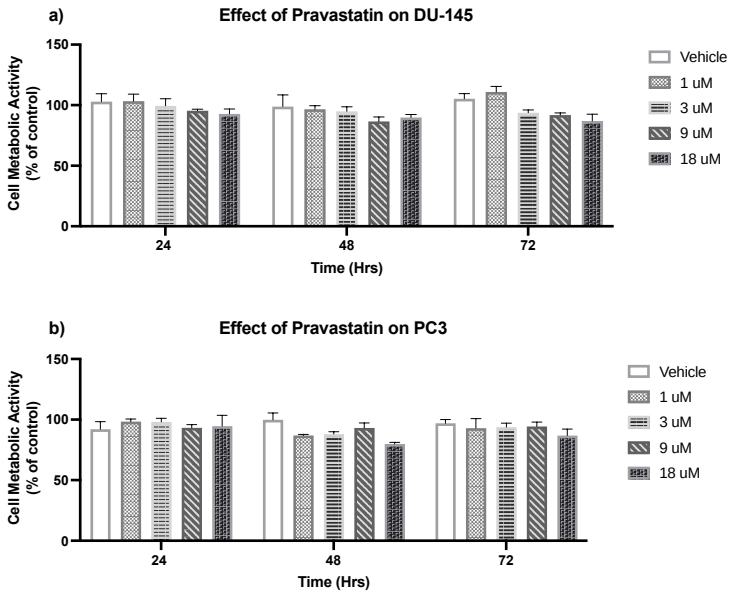

Figure 7. Effect of varying concentrations of Zoledronic acid, on DU-145 and PC3 cell proliferation. After incubation of DU-145 and PC-3 cells for 24, 48, and 72 h with or without treatment (Zo), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM)

The cytotoxicity of the combination of 1 μM Zo and 1 μM Pra (1 μM ZoPra) was then assessed. The results depict a reduction in cell proliferation in DU-145 cells by the vehicle (PBS) 12 hours post-treatment (p=0.0421) and by 1 μM ZoPra 48 hours post-treatment (p=0.0284). A reduction in PC-3 cell proliferation is seen 72 hours post-treatment by both the vehicle and 1 μM ZoPra (p= 0.05 and 0.454 respectively) (**Figure 8**).

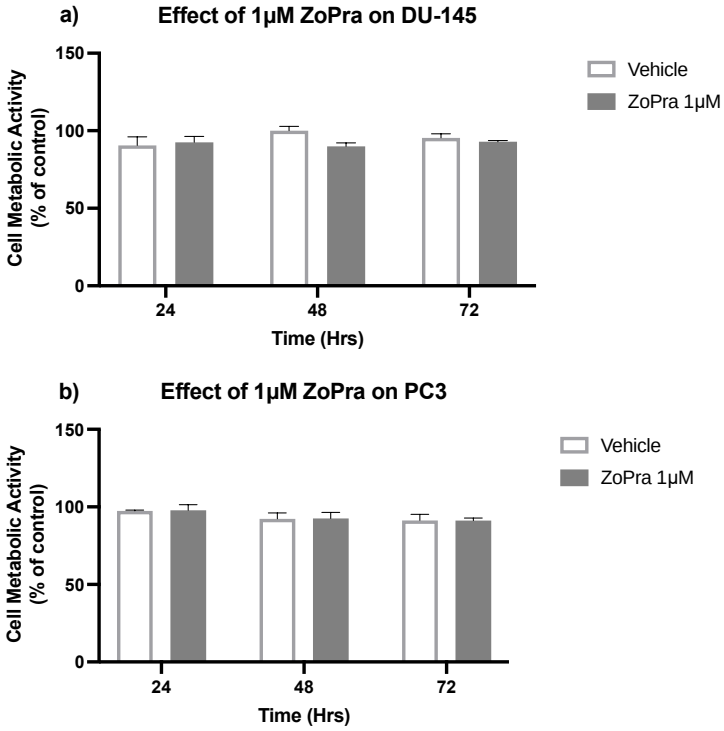

Figure 8. Effect of 1 μM Zo and 1 μM Pra on DU-145 and PC3 cell proliferation. After incubation of DU-145 and PC-3 cells for 24, 48, and 72 h with or without treatment (Pra), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM).

Following, the combinatorial effect of 1 μM Zo, 1 μM Pra, and 1 μM ZoPra with a 2 Gy irradiation to assess their effect on cell proliferation of DU-145 and PC-3 cells. DU-145 cell proliferation was reduced 24 hours post-treatment with 1 μM ZoPra + 2 Gy ($p=0.0023$) and 72 hours post-treatment with 1 1 μM Zo, 1 μM Pra and 1 μM ZoPra + 2 Gy ($p=0.0021$, 0.0081, and 0.0042 respectively). PC-3 cell proliferation was not significantly affected at any time point (**Figure 9**).

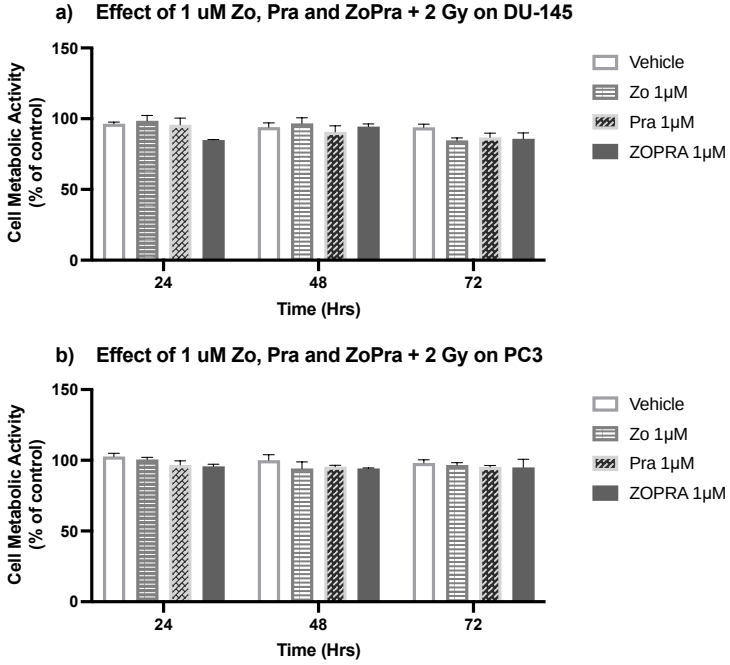

Figure 9. Effect of 1 μM Zo, 1 μM Pra, and 1 μM ZoPra followed by a 2 Gy irradiation on DU-145 and PC3 cell proliferation. After incubation of DU-145 and PC-3 cells for 24, 48, and 72 h with or without treatment followed by a 2 Gy irradiation, cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM).

B. Zoledronic acid and Pravastatin sensitize DU-145 and PC-3 human prostate cancer cells to radiation and reduce the Surviving Fraction (SF)

The clonogenic assay was implemented to determine cell reproductive survival after a 2 Gy irradiation alone, and with pre-treatment using 1 μM Zo, 1 μM Pra, and 1 μM ZoPra. IR alone reduced the SF of DU-145 cells by approximately 57% ± 9.9 colonies (p= 0.0004). When comparing experimental groups (1 μM Zo, Pra, and ZoPra) to 2 Gy alone, 1 μM ZoPra + 2 Gy induced a significantly lower SF (17.8 ± 3.3) than 2 Gy alone (42.8 ± 8.8) (p=0.0448), a reduction of 58.4% in SF (**Figure 10).**

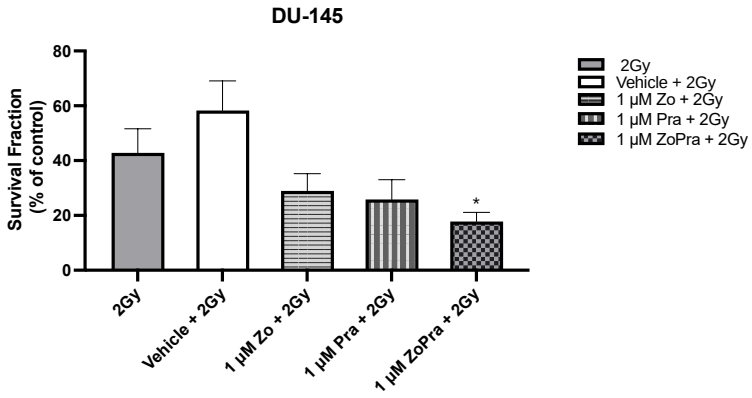

Figure 10. IR and 1 μM ZoPra reduce the survival fraction of DU-145 cells. Cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 μM Zo, Pra, and ZoPra. 24 hours post-IR, cells were seeded with the predetermined density derived from the plating efficiency experiment and incubated for 6-8 days. Cells were then stained and counted for colonies formed by >50 cells. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM) (*P<0.05).

In PC-3 cell lines as well, IR reduced the CFA by approximately 59% ± 9.4 colonies (p= 0.0002). When comparing experimental groups (1 μM Zo, Pra, and ZoPra) to 2 Gy alone, 1 μM ZoPra + 2 Gy induced a significantly lower SF (18.1 ± 4.6) than 2 Gy alone (40.6 ± 6.4) (p=0.0262), a reduction of 55.4% in SF (**Figure 11**).

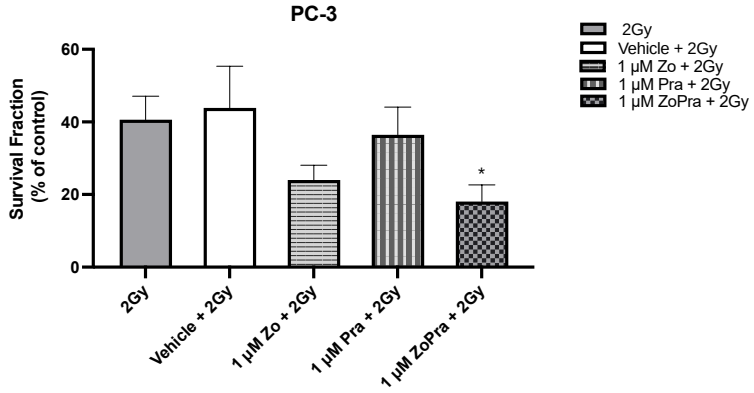

Figure 11. IR and 1 μM ZoPra reduce the survival fraction of PC-3 cells. Cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 μM Zo, Pra, and ZoPra. 24 hours post-IR, cells were seeded with the predetermined density derived from the plating efficiency experiment and incubated for 6-8 days. Cells were then stained and counted for colonies formed by >50 cells. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM) (*P<0.05).

C. Effect of 1 μM ZoPra on residual pATM foci in DU-145 and PC-3 cell lines.

To assess the effect of ZoPra on the pATM kinetics in PCa cell lines, immunofluorescence using anti-pATM was employed. A similar trend was seen in both cell lines. A low number of spontaneous foci (<4), peak at 10 minutes with 36 ± 1.4 and 55 ± 8.6 foci, resolving to 6.6 ± 1.4 and 6 ± 1.1 foci 24 hrs post-IR in both untreated and treated groups with 1 μM ZoPra respectively. A similar trend was observed in PC-3 cells. A low number of spontaneous foci (<3), peak at 10 minutes with 31.6 ± 1.6 and 1 hour with 47.5 ± 10 foci, resolving to 5.6 ± 0.3 and 5.5 ± 0.8 foci 24 hrs post-IR in both untreated and treated groups with 1 μM ZoPra respectively (**Figure 12 a and c)**. There was no significant effect in pATM dynamics 10 mins post-IR in groups pre-treated with 1 μM ZoPra compared to those that were not (**Figures 12 b and d)**.

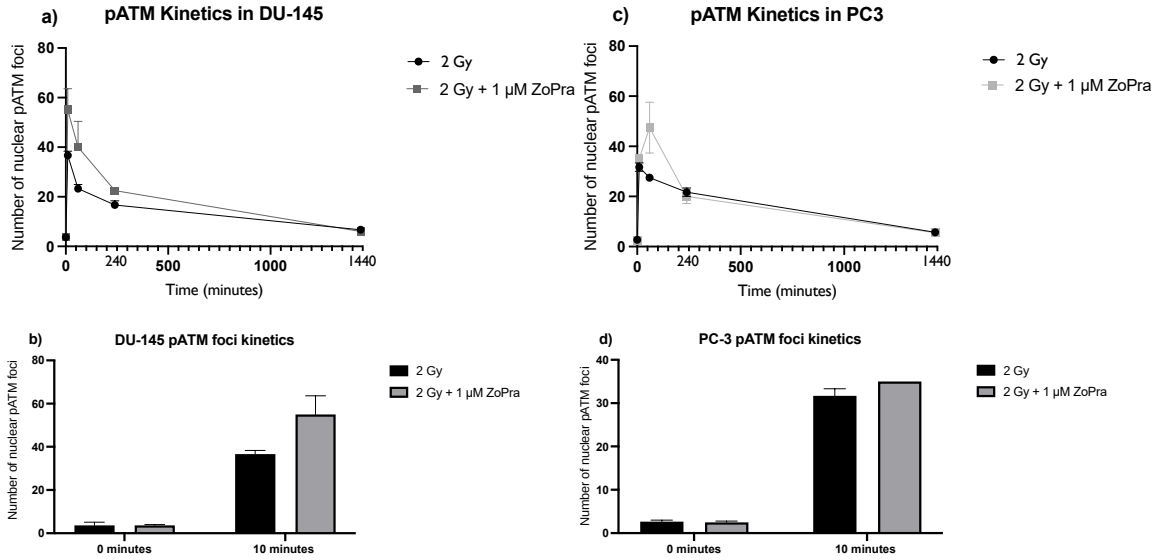

Figure 12. Effect of IR and 1 μM ZoPra on pATM kinetics in DU-145 and PC-3cell lines. DU-145 (a and b) and PC-3 (c and d) cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 μM ZoPra. Cells were fixed 0, 10-, 60-, 240- and 1440-minutes post-IR and stained with anti- pATM to visualize protein kinetics. 30 nuclei were analyzed and pATM foci were counted and plotted. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM).

D. Effect of 1 μM ZoPra on residual γH2AX foci in DU-145 and PC-3 cell lines.

To assess the effect of ZoPra on the γH2AX kinetics in PCa cell lines, anti-γH2AX immunofluorescence was employed. A similar trend was seen in both cell lines. DU-145 γH2AX kinetics show a low number of spontaneous foci 4.2 ± 0.6 and 2.9 ± 0.7 without IR, which peaks 10 mins post radiation with 72.5 ± 1.4 and 75 ± 2.9 foci, and declines to 11.3 ± 0.7 and 17.3 ± 1.4 foci 24 hrs post-IR in 1 μM ZoPra pre-treated and untreated groups respectively. DU-145 cells pre-treated with 1 μM ZoPra show a significant increase in residual γH2AX foci 24 hrs (1440 mins) post-IR when compared with those only treated with IR (p=0.0097) (**Figure 13 a and b)**. PC-3 γH2AX kinetics show a low number of spontaneous foci 2.5 ± 0.4 and 2.7 ± 1.8 without IR, which peaks 1 hr post radiation with 72.5 ± 1.4 and 10-mins post-IR with 73 ± 3.3 foci, and declines to 7.1 ± 1.3 and 12.7 ± 0.9 foci 24 hrs post-IR in 1 μM ZoPra pre-treated and untreated groups respectively. There significant increase in residual γH2AX foci 24 hrs (1440 mins) post-IR (12.7 ± 0.9) when compared with those only treated with 2 Gy (7.1 ± 1.3) (p=0.0070) (**Figure 13 c and d)**.

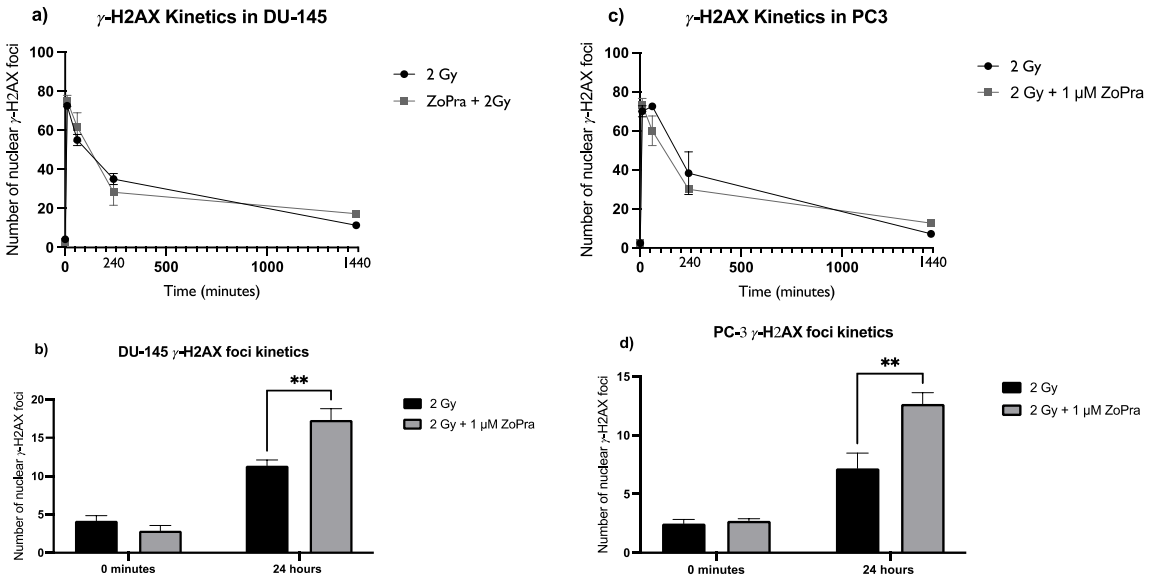

Figure 13. Effect of IR and 1 μM ZoPra on γH2AX kinetics in DU-145 and PC-3 cell lines. DU-145 (a and b) and PC-3 (c and d) cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 μM ZoPra. Cells were fixed 0, 10-, 60-, 240- and 1440-minutes post-IR and stained with anti- γH2AX to visualize protein kinetics. 30 nuclei were analyzed and γH2AX foci were counted and plotted. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM) (*P<0.05; ** P<0.01).

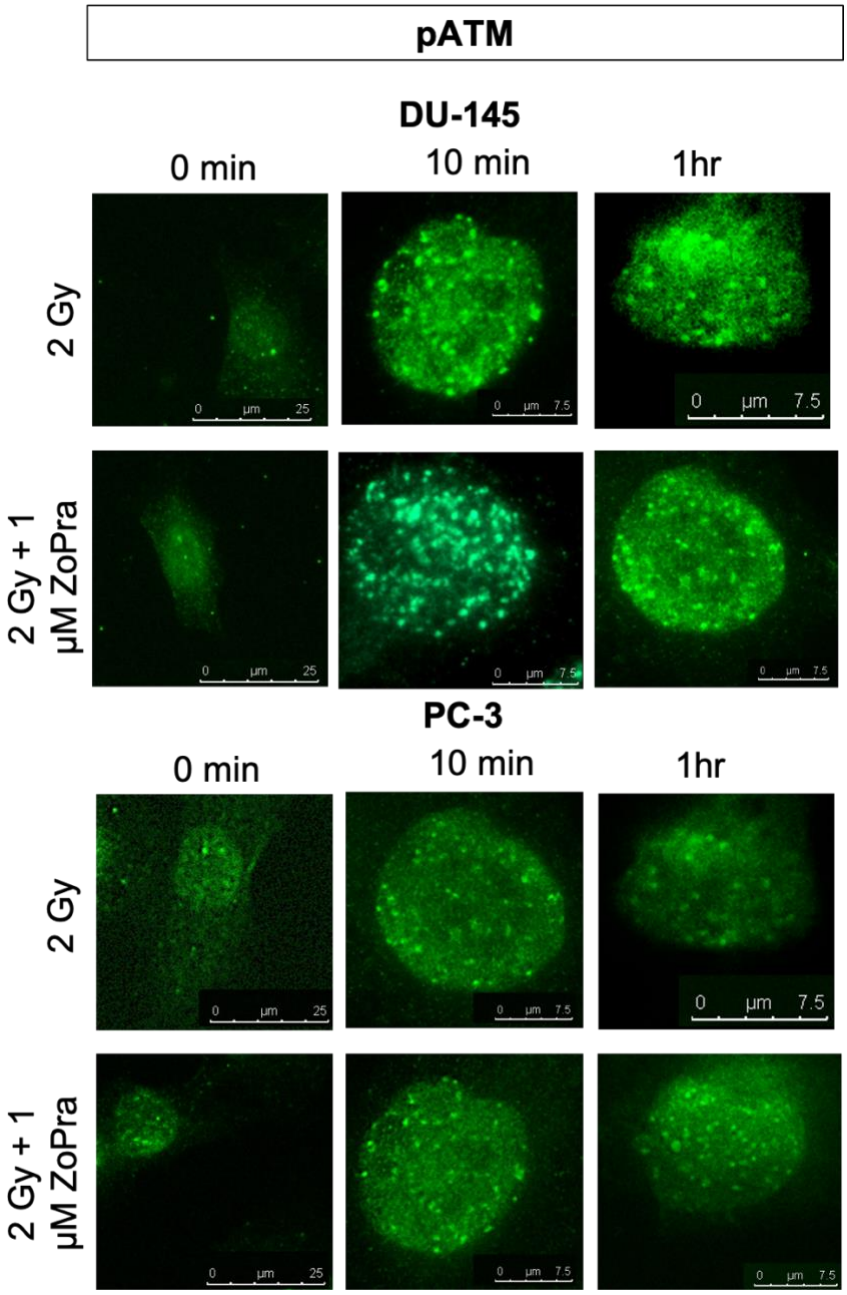

Figure 14. IF images of pATM foci in DU-145 and PC-3 cells. Cells were either treated with a 2 Gy IR alone or pretreated with 1 µM ZoPra. They were then fixed on 12mm coverslips 0-, 10- or 1440-minutes post-IR. Cells were then stained with anti-pATM monoclonal anti-mouse antibody followed by secondary antibody Alexa Fluor 488 goat anti-mouse IgG.

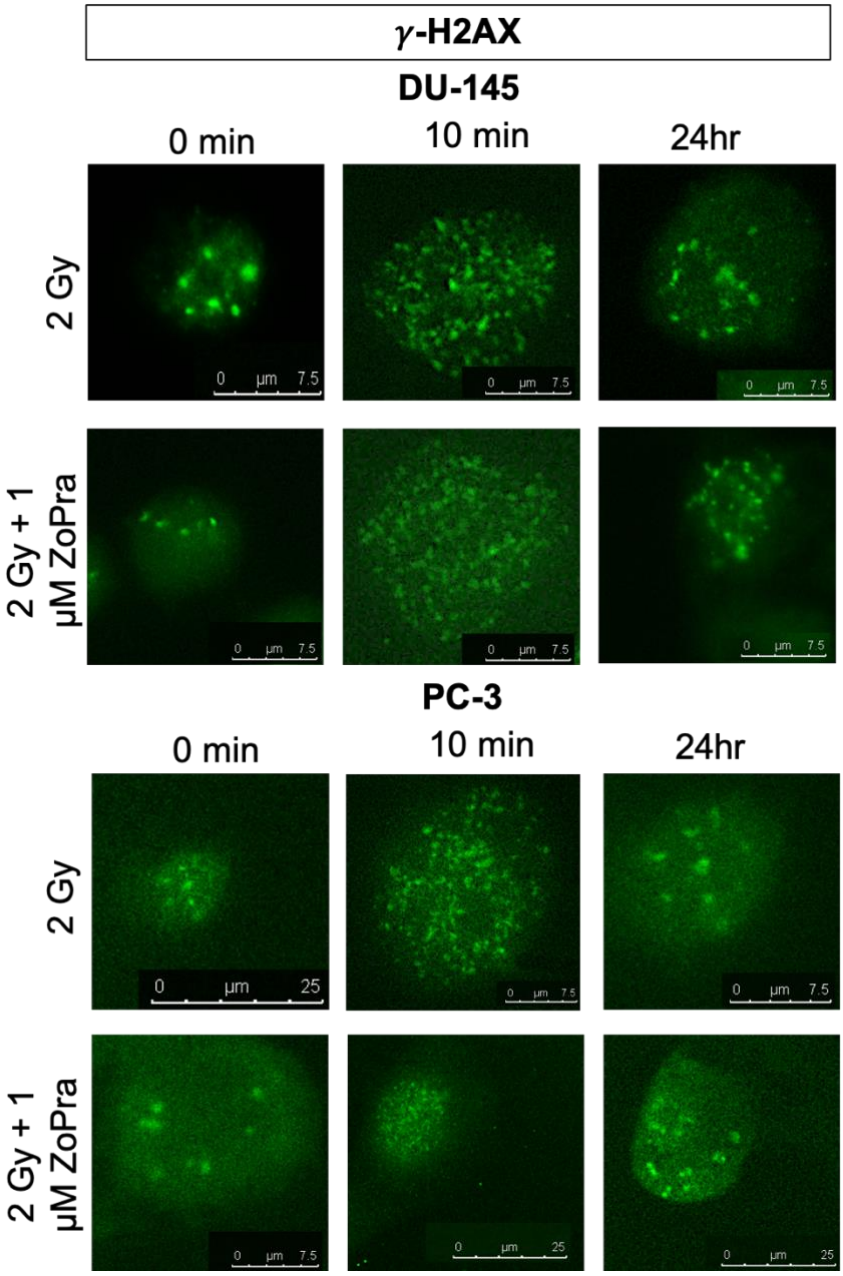

Figure 15. IF images of γH2AX foci in DU-145 and PC-3 cells. Cells were either treated with a 2 Gy IR alone or pretreated with 1 μM ZoPra. They were then fixed on 12mm coverslips 0-, 10- or 1440-minutes post-IR. Cells were then stained with anti-γH2AX monoclonal anti-mouse antibody followed by secondary antibody Alexa Fluor 488 goat anti-mouse IgG.

E. Effect of 1 μM ZoPra on the percentage of radio-induced micronuclei (MN%)

This experiment was used to quantify the percentage of cells with chromosomal aberrations discarded from the nucleus with each condition. Cells were treated with a 2 Gy irradiation, with and without a pre-treatment with 1 μM ZoPra. In DU-145 cells (Figure 14a) ZoPra did not induce any significant changes in MN% when comparing those pre-treated with ZoPra and those that were not. However, in PC-3 cells, there was a significant increase of 2.7% ± 0.78 (p=0.183) in MN% in those pre-treated with 1 μM ZoPra (6.3%) when compared to those that were not (3.6%) (Figure 14b).

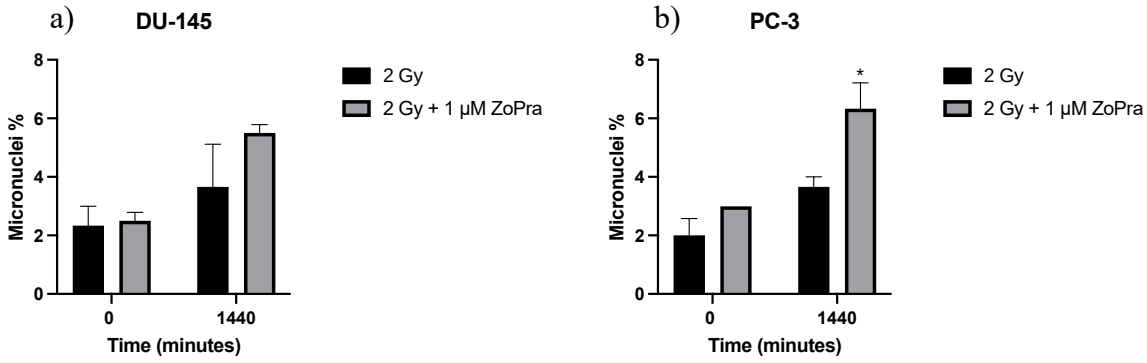

Figure 16. Effect of IR and 1 μM ZoPra on percentage of IR-induced micronuclei in DU-145 and PC-3 cell lines. DU-145 (a) and PC-3 (b) cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 μM ZoPra. DAPI counterstaining performed during immunofluorescence permitted the quantification of micronuclei. Percentage of cells with micronuclei was quantified for each condition. Each plot represents the mean of three independent experiments ± the Standard Error of the Mean (SEM) (*P<0.05).

CHAPTER III

DISCUSSION

On a cellular and molecular level, cell proliferation, cell survival, and DDR proteins' (such as pATM and γH2AX) kinetics can be used to describe cells' radioreponse. The radiosensitizing effect of 1 μM Zo, Pra, and ZoPra was tested on the two human prostate cancer cell lines DU-145 and PC-3. 1 μM ZoPra with IR was shown to reduce cell survival and residual γH2AX 24 hours post-IR in both DU-145 and PC-3 cell lines. The percentage of micronuclei was also shown to be significantly increased by 1 μM ZoPra with IR.

We tested the effect of 1 μM Zo, Pra, and ZoPra on the cell survival of each cell line using the clonogenic assay. Using this assay, cells are incubated with 1 μM Pra for 24 hours and/or 1 μM Zo for 12 hours, then treated with a 2 Gy irradiation 24 hours before plating. This describes the delayed plating technique which is known to be used in radiobiological research as it assesses the capacity of cells to repair IR-induced DNA damage (106). Cells are seeded at a relatively low density predetermined using the plating efficiency technique and allowed to grow for 6-8 days. Cells that retain the capacity to reproduce and form colonies of >50 cells, would have effectively repaired the DNA damage induced by IR. Those that do not will lose their ability to reproduce and hence IR would have resulted in their clonogenic death. Clonogenic death is the desired outcome of RT as when a tumor cell loses its capacity to reproduce it is no longer clonogenically viable (107). Although 1 μM Zo and Pra alone reduced the CFA of both cell lines, only a combination resulted in a significant reduction rendering them radiosensitized.

The main DDR mechanism activated in G0/G1 cells post-IR is the NHEJ pathway. The key players involved in this pathway are the cytoplasmic signaling protein ATM,

which autophosphorylates immediately upon IR, and nucleoshuttles where it phosphorylates and activates histone H2AX forming γH2AX flanking DSB sites. This establishes the visualization of γH2AX foci by immunofluorescence a sensitive method for evaluating DSB formation and assessment of DNA repair kinetics (108-111). γH2AX and pATM foci formation and disappearance kinetics has been shown to predict cell and clinical radiosensitivity (51, 52, 105). Therefore, to further investigate the radio-response of DU-145 and PC-3 cells, cells were pre-treated with 1 μM ZoPra and fixed 0, 10 minutes, 1-, 4-, and 24 hours post-IR. They were stained with anti- γH2AX and anti-pATM to study their kinetics.

First, an IF experiment assessing pATM was conducted to assess the effect of ZoPra on the pATM signaling in DDR. Radiosensitivity of cells was shown to be deduced by a decrease in the maximal number of pATM foci (52). The kinetics of pATM is presented in figures 12a and 12c in DU-145 and PC-3 cells respectively. There was also no significant difference between ZoPra pre-treated and untreated groups in the number of pATM foci. This suggests that ZoPra did not affect the signaling of IR-induced DNA damage in both cell lines.

IF assay was employed to assess the kinetics of γH2AX in DU-145 and PC-3 cells treated with a 2 Gy irradiation with and without pre-treatment with 1 μM ZoPra. Each unrepaired DSB is represented by one focus and the fraction of residual γH2AX foci 24 hours post-IR is a measure of radiosensitivity and therefore was the focus. The results showed a higher number of unrepaired DSB (foci) in both cell lines suggesting that they have been sensitized to a 2 Gy IR. The incubation of DU-145 and PC-3 cells with 1 μM ZoPra alone did not affect the number of spontaneous residual foci and therefore can be

deduced that they do not affect DSB recognition and repair without IR (Figures 13 b and d).

Finally, the effect of the ZoPra combination on the percentage formation of irreversibly damaged chromosomal fragments expelled from the nucleus was assessed using DAPI counterstaining from the IF assay. Figure 15 represents the data gathered from this assay, and shows ZoPra alone in both cell lines did not affect the % of micronuclei. It also shows that 2 Gy + 1 μM ZoPra significantly increased micronuclei 24 hours post-IR. The suggested reason may be that DU-145 cells are considered more radioresistant than PC-3 cells and therefore more sensitive to chromosomal aberrations (112).

The elusive etiology of prostate cancer and the inability to control most of its risk factors emphasize the importance of effective therapy. Radiotherapy is a common therapy option in more than 50% of PCa patients. The efficacy of treatment depends on both tumor response and the radiosensitivity of the patient (105, 113, 114). The dosage required is dependent on the relationship between the lethal dose necessary to eradicate or halt tumor cell progression and tolerance of normal surrounding cells (115-118). There are several studies assessing the effect of bisphosphonates and statins on the radio-response of PCa cells, however, there have been no studies combining both drugs. Thus, assessing the effect of commercial FDA-approved drugs, Zoledronic acid, and Pravastatin, with the hope of repurposing them was highly convenient, especially since Zoledronic acid is prescribed to 90% PCa patients with metastatic disease (81-83). In a submitted article, our laboratory team tested the effect of a combination of Zoledronic acid and Pravastatin on breast cancer cells in vitro. ZoPra increased the radiosensitivity of breast cancer cell lines by impairing DDR capacity to IR (105). This directed us to

focus on NHEJ DDR repair proteins, γH2AX, and pATM, as they are key players in the activation of NHEJ, the main mode of DDR in G0/G1 cells (65).

The assays performed showed promise regarding the radiosensitizing effect of PCa cells using ZoPra. Nevertheless, the results need to be further confirmed and supported by repeating clonogenic and IF experiments. Different assays, such as Trypan blue and western blotting, could be implemented to assess the effect of ZoPra on cell viability and DDR protein quantification respectively. 3D culture models and human-derived PCa organoids, which better mimic the physiological environment, could be used in the future to assess the effect of ZoPra.

In conclusion, the combination of Zoledronic acid and Pravastatin combination has the potential to be a cost-effective radiosensitizing agent. The preliminary data gathered from cellular and molecular experimentation show its implication in reducing the colony forming ability and DSB recognition in both DU-145 and PC-3 cell lines. The repurposing of commercial FDA-approved drugs to widen the therapeutic window of IR by radiosensitizing tumor cells and improving QoL is highly encouraging and requires further experimentation.

REFERENCES

1. Hassanpour SH, Dehghani M. Review of cancer from perspective of molecular. Journal of Cancer Research and Practice. 2017;4(4):127-9.
2. Mattiuzzi C, Lippi G. Current Cancer Epidemiology. Journal of epidemiology and global health. 2019;9(4):217-22.
3. American Cancer Society. Special Section: COVID-19 and Cancer.
4. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021;71(3):209-49.
5. World Health Organization. Cancer. 2021.
6. World Health Organization. Lebanon. 2021.
7. Coleman WB. Chapter 25 - Molecular Pathogenesis of Prostate Cancer. In: Coleman WB, Tsongalis GJ, editors. Molecular Pathology (Second Edition): Academic Press; 2018. p. 555-68.
8. Singh O, Bolla SR. Anatomy, Abdomen and Pelvis, Prostate. StatPearls. Treasure Island (FL)2021.
9. Aaron L, Franco OE, Hayward SW. Review of Prostate Anatomy and Embryology and the Etiology of Benign Prostatic Hyperplasia. Urol Clin North Am. 2016;43(3):279-88.
10. McNeal JE. The zonal anatomy of the prostate. The Prostate. 1981;2(1):35-49.
11. Banerjee PP, Banerjee S, Brown TR, Zirkin BR. Androgen action in prostate function and disease. Am J Clin Exp Urol. 2018;6(2):62-77.
12. Ng KL. The Etiology of Prostate Cancer. In: Bott SRJ, Ng KL, editors. Prostate Cancer. Brisbane (AU): Exon Publications

13. Delongchamps NB, Singh A, Haas GP. The role of prevalence in the diagnosis of prostate cancer. Cancer Control. 2006;13(3):158-68.
14. Perdana NR, Mochtar CA, Umbas R, Hamid AR. The Risk Factors of Prostate Cancer and Its Prevention: A Literature Review. Acta Med Indones. 2016;48(3):228-38.
15. Carter HB AP, Barry MJ, Etzioni R, Freedland SJ, Greene KL, Holmberg L, Kantoff P, Konety BR, Murad MH, Penson DF and Zietman AL, . Early detection of prostate cancer: American Urological Association Guideline. . The Journal of Urology 2018.
16. Board PDQATE. Prostate Cancer Treatment (PDQ®): Patient Version. PDQ Cancer Information Summaries. Bethesda (MD): National Cancer Institute (US); 2002.
17. Society AC. American Cancer Society Recommendations for Prostate Cancer Early Detection. 2021.
18. Eastham J. Prostate cancer screening. Investig Clin Urol. 2017;58(4):217-9.
19. Descotes J-L. Diagnosis of prostate cancer. Asian J Urol. 2019;6(2):129-36.
20. Prostate. In: Greene FL, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG, et al., editors. AJCC Cancer Staging Manual. New York, NY: Springer New York; 2002. p. 309-16.
21. Hermanek P. ["Grading" and "staging". Significance for clinical oncology]. Fortschr Med. 1978;96(10):520-4.

22. Leslie SW, Soon-Sutton TL, Sajjad H, Siref LE. Prostate Cancer. StatPearls. Treasure Island (FL): StatPearls Publishing

23. Merseburger AS, Burger M, SpringerLink. Urologic Oncology. 1st 2019. ed. Cham: Springer International Publishing; 2019.
24. Ritch CR, Graves AJ, Keegan KA, Ni S, Bassett JC, Chang SS, et al. Increasing use of observation among men at low risk for prostate cancer mortality. J Urol. 2015;193(3):801-6.
25. Schatten H, SpringerLink. Molecular & Diagnostic Imaging in Prostate Cancer: Clinical Applications and Treatment Strategies. Cham: Springer International Publishing; 2018.
26. Chargari C, Deutsch E, Blanchard P, Gouy S, Martelli H, Guérin F, et al. Brachytherapy: An overview for clinicians. CA: A Cancer Journal for Clinicians. 2019;69(5):386-401.
27. Thüroff S, Chaussy CG, SpringerLink. Focal Therapy of Prostate Cancer: An Emerging Strategy for Minimally Invasive, Staged Treatment. Cham: Springer International Publishing; 2015.
28. Horwich A. Systemic Treatment of Prostate Cancer. Oxford, UNITED KINGDOM: Oxford University Press, Incorporated; 2010.
29. Costello AJ. Considering the role of radical prostatectomy in 21st century prostate cancer care. Nat Rev Urol. 2020;17(3):177-88.
30. Nader R, El Amm J, Aragon-Ching JB. Role of chemotherapy in prostate cancer. Asian J Androl. 2018;20(3):221-9.
31. Prevention of accidental exposures to patients undergoing radiation therapy. A report of the International Commission on Radiological Protection. Ann ICRP. 2000;30(3):7-70.
32. Wang J-S, Wang H-J, Qian H-L. Biological effects of radiation on cancer cells. Mil Med Res. 2018;5(1):20-.
33. van der Wielen GJ, Mulhall JP, Incrocci L. Erectile dysfunction after radiotherapy for prostate cancer and radiation dose to the penile structures: a critical review. Radiother Oncol. 2007;84(2):107-13.
34. Jhons Hopkins Medicine. Urinary Dysfunction After Prostate Cancer Treatment.
35. Nolsøe AB, Jensen CFS, Østergren PB, Fode M. Neglected side effects to curative prostate cancer treatments. International Journal of Impotence Research. 2021;33(4):428-38.
36. O'Neil BB, Presson A, Gannon J, Stephenson RA, Lowrance W, Dechet CB, et al. Climacturia after definitive treatment of prostate cancer. J Urol. 2014;191(1):159-63.
37. Arno J. Mundt M, John C. Roeske, PhD, Theodore D. Chung, MD, PhD, and Ralph R. Weichselbaum, MD. Physical Basis of Radiation Therapy. 2003.
38. Danzker M, Kessaris ND, Laughlin JS. Absorbed Dose and Linear Energy Transfer in Radiation Experiments. Radiology. 1959;72(1):51-61.
39. Baskar R, Dai J, Wenlong N, Yeo R, Yeoh K-W. Biological response of cancer cells to radiation treatment. Front Mol Biosci. 2014;1:24-.
40. Kadhim M, Tuncay Cagatay S, Elbakrawy EM. Non-targeted effects of radiation: a personal perspective on the role of exosomes in an evolving paradigm. International Journal of Radiation Biology. 2021:1-11.
41. Jeggo P, Löbrich M. Radiation-induced DNA damage responses. Radiation Protection Dosimetry. 2007;122(1-4):124-7.

42. Caldecott KW. Single-strand break repair and genetic disease. Nature Reviews Genetics. 2008;9(8):619-31.
43. Joubert A, Zimmerman KM, Bencokova Z, Gastaldo J, Chavaudra N, Favaudon V, et al. DNA double-strand break repair defects in syndromes associated with acute radiation response: At least two different assays to predict intrinsic radiosensitivity? International journal of radiation biology. 2008;84(2):107-25.
44. Mahaney BL, Meek K, Lees-Miller SP. Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. Biochem J. 2009;417(3):639-50.
45. Shibata A. Regulation of repair pathway choice at two-ended DNA double-strand breaks. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2017;803:51-5.
46. Lomax ME, Folkes LK, O'Neill P. Biological Consequences of Radiation-induced DNA Damage: Relevance to Radiotherapy. Clinical Oncology. 2013;25(10):578-85.
47. Mladenov E, Magin S, Soni A, Iliakis G. DNA double-strand break repair as determinant of cellular radiosensitivity to killing and target in radiation therapy. Front Oncol. 2013; 3: 113. Interesting overview of the importance of DNA double-strand break repair in radiosensitivity. 2013.
48. Willers H, Dahm-Daphi J, Powell SN. Repair of radiation damage to DNA. British Journal of Cancer. 2004;90(7):1297-301.
49. Borrego-Soto G, Ortiz-López R, Rojas-Martínez A. Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer. Genet Mol Biol. 2015;38(4):420-32.
50. Yano S, Miwa S, Mii S, Hiroshima Y, Uehara F, Yamamoto M, et al. Invading cancer cells are predominantly in G0/G1 resulting in chemoresistance demonstrated by real-time FUCCI imaging. Cell Cycle. 2014;13(6):953-60.
51. Bodgi L, Foray N. The nucleo-shuttling of the ATM protein as a basis for a novel theory of radiation response: resolution of the linear-quadratic model*. International Journal of Radiation Biology. 2016;92(3):117-31.
52. Granzotto A, Benadjaoud MA, Vogin G, Devic C, Ferlazzo ML, Bodgi L, et al. Influence of Nucleoshuttling of the ATM Protein in the Healthy Tissues Response to Radiation Therapy: Toward a Molecular Classification of Human Radiosensitivity. International Journal of Radiation Oncology*Biology*Physics. 2016;94(3):450-60.
53. Maréchal A, Zou L. DNA damage sensing by the ATM and ATR kinases. Cold Spring Harb Perspect Biol. 2013;5(9):a012716.
54. Falck J, Coates J, Jackson SP. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature. 2005;434(7033):605-11.
55. Berthel E, Foray N, Ferlazzo ML. The Nucleoshuttling of the ATM Protein: A Unified Model to Describe the Individual Response to High- and Low-Dose of Radiation? Cancers (Basel). 2019;11(7):905.
56. Hall EJ. The bystander effect. Health Phys. 2003;85(1):31-5.
57. Cheng Q, Chen J. Mechanism of p53 stabilization by ATM after DNA damage. Cell Cycle. 2010;9(3):472-8.
58. Bataini JP, Belloir C, Mazabraud A, Pilleron JP, Cartigny A, Jaulerry C, et al. Desmoid tumors in adults: the role of radiotherapy in their management. Am J Surg. 1988;155(6):754-60.

59. Cox JD, Kline RW. Do prostatic biopsies 12 months or more after external irradiation for adenocarcinoma, Stage III, predict long-term survival? Int J Radiat Oncol Biol Phys. 1983;9(3):299-303.
60. Shay JW, Roninson IB. Hallmarks of senescence in carcinogenesis and cancer therapy. Oncogene. 2004;23(16):2919-33.
61. Eriksson D, Stigbrand T. Radiation-induced cell death mechanisms. Tumor Biology. 2010;31(4):363-72.
62. Au - Kobayashi D, Au - Shibata A, Au - Oike T, Au - Nakano T. One-step Protocol for Evaluation of the Mode of Radiation-induced Clonogenic Cell Death by Fluorescence Microscopy. JoVE. 2017(128):e56338.
63. Thoma C. Postoperative RT response prediction. Nature Reviews Urology. 2016;13(12):694-.
64. Chaiswing L, Weiss HL, Jayswal RD, Clair DKS, Kyprianou N. Profiles of Radioresistance Mechanisms in Prostate Cancer. Crit Rev Oncog. 2018;23(1-2):39-67.
65. Chang L, Graham PH, Hao J, Ni J, Bucci J, Cozzi PJ, et al. PI3K/Akt/mTOR pathway inhibitors enhance radiosensitivity in radioresistant prostate cancer cells through inducing apoptosis, reducing autophagy, suppressing NHEJ and HR repair pathways. Cell Death & Disease. 2014;5(10):e1437-e.
66. Carlos-Reyes A, Muñiz-Lino MA, Romero-Garcia S, López-Camarillo C, Hernández-de la Cruz ON. Biological Adaptations of Tumor Cells to Radiation Therapy. Frontiers in Oncology. 2021;11.
67. Bromfield GP, Meng A, Warde P, Bristow RG. Cell death in irradiated prostate epithelial cells: role of apoptotic and clonogenic cell kill. Prostate Cancer Prostatic Dis. 2003;6(1):73-85.
68. Joshi S, Yu D. Chapter 8 - Immunofluorescence. In: Jalali M, Saldanha FYL, Jalali M, editors. Basic Science Methods for Clinical Researchers. Boston: Academic Press; 2017. p. 135-50.
69. Wang H, Mu X, He H, Zhang XD. Cancer Radiosensitizers. Trends Pharmacol Sci. 2018;39(1):24-48.
70. Tan P, Wei S, Yang L, Tang Z, Cao D, Liu L, et al. The effect of statins on prostate cancer recurrence and mortality after definitive therapy: a systematic review and meta-analysis. Sci Rep. 2016;6:29106-.
71. Aschenbrenner B, Negro G, Savic D, Sorokin M, Buzdin A, Ganswindt U, et al. Simvastatin is effective in killing the radioresistant breast carcinoma cells. Radiol Oncol. 2021;55(3):305-16.
72. Kim EH, Kim M-S, Lee K-H, Koh J-S, Jung W-G, Kong C-B. Zoledronic acid is an effective radiosensitizer in the treatment of osteosarcoma. Oncotarget. 2016;7(43).
73. Varela I, Pereira S, Ugalde AP, Navarro CL, Suárez MF, Cau P, et al. Combined treatment with statins and aminobisphosphonates extends longevity in a mouse model of human premature aging. Nat Med. 2008;14(7):767-72.
74. Ferlazzo ML, Sonzogni L, Granzotto A, Bodgi L, Lartin O, Devic C, et al. Mutations of the Huntington's disease protein impact on the ATM-dependent signaling and repair pathways of the radiation-induced DNA double-strand breaks: corrective effect of statins and bisphosphonates. Mol Neurobiol. 2014;49(3):1200-11.
75. Combemale P, Sonzogni L, Devic C, Bencokova Z, Ferlazzo ML, Granzotto A, et al. Individual Response to Radiation of Individuals with Neurofibromatosis Type I: Role of the ATM Protein and Influence of Statins and Bisphosphonates. Molecular Neurobiology. 2021.

76. Ferlazzo ML, Bach-Tobdji MKE, Djerad A, Sonzogni L, Devic C, Granzotto A, et al. Radiobiological Characterization of Tuberous Sclerosis: a Delay in the Nucleo-Shuttling of ATM May Be Responsible for Radiosensitivity. Molecular Neurobiology. 2018;55(6):4973-83.
77. Misra J, Mohanty ST, Madan S, Fernandes JA, Hal Ebetino F, Russell RG, et al. Zoledronate Attenuates Accumulation of DNA Damage in Mesenchymal Stem Cells and Protects Their Function. Stem Cells. 2016;34(3):756-67.
78. Soto DE, Daignault S, Sandler HM, Ray ME. No Effect of Statins on Biochemical Outcomes After Radiotherapy for Localized Prostate Cancer. Urology. 2009;73(1):158-62.
79. Iguchi T, Miyakawa Y, Saito K, Nakabayashi C, Nakanishi M, Saya H, et al. Zoledronate-induced S phase arrest and apoptosis accompanied by DNA damage and activation of the ATM/Chk1/cdc25 pathway in human osteosarcoma cells. Int J Oncol. 2007;31(2):285-91.
80. Moriceau G, Ory B, Mitrofan L, Riganti C, Blanchard F, Brion R, et al. Zoledronic Acid Potentiates mTOR Inhibition and Abolishes the Resistance of Osteosarcoma Cells to RAD001 (Everolimus): Pivotal Role of the Prenylation Process. Cancer Research. 2010;70(24):10329-39.
81. Drake MT, Clarke BL, Khosla S. Bisphosphonates: mechanism of action and role in clinical practice. Mayo Clin Proc. 2008;83(9):1032-45.
82. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, et al. A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. J Natl Cancer Inst. 2002;94(19):1458-68.
83. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, et al. Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer. J Natl Cancer Inst. 2004;96(11):879-82.
84. Michaelson MD, Kaufman DS, Lee H, McGovern FJ, Kantoff PW, Fallon MA, et al. Randomized controlled trial of annual zoledronic acid to prevent gonadotropin-releasing hormone agonist-induced bone loss in men with prostate cancer. J Clin Oncol. 2007;25(9):1038-42.
85. Vogt U, Bielawski KP, Bosse U, Schlotter CM. Breast tumour growth inhibition in vitro through the combination of cyclophosphamide/metotrexate/5-fluorouracil, epirubicin/cyclophosphamide, epirubicin/paclitaxel, and epirubicin/docetaxel with the bisphosphonates ibandronate and zoledronic acid. Oncol Rep. 2004;12(5):1109-14.
86. Algur E, Macklis RM, Häfeli UO. Synergistic cytotoxic effects of zoledronic acid and radiation in human prostate cancer and myeloma cell lines. International journal of radiation oncology, biology, physics. 2005;61(2):535.
87. Sidhu G, Sapra A. Pravastatin. StatPearls. Treasure Island (FL)2021.
88. Tsai HK, Katz MS, Coen JJ, Zietman AL, Kaufman DS, Shipley WU. Association of statin use with improved local control in patients treated with selective bladder preservation for muscle-invasive bladder cancer. Urology. 2006;68(6):1188-92.
89. Katz MS, Minsky BD, Saltz LB, Riedel E, Chessin DB, Guillem JG. Association of statin use with a pathologic complete response to neoadjuvant chemoradiation for rectal cancer. International Journal of Radiation Oncology*Biology*Physics. 2005;62(5):1363-70.

90. Nakamura H, Arakawa K, Itakura H, Kitabatake A, Goto Y, Toyota T, et al. Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA Study): a prospective randomised controlled trial. Lancet. 2006;368(9542):1155-63.
91. Rosenson RS, Tangney CC, Casey LC. Inhibition of proinflammatory cytokine production by pravastatin. Lancet. 1999;353(9157):983-4.
92. Holler V, Buard V, Gaugler MH, Guipaud O, Baudelin C, Sache A, et al. Pravastatin limits radiation-induced vascular dysfunction in the skin. J Invest Dermatol. 2009;129(5):1280-91.
93. Haydont V, Bourgier C, Pocard M, Lusinchi A, Aigueperse J, Mathé D, et al. Pravastatin Inhibits the Rho/CCN2/extracellular matrix cascade in human fibrosis explants and improves radiation-induced intestinal fibrosis in rats. Clin Cancer Res. 2007;13(18 Pt 1):5331-40.
94. Haydont V, Gilliot O, Rivera S, Bourgier C, François A, Aigueperse J, et al. Successful mitigation of delayed intestinal radiation injury using pravastatin is not associated with acute injury improvement or tumor protection. Int J Radiat Oncol Biol Phys. 2007;68(5):1471-82.
95. Gaugler MH, Vereycken-Holler V, Squiban C, Vandamme M, Vozenin-Brotons MC, Benderitter M. Pravastatin limits endothelial activation after irradiation and decreases the resulting inflammatory and thrombotic responses. Radiat Res. 2005;163(5):479-87.
96. Mahmoudi M, Gorenne I, Mercer J, Figg N, Littlewood T, Bennett M. Statins use a novel Nijmegen breakage syndrome-1-dependent pathway to accelerate DNA repair in vascular smooth muscle cells. Circ Res. 2008;103(7):717-25.
97. Nübel T, Damrot J, Roos WP, Kaina B, Fritz G. Lovastatin protects human endothelial cells from killing by ionizing radiation without impairing induction and repair of DNA double-strand breaks. Clin Cancer Res. 2006;12(3 Pt 1):933-9.
98. Ostrau C, Hülsenbeck J, Herzog M, Schad A, Torzewski M, Lackner KJ, et al. Lovastatin attenuates ionizing radiation-induced normal tissue damage in vivo. Radiother Oncol. 2009;92(3):492-9.
99. Jenrow KA, Liu J, Brown SL, Kolozsvary A, Lapanowski K, Kim JH. Combined atorvastatin and ramipril mitigate radiation-induced impairment of dentate gyrus neurogenesis. J Neurooncol. 2011;101(3):449-56.
100. Wedlake LJ, Silia F, Benton B, Lalji A, Thomas K, Dearnaley DP, et al. Evaluating the efficacy of statins and ACE-inhibitors in reducing gastrointestinal toxicity in patients receiving radiotherapy for pelvic malignancies. Eur J Cancer. 2012;48(14):2117-24.
101. Doi H, Matsumoto S, Odawara S, Shikata T, Kitajima K, Tanooka M, et al. Pravastatin reduces radiation-induced damage in normal tissues. Exp Ther Med. 2017;13(5):1765-72.
102. Miller AC, Kariko K, Myers CE, Clark EP, Samid D. Increased radioresistance of ejras-transformed human osteosarcoma cells and its modulation by lovastatin, an inhibitor of p21ras isoprenylation. International Journal of Cancer. 1993;53(2):302-7.
103. Fritz G, Brachetti C, Kaina B. Lovastatin causes sensitization of HeLa cells to ionizing radiation-induced apoptosis by the abrogation of G2 blockage. International Journal of Radiation Biology. 2003;79(8):601-10.
104. Ferlazzo ML, Sonzogni L, Granzotto A, Bodgi L, Lartin O, Devic C, et al. Mutations of the Huntington's disease protein impact on the ATM-dependent signaling

and repair pathways of the radiation-induced DNA double-strand breaks: Corrective effect of statins and bisphosphonates. Molecular neurobiology. 2014;49(3):1200-11.
105. **Bodgi L B-GJ, Azzi J, Baalbaki K, Kharroubi H, Chhade F, Geara F, Abou-Kheir W, and Ayoub Z**. ZOledronate and PRAvastatin (ZOPRA) in the Treatment of Breast Cancer: Assessing the Effect of Radiosensitivity *In Vitro*. *Submitted*.
106. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nature Protocols. 2006;1(5):2315-9.
107. Chang DS, Lasley FD, Das IJ, Mendonca MS, Dynlacht JR. Cell Death and Survival Assays. Basic Radiotherapy Physics and Biology. Cham: Springer International Publishing; 2014. p. 211-9.
108. Flegal M, Blimkie MS, Wyatt H, Bugden M, Surette J, Klokov D. Measuring DNA damage and repair in mouse splenocytes after chronic in vivo exposure to very low doses of beta-and gamma-radiation. JoVE (Journal of Visualized Experiments). 2015(101):e52912.
109. Blimkie MS, Fung LC, Petoukhov ES, Girard C, Klokov D. Repair of DNA double-strand breaks is not modulated by low-dose gamma radiation in C57BL/6J mice. Radiation research. 2014;181(5):548-59.
110. Osipov AN, Pustovalova M, Grekhova A, Eremin P, Vorobyova N, Pulin A, et al. Low doses of X-rays induce prolonged and ATM-independent persistence of γH2AX foci in human gingival mesenchymal stem cells. Oncotarget. 2015;6(29):27275.
111. Ulyanenko S, Pustovalova M, Koryakin S, Beketov E, Lychagin A, Ulyanenko L, et al. Formation of γH2AX and pATM Foci in Human Mesenchymal Stem Cells Exposed to Low Dose-Rate Gamma-Radiation. Int J Mol Sci. 2019;20(11).
112. Jayakumar S, Kunwar A, Sandur SK, Pandey BN, Chaubey RC. Differential response of DU145 and PC3 prostate cancer cells to ionizing radiation: Role of reactive oxygen species, GSH and Nrf2 in radiosensitivity. Biochimica et Biophysica Acta (BBA) - General Subjects. 2014;1840(1):485-94.
113. Valentin J. Avoidance of radiation injuries from medical interventional procedures, ICRP Publication 85. Annals of the ICRP. 2000;30(2):7-.
114. Hu Q, Hill RP. Radiosensitivity, apoptosis and repair of DNA double-strand breaks in radiation-sensitive Chinese hamster ovary cell mutants treated at different dose rates. Radiation research. 1996;146(6):636-45.
115. Abshire D, Lang MK, editors. The evolution of radiation therapy in treating cancer. Seminars in oncology nursing; 2018: Elsevier.
116. Desouky O, Ding N, Zhou G. Targeted and non-targeted effects of ionizing radiation. Journal of Radiation Research and Applied Sciences. 2015;8(2):247-54.
117. Hughes JR, Parsons JL. FLASH radiotherapy: current knowledge and future insights using proton-beam therapy. International journal of molecular sciences. 2020;21(18):6492.
118. Mehta S, Suhag V, Semwal M, Sharma N. Radiotherapy: Basic concepts and recent advances. Medical Journal Armed Forces India. 2010;66(2):158-62.