AMERICAN UNIVERSITY OF BEIRUT

ASSESSING THE RADIOPROTECTING EFFECT OF ZOPRA ON HUMAN PROSTATE EPITHELIAL CELLS AND HUMAN FIBROBLAST CELLS

by
JHONNY EDMOND FAWAZ

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
September 2022

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ACKNOWLEDGEMENTS

Although the following thesis is an individual work, I could never have accomplished it without the assistance, direction, support, and efforts of a lot people.

First of all, I want to thank my advisor, Dr. Wassim Abou-Kheir, a superb mentor and role model. He not only shared knowledge, but he also fueled my interest in learning new skills. His wisdom and assistance, helped me grow both mentally and spiritually. He always pushed me to the top. I want to extend my gratitude to Dr. Larry Bodgi, my co-advisor. Many appreciations for his kind, friendly, and motivating personality. He was always positive and gave generously of his time and vast knowledge.

Additionally I am thankful for the efforts and willingness of my committee members, Dr. Assaad Eid and Dr. Georges Daoud, who guided me through the process of writing my thesis. I want to thank them for having faith in me and for pushing me to do my absolute best.

This project would not have been possible without the generous funding from the Diana Tamari Sabbagh Scholars Program (DTSSP).

I am grateful to my fellow WAK Lab members for their encouraging friendship, constant support, and restless nights. They have shown me that anything is possible with perseverance, patience, and hard work. I feel privileged to be a part of this family.

Finally, I want to say how thankful I am to my parents and my friends for their unwavering support and continous encouragement throughout the year. Without them, this accomplishment would not have been possible.
Thank you everyone.

ABSTRACT
OF THE THESIS OF

Jhonny Edmond Fawaz for Master of Science
Major: Physiology

Title: Assessing the Radioprotecting Effect of ZoPra on Human Prostate Epithelial Cells and Human Fibroblast Cells.

Prostate cancer is the most prevalent cancer in men and the second leading cause of cancer-related deaths in men around the world. One of the first-line treatments for lowering tumor size and preventing further cancer development is radiotherapy. Its effectiveness is based on the patient's and tumor cells' radiosensitivity. The radioresistance of tumor cells and harmful effects on the surrounding normal cells, however, greatly limit it.

Radiation exposure to normal tissue can cause both acute and chronic toxicities. Radiotherapy induces DNA double strand breaks and the cell's response translates into the activation of DSB repair through ATM nucleoshuttling and phosphorylation of H2AX Radioprotectors are chemicals that are meant to decrease the effects of radiation on normal tissues. Numerous disorders linked to bone loss are treated using bisphosphonates, such as zoledronic acid (Zo). Pravastatin (Pra) is one of many statins that are used to control lipid levels.

The ZoPra combination has been found to improve the speed of ATM nucleo-shuttling by blocking nucleus membrane farnesylation. This results in faster and better DSB signaling, which improves the cell's ability to repair DNA. As a result, the combination of Zoledronate and Pravastatin may have a radio protecting effect on normal prostate and fibroblast cells. Therefore, the aim of this study is to assess the radioprotecting effect of Zoledronic acid and Pravastatin, alone and in combination on normal epithelial human prostate cell line, RWPE-1, in vitro, on a cellular and molecular level.

The cytotoxic effect of different concentrations of Zo and Pra 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 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 decrease the residual number of γH2AX foci. However no significant change was observed in cell survival of RWPE-1 cells. This study presents novel findings on the potential use of ZoPra as a radioprotecting agent for normal human prostate epithelial cells.

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ABBREVIATIONS

ACS: American Cancer Society

ADT: Androgen deprivation therapy

ATM: Ataxia Telangiectasia Mutated

pATM: phosphorylated ATM

ATR: ATM and Rad3 related

CT: Chemotherapy

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

HMGCo-A: Hydroxymethyl glutaryl coenzyme-A

HR: Homologous recombination

HSP90: Heat Shock Protein 90

γH2AX: phosphorylated histone H2AX

IR: Ionizing Radiation

IF: immunofluorescence

Gy: Gray (absorbed dose)

MRN: MRE11-Rad50-Nbs1

NF1: Neurofibromatosis type 1

NHEJ: Non-homologous endjoining

PCa: Prostate cancer

PRA: pravastatin

PSA: Prostate specific antigen

Qol: Quality of Life

RIANS: Radiation-induced nucleoshuttling of the ATM kinase

ROS: Reactive oxygen species

RT: Radiotherapy

SF: Surviving Fraction

SSB: Single strand breaks

ssDNA: Single strand DNA

TGF-β: Transforming Growth Factor beta

ZO: zoledronic acid

ZoPra: zoledronic acid and pravastatin

SEM: the Standard Error of the Mean

CHAPTER I

INTRODUCTION

A. Cancer overview: Epidemiology

Cancer is considered to be a prominent cause of morbidity and mortality in the world. According to estimates from the World Health Organization (WHO) in 2019, cancer is the first or second leading cause of death before the age of 70 years in 112 of 183 countries and ranks third or fourth in a further 23 countries (1). Based on GLOBOCAN 2020 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer (IARC), an estimated 19.3 million new cancer cases and about 10 million cancer deaths occurred in 2020 worldwide.

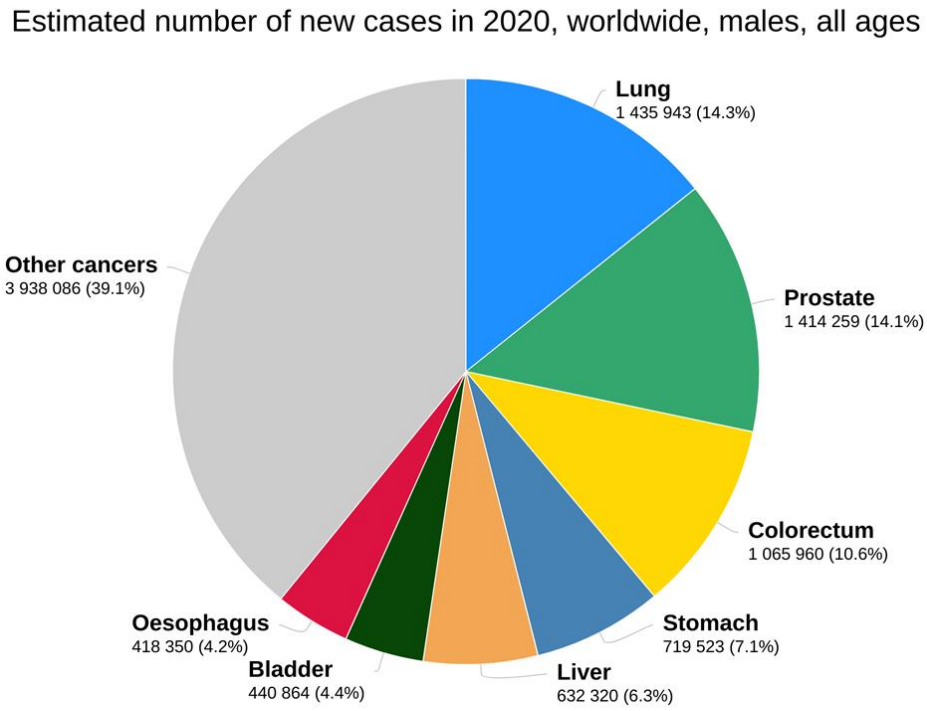
During the pandemic, cancer patients faced a difficult dilemma: staying at home may hasten tumor progression, but going to the hospital for treatment may raise the danger of spreading COVID-19 (2). The coronavirus disease 2019 (COVID-19) outbreak impeded cancer detection and therapy in 2020. Reduced access to care as a result of health-care facility closures, for example, resulted in delays in diagnosis and treatment, which could result in a short-term decrease in cancer incidence followed by a rise in advanced-stage disease and, eventually, increased mortality (3).

Additionally, cancer is a broad term that refers to a collection of illnesses that invade and destroy any normal bodily tissue and have the same phenotype: aberrant cell proliferation and growth (4). Chemical substances are well known for their function in the formation of gene mutations and cancer cells. Moreover, carcinogenic environmental chemical compounds affect the cytoplasm and nucleus of cells directly or indirectly, resulting in genetic abnormalities and gene alterations. Other carcinogens include viruses, bacteria, and radiation rays, which account for around 7% of all cancers (5).

Cancer is caused by a succession of gene mutations resulting in the cell's functions to be altered (6). This disruption affects the cell cycle, resulting in aberrant proliferation (7).In like manner, cancer is a major issue that has an impact on the health of all human societies. Unfortunately, at the tissue level, there is a wide variety of cancer types, and this variation is a limiting factor for both diagnosis and treatments (8).

The prostate, lung and bronchus, colon and rectum, and urinary bladder all have the highest percentages of cancer types in men, respectively. Breast cancer, lung and bronchus cancer, colon and rectum cancer, uterine corpus cancer, and thyroid cancer are the most common cancers in women, respectively (9). According to this data, prostate and breast cancer account for a significant part of cancer in men and women, respectively.

In this study, we will be focusing on prostate cancer's development, progression, available treatments and potential novel therapeutic drugs.



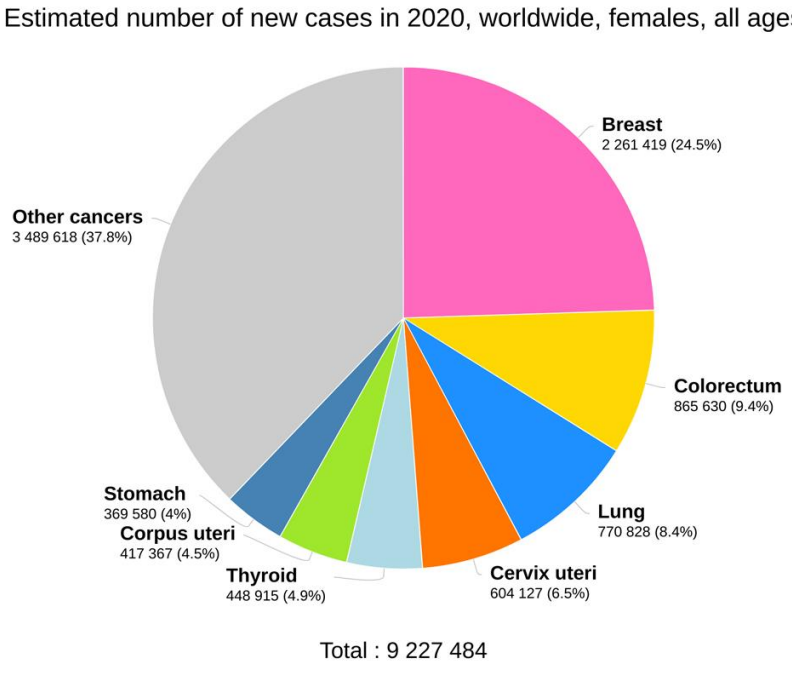


Figure 1: Estimates of new cancer cases in 2020, worldwide, females and males, all ages, produced by the International Agency for Research on Cancer (IARC)

B. The prostate gland

1. Gland anatomy and physiology

The human prostate is an exocrine part of the male reproductive system which makes the fluid component of the semen (10). The prostate, having the size of a walnut, is a glandular and muscular tissue located just below the bladder's neck and around the beginning of the urethra. It is found anterior to the rectum, within the pelvic cavity, below the inferior edge of the symphysis pubis and above the triangular ligament. Its base is below the bladder's neck and points upwards, whereas the tip reaches the ligament and points downwards. Its posterior surface is adjacent to the second section of the rectum, and puboprostatic ligaments attach its anterior surface to the pubis. The levator ani muscles are in contact with its lateral surfaces (11). The prostate is encased in a thin, fibrous capsule that is separated from the rectovesical fascia by a plexus of veins. Muscle tissue can be observed just under the capsule and surrounding the urethra. The internal pudic, vesical, and haemorrhoidal arteries provide arterial feed to the prostate, whereas venous drainage begins in the dorsal vein of the penis and ends in the internal iliac vein. The prostate receives its nerve supply from the pelvic plexus (inferior hypogastric plexus). The most dilatable section of the urethra is the intraprostatic segment (12).

McNeal and his colleagues analyzed the normal and pathological anatomy of the prostate and established an anatomical zone concept. The prostate gland has four unique anatomical zones. However they can be categorized into three major parts, each of which is histologically and anatomically distinct: the non-glandular fibromuscular stroma and two glandular regions known as the peripheral and central zones, which comprise a complex and histologically distinct ductal system (13).

- The central zone is a layer of tissue that originates near the orifices of the ejaculatory ducts and follows them proximally before branching laterally towards the prostate base. Central zone cancers are uncommon, however, they are usually linked to a higher risk of death.
- The peripheral zone which surrounds the core zone makes up the rest of the gland. The peripheral zone accounts for more than 70% of the glandular prostate. This region is the primary site of prostatitis and prostate cancer. Almost every carcinoma begins here, but not benign prostatic hyperplasia (BPH).
- The preprostatic zone. The proximal segment has no main ducts, but the lateral rows of peripheral zone orifices persist. The development of the ducts is halted, leaving just a tiny transition zone and a few smaller periurethral ducts.
- The anterior fibromuscular stroma is a layer of fibromuscular tissue. It produces a thick, non-glandular apron that covers the whole anterior and anterolateral surfaces of the prostate, hiding the three glandular regions (14).

The prostate gland is made up of epithelial and stromal cells at the cellular level. Secretory columnar epithelial and basal epithelial cells, as well as rare neuroendocrine cells, make up the epithelium. Smooth muscle cells and fibroblasts make up the stroma that surrounds the prostatic glands. Additional constituent cell parts of the typical adult human prostate include blood arteries, peripheral nerves and ganglia, and tissue invading white blood cells (15).

2. Prostate Cancer

Benign prostatic hyperplasia and prostate cancer are two prevalent and severe disorders that affect aging men's prostate glands (16).

With an anticipated 1,414,259 new cancer cases and 375,304 deaths in 2020, prostate cancer is the second most commonly diagnosed cancer and the fifth leading cause of cancer mortality among men globally. It is the most commonly diagnosed cancer in over 50% of countries in the world (112 of 185) (17).

Lebanon is one of the countries with the highest incidence of prostate cancer among men. Prostate cancer is the most frequent cancer in men in the United States, aside from skin cancer. According to the American Cancer Society, there will be roughly 248,530 new cases of prostate cancer in the United States in 2021. In the US, prostate cancer claims the lives of approximately 34,130 men each year.

By 2040, the global burden of prostate cancer is anticipated to rise to over 2.3 million new cases and 740,000 deaths, owing to population expansion and aging (18).

In comparison to men of other ethnicities, African-American men are more susceptible to the disease. The prevalence of prostate cancer is thought to be high in Africa. Prostate cancer is the leading malignancy in terms of incidence and mortality among males of African origin, as evidenced by numerous articles. Since the majority of new diagnoses are advanced/metastatic tumors with poor prognoses and limited prospects of long-term survival, prostate cancer is becoming a growing source of public concern in Africa.(19)

Prostate cancer is characterized by a high level of inter/intra tumor heterogeneity, making clinical management challenging. The majority of prostate tumors grow slowly and are low-grade, posing little concern, but around 10% progress to aggressive stages quickly, providing a poor prognosis and limited survival. A complicated sequence of intrinsic cellular changes

and microenvironment alterations mediates the progression of prostate cancer from primary to advanced stages (20).

3. PCa Screening

Screening is a method of detecting cancer before any symptoms or indicators appear. When cancer is detected in time of its progression, it is usually at a more early state. This suggests that PCa cancer has a better probability of being effectively treated. Scientists have created and are continuing to develop tests that can be used to screen people for specific cancers. Cancer screening's overall goals are to:

- Limit or eliminate cancer-related deaths.
- Limit the number of patients suffering from cancer (21).

PSA, or prostate-specific antigen, is a protein produced by both normal and cancerous cells in the prostate gland. The PSA test evaluates the level of PSA present in a man's blood. A blood sample is submitted to a laboratory for analysis in this procedure. PSA levels are commonly expressed in nanograms per milliliter of blood (ng/mL) (22). In men with a normal prostate, only small amounts of PSA leak into circulation, but an abnormal prostate leaks much larger amounts of the antigen, hence the blood level of PSA in men with prostate cancer are frequently elevated (23). The PSA test was first approved by the US Food and Drug Administration (FDA) in 1986 to track the progression of prostate cancer in men who had previously been diagnosed with the condition. The PSA test, in combination with a digital rectal exam (DRE), was approved by the FDA in 1994 to screen asymptomatic men for prostate cancer. PSA testing is commonly performed on men who report prostate symptoms to assist doctors in locating the nature of the disease (24). A number of benign (non-cancerous) illnesses can cause a man's PSA level to rise, in addition to prostate cancer. Prostatitis (prostate inflammation) and benign prostatic hyperplasia (BPH) (enlargement of

the prostate) are the two most common benign prostate disorders that induce an increase in PSA levels. Although there is no evidence that prostatitis or BPH promote prostate cancer, it is possible for a man to develop prostate cancer while suffering from one or both of these disorders (25).

On the other hand, the rectum is examined using a digital rectal exam (DRE). A greased, gloved finger is inserted into the bottom part of the rectum by the doctor or nurse to feel the prostate for lumps or anything abnormal. A physician may request a PSA test after a concerning DRE or prefer that the two tests be done at the same time (26). However, some evidence suggests that the DRE may not significantly reduce mortality, but rather may result in a high number of false-positives, leading to unnecessary invasive diagnostic tests that can lead to pain, erectile dysfunction, and urinary incontinence, as well as prostate cancer over diagnosis and overtreatment (27).

Further, the prostate cancer antigen 3 test has been validated in this cohort, demonstrating an 88 % negative predictive value for subsequent biopsy. (28) Over and above, a biomarker is a biological molecule found in blood, body fluids, or tissues that indicates whether a condition or disease is normal or pathological. Markers can also be used to assess how the body reacts to a disease treatment. New molecular biomarkers that define tumor aggressiveness (e.g., Decipher, Prolaris, and Oncotype DX) have become available and may aid in the identification of PCa. A cell cycle progression score based on 31 genes can predict clinical progression and prostate cancer mortality using biopsy tissue (29). A 17-gene assay used on biopsy tissue can predict the probability of adverse pathology, biochemical recurrence, and metastasis after prostatectomy. The Prostate Health Index (PHI) provides an effective way to integrate total PSA, free PSA, and p2PSA to predict the risk of a future biopsy diagnosing prostate cancer (30). Prognostic information is also provided by a 22-

marker genetic classifier test developed to evaluate metastatic risk based on the prostatectomy specimen. These and other molecular biomarkers could help doctors distinguish between indolent illness and aggressive tumors discovered after a biopsy. These methods may provide prognostic information that is useful (31).

4. PCa diagnosis

Many tests can indicate the presence of cancer, but only a biopsy can confirm the diagnosis. A biopsy is a procedure in which a small piece of tissue is removed and examined under a microscope. A surgeon will most likely employ transrectal ultrasound (TRUS) and a biopsy tool to get a tissue sample from the prostate. Several regions of the prostate will be sampled for biopsies (32). Although the transrectal US-guided technique aids in the visualization of the prostate gland and the systematic sampling of various parts of the prostate, it is unable to properly pinpoint prostate cancer for targeting.

The traditional transrectal US-guided method is particularly ineffective at sampling malignancies in the anterior and apical regions, resulting in clinically significant illness being missed. One of the most significant drawbacks of transrectal US-guided biopsy is that up to 40% of cases categorized as low grade on transrectal US-guided biopsy are found to have higher grade disease in surgical histologic specimens. Given a reasonable fear that the biopsy is underestimating the disease, uncertainty over the results of a transrectal US-guided biopsy can lead to more severe therapy than is necessary. As a result of this ambiguity, patients are more likely to choose needless therapies that result in increased morbidity, lower quality of life, and higher healthcare costs (33).

Targeted biopsy of the prostate utilizing multiparametric magnetic resonance imaging (MR imaging) is one prospective method for increasing prostate cancer detection.

Multiparametric MR imaging combines anatomic and functional imaging to improve the diagnosis of clinically important disease while decreasing the detection of non-clinically significant malignancies. Multiparametric MR imaging can better depict the underlying tumor location and volume than traditional transrectal US-guided biopsy in defining a suspicious area for targeted prostate biopsy. Multiparametric MR imaging–targeted biopsies result in better detection rates of clinically significant malignancy and less upgrading of tumors after surgery, boosting biopsy confidence (34). Hence, Prostate MRI is becoming a routine imaging method for prostate cancer diagnosis. It can help with staging and localization by identifying and grading suspicious prostate nodules, checking for extra capsular extension, evaluating the seminal vesicles for suspected tumor involvement, and determining enlargement of surrounding lymph nodes that could signal early metastatic illness (35).

5. PCa stages

Other tests are performed if prostate cancer is diagnosed to determine if cancer cells have spread within the prostate or to other parts of the body. This is referred to as staging. The stage of prostate cancer is determined by whether the cancer is contained within the prostate or has spread to other parts of the body. The stage of prostate cancer determines the type of treatment required. Doctors conduct diagnostic tests to determine the cancer's stage, therefore it's possible that the staging won't be complete until all of the tests have been completed. Knowing the stage assists the doctor in determining the best course of therapy and can aid in predicting a patient's prognosis, or possibility of recovery. Distinct forms of cancer have different stage descriptions (36).

There are two forms of prostate cancer staging:

- Clinical staging based on DRE, PSA tests, and Gleason score results.

- Pathologic staging based on information gathered during surgery, as well as the pathology (lab results) of prostate tissue removed during surgery (37).

The TNM classification system is the most frequently used cancer staging system. The TNM system assists in determining the disease's anatomic extent. T for tumor, N for nodes, and M for metastasis (spread) are the three primary categories that might be assigned. The three factors can be used to determine the tumor's overall stage. With tumors staged from I through IV, with stage IV being the most aggressive, this method allows for simplification. Carcinoma in situ (abnormal cells present but not spread to neighboring tissue) is classified as stage 0 and is not considered malignant but may develop into cancer in the future (38).

6. PCa grading system

The microscopic appearance of the tumor's cells and tissue is characterized by cancer grading. Prostate cancer grades were formerly classified using the Gleason Score, a method named after the pathologist who created it in the 1960s. As malignant cells transition from normal cells to tumor cells, physicians noticed that they fall into five distinct patterns (39).The pathologist examines how the cancer cells are organized in the prostate and assigns a score from 2 distinct spots on a scale of 3 to 5.

Cancer cells that resemble healthy cells are given a low score. Cancer cells that resemble healthy cells less or appear to be more aggressive are given a higher score. To assign the numbers, the pathologist first determines the primary pattern of cell development, which is the most evident area of cancer, and then searches for another area of growth. The doctor then assigns a score from 3 to 5 to each location. The scores are combined together to provide a final score between 6 and 10 on a scale of 1 to 10 (40, 41).

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

Gleason score	Grade group	Characteristics	Description	Prognosis
6	Grade Group 1	Less aggressive/ Low risk/ Very slow growing	Small uniform gland	Well differentiated
3 + 4 = 7	Grade Group 2	Slightly aggressive/ Low to intermediate risk/ Slow growing	More stoma between glands	
4 + 3 = 7	Grade Group 3	Moderately aggressive/ Intermediate to high risk/ Fast growing	Distinctly infiltrative margins	Moderately differentiated
8	Grade Group 4	Aggressive/ High risk/ Rapidly growing	Irregular masses of neoplastic	Poorly differentiated/ Anaplastic
9 -10	Grade Group 5	Highly aggressive / High risk/ Rapidly growing	Periodic gland formation	

7. PCa types

Adenocarcinoma is the most frequent type of prostate cancer, with squamous cell carcinoma, Transitional Cell Carcinoma, small cell carcinoma, and prostate sarcomas accounting for fewer than 5% of identified cases (42).

Table 2: Types of PCa and its prevalence

Types of Prostate Cancer	Origin	Percentage of all prostate Cancer
Adenocarcinomas	Glandular cells	95 %
Squamous cell carcinoma	Flat cells that cover the prostate	0.5 – 1 %
Transitional cell carcinoma	Epithelium of urethra	Rare
Small cell carcinoma	Small round cells in the prostate	1 %
Sarcoma	Soft and supportive tissues	Very rare

C. Treatments

Following the diagnosis, clinicians have a variety of therapeutic interventions that may be appropriate for each type and grade of prostate cancer, but these options are also influenced by the patient's overall health.

Surgery, radiotherapy, secondary hormonal treatments, chemotherapy, vaccine-based immunotherapy, and novel targeted therapeutic applications are some of the treatment options available to patients with prostate cancer. Treatment options for prostate cancer are mostly determined by histological architecture, PSA levels, and the degree of local illness (43).

Treatments for prostate cancer can have a significant impact on a person's quality of life. Erectile dysfunction, failure to develop and sustain an erection, and the inability to control urine flow or bowel function, are all possible side effects of these therapies.

1. Active surveillance

Furthermore, many prostate tumors progress slowly and generate no signs or complications. As a result, many patients may think of postponing cancer treatment rather than pursuing it immediately. This is referred to as "active surveillance." The malignancy is continuously checked for symptoms of worsening during active surveillance. Treatment will begin if the cancer is proven to be worsening (44).

2. Local therapy

Cancer is eradicated from a specific, confined area of the body using local therapy. Surgical and radiation therapy are examples of such procedures. Local therapy for early-stage prostate cancer may be able to totally remove the malignancy (45). If the disease has gone beyond the prostate gland, further treatments known as systemic treatments (the use

of medication to fight cancer cells) may be required to eliminate cancer cells in other parts of the body. A medical oncologist, a clinician who specializes in using medications to treat cancer, usually prescribes systemic therapy.

a) Surgery

During surgery, the prostate and some lymph nodes in the surrounding area are removed. In males with prostate cancer, surgery is not considered a monotherapy; rather, it is part of a multimodality treatment. Surgery is recommended mostly for high-risk, locally advanced prostate cancer (46).

In the case of prostate cancer, radical prostatectomy is one of the most commonly used surgical procedures. The entire prostate as well as the seminal vesicles are surgically removed in a radical prostatectomy. It is also possible to remove lymph nodes in the pelvic area. Sexual function may be harmed as a result of this procedure (47). Due to various concerns about side effects such as high rates of positive surgical margins, danger of lymph node metastases, and high rates of PSA recurrence, RP has traditionally been discouraged for high-risk prostate cancer (48).

b) Radiotherapy

External beam radiation (EBRT) and brachytherapy are the two most widely used types of radiation therapy, according the AUA. In EBRT, tumor cells are exposed to high intensity rays, typically Xrays, to either kill them or stop their growth with the least amount of harm to healthy tissue.

Brachytherapy is the implantation of radioactive seeds or particles within or close to a tumor. It enables the direct application of a very high radiation dose to the tumor while lessening the impact on surrounding cells (49).

3. Systemic therapy

Hormonal therapy, Targeted therapy, Chemotherapy, and Immunotherapy are examples of systemic therapies for prostate cancer (50).

i. Androgen Deprivation Therapy

For locally advanced and metastatic prostate cancer, androgen deprivation therapy (ADT) is the first line of treatment. The foundation of treatment is the inhibition of numerous hormones, receptors, or enzymes involved in the androgen synthesis pathway. ADT's therapeutic benefits in males with symptomatic metastatic prostate cancer are immediate and significant (51).

ii. AR-targeting vaccines

The use of AR-targeting vaccines is a novel technique being investigated in prostate cancer. Since the FDA authorized sipuleucel-T as the first immunotherapy for prostate cancer in 2010, researchers have been working to find new ways to generate an immune response that could lead to therapeutic benefit in patients with advanced illness (52).

iii. Chemotherapy

Chemotherapy is the use of medications to kill cancer cells by preventing them from growing, dividing, and producing new ones.

In many circumstances, the use of medications to treat cancer has proven to be effective. Patients may receive a combination of one or two medications at a time (53).

Chemotherapy may benefit people with advanced or castration-resistant prostate cancer, as well as those who have recently been diagnosed or have castration-sensitive metastatic

prostate cancer. A chemotherapy regimen, often known as a schedule, consists of a defined number of cycles administered over a set length of time (54).

Chemotherapy has been proven to enhance quality of life in patients with hormone-refractory prostate cancer (HRPC), as well as lowering PSA levels. Mitoxantrone, doxorubicin, vinblastine, paclitaxel, docetaxel, and other chemotherapeutic pharmaceuticals are frequently prescribed to treat advanced prostate cancer (55).

Recent prostate-preserving treatment techniques, on the other hand, are beginning to emerge, with the majority of them focusing on a radio-chemotherapy combination. This emphasizes the importance of recognizing and quantifying the effects of each therapy separately and in combination (56).

Docetaxel, which is commonly administered with prednisone, is the only licensed medicine that has been demonstrated to prolong survival in males with CRPC. Cabazitaxel is also utilized in the treatment of docetaxel-resistant CRPC (57).

The value of these conventional medications in terms of survival is yet unknown, and they do not have a satisfying effect. As a result of efforts to slow disease progression associated with treatment resistance, more patient-specific and disease-targeted medication is becoming available. NSAIDS and retinoids were among the targeted medicines explored against PCa (58).

D. Radiotherapy

Radiotherapies are the second most common treatment option for localized high-risk prostate tumors, after Radical Prostatectomy. The use of high-energy rays to destroy cancer cells is known as radiation therapy. Radiation is one of the most common anti-cancer treatments, with more than half of cancer patients receiving it. Prostate cancer treatment options such as external-beam radiation (EBRT) and brachytherapy have seen substantial clinical and technological advancement in recent decades (59). The most prevalent type of radiation treatment is external-beam radiation therapy. Every patient with no distant metastases and a life expectancy of at least 5-10 years may benefit from EBRT (60).

Internal radiation therapy, also known as brachytherapy, involves inserting radioactive sources directly into the prostate. The source emits radiation just around the targeted area and can be left there for a short(high-dose rate) or a long time (low-dose rate). Other therapies, such as external-beam radiation therapy and/or hormone therapy, may be combined with brachytherapy (61).

In EBRT, a machine outside the body is used by the radiation oncologist to focus a beam of x-rays on the affected area. X-rays are the most prevalent type of radiation employed, and they are the only radiation technique available in Lebanon.

Production of X-rays

X-rays are a form of electromagnetic radiation. The amount of energy carried by each photons distinguishes the different forms of radiation.

By speeding electrons across an electrical voltage potential and stopping them in a target, radiation-producing devices produce X-rays. Most X-ray devices start with a cathode that

emits electrons, which are then accelerated by a voltage before hitting an anode that emits X-ray photons: Bremsstrahlung and characteristic X-ray photons (62).

a) Bremsstrahlung effect

When electrons collide with the anode, they decelerate or brake, emitting Bremsstrahlung (German for "braking radiation"). When small charged particles collide with massive atoms, bremsstrahlung is produced most effectively.

Hence The Bremsstrahlung effect is a two-step process that produces X-rays. When a fast-circulating electron collides with an atom, it creates a plasma. The negative force from the cloud of electrons surrounding the atom interacts with this electron as a result of the atom collapsing. Either the electron is slowed or stopped. The exiting electron becomes slower and has less energy. An X-ray photon with the same energy is emitted according to the law of "energy conservation."(63). 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) (64).

b) Characteristic X-rays

When a high-energy electron collides with an electron from the inner shell, both are ejected from the tungsten atom, leaving a hole in the inner layer. An outer shell electron fills this with a loss of energy emitted as an X-ray photon.

Characteristic X-rays are produced when electrons flow from one atomic orbit to another. Individual photon energies are unique to each atom type and can be used to identify very small amounts of a certain element. As a result, they're critical in analytical X-ray applications in research labs (65).

E. Radio-induced DNA damage

Radiation damages cells either directly or indirectly through its effect on DNA. Direct effect: Radiation directly damages DNA molecules by disrupting their structural integrity, resulting in DNA damage.

Indirect effect: When radiation strikes some of the cell's most important constituents, such as water or organic molecules, it causes them to ionize. The water molecules are subsequently converted into free radicals such hydroxyl (OH•) and alkoxy, which attack the DNA and cause DNA damage. According to studies, this pathway is responsible for roughly 70% of radio-induced DNA damage (66).

Different forms of DNA damage are caused by IR or exposure to genotoxic substances. Base damage (BD), single-strand breaks (SSB), and double-strand breaks (DSB) are the most common radio and chemo-induced DNA damages:

A. Base damage, also known as chemical lesions, is the most prevalent type of genomic damage and can have major ramifications for a variety of biological functions, including genomic mutation, transcriptional mutagenesis, and regulatory DNA element disruption. There are four types of base lesions: oxidation (by ROS), deamination (where an oxygen atom replaces the nitrogen in basses), alkylation (where substrates such as methyl groups are added to purines and pyrimidines), and hydrolysis (generation of abasic sites by hydrolysis of N-glycosidic bond) (67).
B. Single strand breaks: a single strand break in the DNA backbone occurs when phosphodiester links in one of the strands break. The two strands do not separate from each other in this scenario. Approximately 1000 SSB are induced per Gy, with 50% of them being corrected within 10-20 minutes after IR (68).

C. Double strand breaks: A double strand break occurs when the broken links occur in both strands of DNA. Although DSBs are uncommon (40 per Gy), they are more difficult to repair (more than 50 min to repair half of them) (69).

DSBs are considered the most damaging of all DNA damages. Indeed, if left unaddressed, they may induce cell death, as well as deletions, translocations, and fusions in the DNA. These changes are known as genomic rearrangements, and they're very common in malignant cells (70).

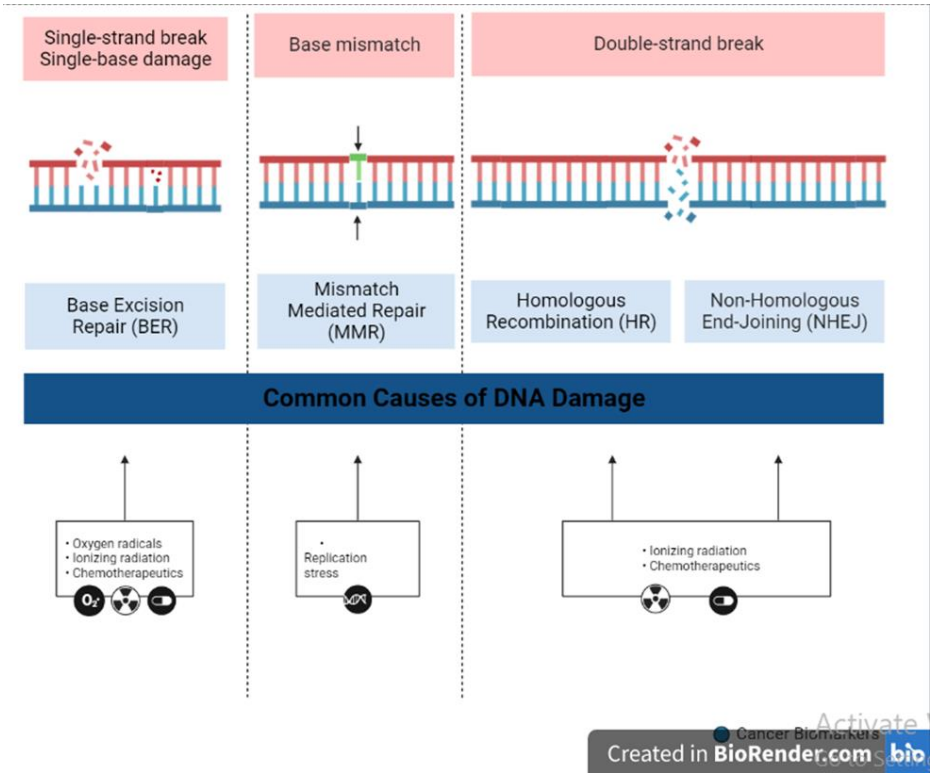


Figure 2: Main DNA damage and their Causes. Different forms of DNA damage are caused by IR or exposure to genotoxic substances. Base damage (BD), single-strand breaks (SSB), and double-strand breaks (DSB)

F. DNA DSB signaling and repair

In response to DNA damage induction, eukaryotic cells activate the DNA damage response (DDR), a signaling pathway that detects and signals damaged DNA, first to stop cell cycle progression (activation of checkpoints) and then to start a suitable repair mechanism

(71). DDR is primarily activated by DNA DSB, and sensors in this route are able to recognize these abnormal DNA structures directly and activate DDR upstream kinases, which in turn activate DDR effectors to control cell cycle progression and commence repair. The MRN complex (MRE11-Rad50-Nbs1) is the most significant DDR pathway sensor, as it can directly attach to the DSB's double-stranded DNA ends (72).

MRN stimulates the ATM protein, which is the DDR pathway's transducer. ATM belongs to the phosphatidylinositol-3-kinase-like kinase (PIKK) family, which can phosphorylate target proteins, triggering the NHEJ repair pathway as well as G1/S checkpoints, causing cell cycle arrest (73). ATM is thus localized to regions of DNA damage in response to DSB, and its kinase activity is increased. ATM is located in dimers in the cytoplasm, and when activated by DNA DSB, it auto-phosphorylates, allowing it to enter the nucleus in a process known as pATM nucleoshuttling (74).

ATM activates a series of DDR events at DSB sites when it has been activated. The phosphorylation of the histone variation H2AX by ATM within minutes of the damage at the site and near DSBs is the main mechanism driving this process. The buildup of many DNA repair proteins and chromatin-remodeling complexes near DSBs is prompted by phosphorylated H2AX (γH2AX) (75).

Hyper radiosensitivity is a side effect of ATM gene mutations, as seen in AT (ataxia-telangiectasia) patients with a faulty DSB repair pathway. As a result, any delay in pATM nucleoshuttling favors a delay in DSB identification, resulting in DSB misrepair that is not regulated by the NHEJ pathway, and hence radio or chemosensitivity (76).

The DNA repair system corrects damages in the DNA caused by radiotherapy and chemotherapy. Genetic stability is maintained as a result of this event, which is critical

for cell survival. Unrepaired DNA DSB causes cell death, whereas misrepaired DSB causes mutations to spread through subsequent cell generations after each replication cycle (77).

G. Radio induced cell death

Unrepaired DSBs were found to be linked to cell death, as previously stated. After unrepaired damage, various cell death types can be generated, with apoptosis, mitotic catastrophe, and senescence being the most common cell death pathways (78):

1. Apoptosis, also known as programmed cell death, is a process in which a cell "decides" to die as a result of irreversible damage, stress, or even to avoid cancer.
2. Another putative genetically controlled pathway for cell death following irradiation is Mitotic Catastrophe. A mitotic catastrophe is defined by the accumulation of uncondensed chromosomes in big nuclei, as well as the presence of chromosome abnormalities and micronuclei. Mitotic catastrophe, or the loss of replicative capacity, occurs in cells that enter the cycle with unrepaired or misrepaired DNA.
3. Senescence: also known as pre-mature senescence, in which cells incur a permanent cell cycle arrest as a result of cell cycle inhibitor activation during IR or genotoxic stress, resulting in widespread silencing and an increase in heterochromatin regions. As a result, senescent cells have stopped proliferating and can no longer contribute to tumor repopulation (79).

When it comes to anti-cancer therapy, however, and regardless of the sort of cell death, what matters is the cell's ability to regenerate and form colonies, or clonogenic capacity. As a result, Puck and Marcus developed the clonogenic assay in 1956, which allowed for the assessment of the number of surviving colonies after treatments (80). These

colonies are the offspring of colony-forming cells, also known as cancer stem cells that have acquired stem-like qualities and so can repopulate a tumor following treatment (81).

H. Radiosensitivity

Even though radiation therapy is commonly used to treat prostate cancer. Acute skin toxicity is a typical radiation-related side effect that many patients suffer. It has been proven that radiation therapy improves loco-regional control and lowers mortality (82).

Radiation-induced damage to normal tissues, on the other hand, is linked to a slew of potential acute and chronic side effects. Skin erythema, desquamation, pigmentation, and breast swelling are among the most common acute side effects, and their frequency is dependent on the individual's natural tissue radiosensitivities (83).

The ultimate goal is to increase the radiosensitivity of prostate cancer cells while protecting surrounding normal epithelial tissues.

I. Radioprotection

Radioprotection is a science-based subject that develops concepts, methods, and processes for preventing the detrimental effects of ionizing radiation on persons and the environment. Radioprotection aims to reduce the likelihood of radiation-induced stochastic effects, such as cancer, while simultaneously preventing deterministic outcomes, sometimes known as 'tissue reactions.' (84).

The International Commission on Radioactive Protection (ICRP) has been developing recommendations and practical guidance for radiological protection for more than 80 years, and in its 2007 Recommendations, the Commission defined its most recent system of protection with this goal in mind (85).

Almost all regulatory requirements for ionizing radiation-related activities, such as in industry, health, agriculture, and fundamental research, are based on the radiation protection concept, which is dependent on acceptance of the linear non-threshold (LNT) theory.

According to LNT, any dose, no matter how small, can produce genetic abnormalities or cancer. With no threshold, cancer risk is believed to increase linearly with increasing radiation dose (86).

The basic model of radiation protection was based on three physical principles:

I. Shielding (usually with lead) of unexposed areas, particularly radiosensitive organs like bone marrow, gonads, and thyroid;
II. Increased distance between the radiation source and radiation workers or patients;
III. Reduction of exposure time. Each of these aspects has proven to be extremely beneficial, but they are not without flaws. The development of a unique biological protection technique could boost the effectiveness of present efforts to reduce the risk of radiation damage in humans (87).

To protect normal tissues from possible radiation damage, it would be necessary to find biological or chemical treatments that, when administered prior to radiation exposure, would protect all normal tissues (88). Soon after World War II, scientists began looking for non-toxic radioprotective chemicals that could protect normal tissue from radiation harm. Extensive radiobiological research revealed a number of medications that, when administered prior to radiation exposure, protected animals (mostly rats) from radiation harm (89).

One method of reducing radiation's harmful effects on cells has been proposed: using radioprotective chemicals. Antioxidants can operate as free radical scavengers, reducing some of the DNA damage induced by ionizing radiation (90). This intervention

would theoretically allow cellular defenses to keep up with the free radicals produced by radiation exposure (assuming the intracellular level of antioxidants is sufficient at the time of radiation exposure) (91).

Radioprotective compounds may inhibit free radical formation, remove free radicals, induce natural radioprotector production (such as superoxide dismutase, glutathione peroxidase, and catalase), improve DNA repair, reduce the post-radiation inflammatory response, or even delay cellular division to give cells more time to repair or undergo apoptosis. Despite the fact that radioprotective chemicals have been found to reduce the adverse effects of radiation therapy, no radioprotectants are currently employed in patients' treatment (92).

J. Zoledronic acid and Pravastatin

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

Recently, a combination of bi-phosphonates (zoledronic acid) and statins (pravastatin), or ZoPra, was shown to radio-protect normal tissues by enhancing DNA DSB repair mechanism (93). In fact, all radiation treatment targets DNA, and the cell's ability to repair radio-induced DNA double stranded breaks (DSB) is the most significant determinant in determining cell survival and radiosensitivity (94). In the G0/G1 phase of the cell cycle, the non-homologous end-joining repair (NHEJ) pathway is the main DNA DSB repair pathway: after irradiation, protein kinase ataxia-telangiectasia mutated (ATM), a dimer present in the cytoplasm, was shown to autophosphorylate and shuttle to the nucleus in a process known as nucleoshuttling. pATM will phosphorylate histone H2AX on DSB sites once it reaches the nucleus, triggering NHEJ repair (95).

Following RT, these two proteins relocalize as nuclear foci, making them visible and quantifiable by immunofluorescence (IF). This allows for the visual assessment of the number of foci to be used to quantify DNA DSB (96). Previous research has demonstrated that the appearance and disappearance kinetics of both biomarkers (pATM and gH2AX) can predict radiosensitivity at the cellular and clinical levels for a wide variety of doses and radiation types (97).

Many medications have been tried to boost cancer cell radiosensitivity, but their success has been restricted by normal tissue tolerance and radiosensitivity (98). To put it another way, as cancer cell radiosensitivity intensified, normal tissue problems deteriorated as well.Recent research has found that a combination of zoledronate (ZO) and pravastatin (PRA), known as ZOPRA, can protect normal cells from people with radiosensitive hereditary illnesses such Huntington's disease, progeria, and tuberous sclerosis (99).

2. Zoledronic Acid

Zoledronic acid, like other bisphosphonates, is used to treat bone pain caused by malignancies, osteoporosis, Paget's disease, and unusually high blood calcium (hypercalcemia), among other conditions. Bisphosphonates are drugs that are used to treat cancer patients to prevent bone loss and fractures caused by osteoporosis (100).

Bisphosphonates have previously been shown to have localized effects in the presence of irradiation bone, both in the presence of a local tumor and in nontumor affected bone (101). Both zoledronic acid alone and zoledronic acid combined with the anabolic drug parathyroid hormone (PTH) enhanced bone microarchitecture and strength, although adding PTH only improved microCT measurements of bone quality without increasing strength (102).Although the current data are moderated by the lack of pretreatment DXA scans that

would have conclusively removed the potential for baseline differences between the groups, zoledronic acid enhanced BMD over control limbs at each time point, as has been established clinically. In terms of morphology, zoledronic acid treatment reduced the harmful effects of irradiation on the trabecular bone indices (BV/TV, TB.N) while also raising the BMC (103).

Some adjuvant trials of oral clodronate or intravenous zoledronic acid have shown improvements in bone-metastasis-free survival, disease-free survival, and overall survival in women with early breast cancer (104). However, no substantial benefits were identified in analyses that included all randomized participants in other adjuvant bisphosphonate studies, however both planned and exploratory subset analyses revealed benefits in postmenopausal women or older women. This led to the theory that medication is only beneficial in patients who have low levels of reproductive hormones (e.g., those who are postmenopausal or on ovarian suppression therapy) (105).

3. Pravastatin

Statins are a type of lipid-lowering drug that has been found to reduce the risk of cardiovascular death and mortality in people with high blood pressure (106).
Despite the fact that statins have significant antiproliferative and tumoricidal activities in vitro, their anticancer effects in animal models are moderate, and their efficacy in clinical trials is unknown. Statins are inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which have been used to treat hypercholesterolemia in the clinic. They have a high safety profile and are effective in both primary and secondary cardiovascular disease prevention (107).

Statins have been hypothesized to have the capacity to modify the efficacy of anticancer treatment methods, either by increasing or, in rare situations, decreasing their efficacy (108). Statins have been shown to have anti-inflammatory properties, thus it's not

impossible that they could protect against malignancies caused by inflammation. Statins have been shown to have anti-inflammatory properties, thus it's not impossible that they could protect against malignancies caused by inflammation (109).

Statins have also been shown to prevent DNA damage caused by chemical carcinogens and to speed up DNA repair. Pravastatin, fluvastatin, and simvastatin are examples of HMG-CoA reductase inhibitors that are commonly used in clinical therapy for individuals with hyperlipidemia (110).

FV is the first HMG-CoA reductase inhibitor to be completely synthesized, and its chemical structure is distinct from that of other HMG-CoA reductase inhibitors derived by chemical modification of fungal metabolites. FV is commonly used in the treatment of hyperlipidemia patients, and it has recently been discovered to have an antioxidant impact on LDL oxidation and to scavenge ROS.

ROS such as hydroxyl radicals and singlet oxygen enhance the concentration of 8-oxo-29-deoxyguanosine (8-oxodG), a key oxidative product of DNA bases. Carcinogenesis has been linked to oxidative DNA damage, and it is now apparent that several carcinogens cause DNA oxidation in their target tissues (111). The current investigation reveals that stomach intubation with FV 30 minutes before BOP administration effectively reduced the increase in 8-oxodG in the nuclear DNA of the target tissue, the pancreas, produced by BOP administration. FV appears to be beneficial in reducing oxidative DNA damage as well as lipid peroxidation. If the production of oxidative DNA damage is connected with carcinogenesis, FV may limit the occurrence of malignancies by preventing the starting stage in the carcinogenetic pathway (112).

Although statins can prevent DNA damage following ionizing radiation or cytotoxic medicines in cultured cells and in vivo in both animals and humans with

atherosclerosis, the mechanisms behind this effect are unknown and likely to be diverse. Statins can reduce oxidative stress, limit protein prenylation, and block downstream DNA damage signaling, all of which can reduce DNA damage and the damage response (113). Statins can also promote phosphorylation of Ser166 on the ubiquitin ligase mdm2, which improves its nucleus localization and association with p300 while inhibiting its interaction with p19ARF, resulting in increased p53 degradation. Statins caused robust phosphorylation of Hdm2, reduced Hdm2 interaction with NBS-1, and reduced NBS-1 degradation, according to our findings. Despite the fact that statin treatment presumably affects numerous proteins, Hdm2 was required for atorvastatin to expedite DNA repair since Hdm2 knockdown replicated the atorvastatin effect and prevented any additional atorvastatin impact (114).

Statins must block physiologically substantial DNA damage for their effect on DNA repair to be clinically important. ROS cause DNA strand breakage and base and nucleotide changes, especially in guanosine-rich sections like telomeres. Telomere shortening triggers a DNA damage response that includes the activation of proteins like ATM, NBS-1, and H2AX, which are implicated in oxidative DNA damage (115).

In VSMCs, atorvastatin suppresses the appearance of SAG and telomere shortening. Despite the fact that the effect on telomere shortening could be multifactorial, telomerase and telomere-associated protein expression remained unaltered, and cells with constitutive telomerase expression still showed faster DNA repair following atorvastatin treatment.

As a result, statins are expected to speed up the repair of broken telomeres, most likely via NBS-1. NBS-1 is necessary for telomere maintenance in eukaryotic and yeast cells, and it binds with TRF2 in human telomeres (116).

Pretreatment with atorvastatin sped up the restoration to normal -H2AX expression and decreased apoptosis in the intestinal mucosa. Although statins have been shown to lessen the long-term effects of radiation, this is the first study to show that rapid DNA repair may be a factor. In addition, long-term statin treatment lowers expression of ATM/ATR substrate activity in rabbits with established neointimal lesions (117).

In irradiated mice, Naeimi et al. looked at the effect of atorvastatin as a potential radioprotectant in pelvic cancer to prevent radiation damage to testicular tissue (118). Mice were given varied doses of atorvastatin seven days before being irradiated in this study. The possible radioprotective effects were assessed using biochemical, histological, and immunohistological markers. The protective effect of atorvastatin was dose-dependent, with the highest dosages providing the most protection. Atherosclerosis was significantly reduced in mice given atorvastatin, and total serum testosterone levels were significantly greater. In irradiated controls, histologic investigation revealed a decrease in testicular epithelial thickness and atrophy of the seminiferous tubules (119).

Although atorvastatin pretreatment increased epithelial thickness and seminiferous tubule diameter in mice, the increased diameter of the seminiferous tubules was not statistically significant, according to the authors. Atherosclerosis was reduced in mice pretreated with atorvastatin, implying that atorvastatin works via lowering apoptosis after irradiation (120).

According to Combemale et al. 47 Fibroblast cell lines show that cells from people with NF1 have an aberrant sensitivity to radiation. A cellular radiosensitivity with unfavorable effects after radiotherapy and an elevated risk of radiation-induced cancer after radiodiagnosis could be the result of an IR exposure. The altered NF1 proteins may sequester the ATM kinase in the cytoplasm, explaining both the radiosensitivity and radiosusceptibility of NF1 cells. A DSB repair deficiency and enhanced genomic instability could be the direct

effects of an RIANS delay. The combination of zoledronate and pravastatin may greatly minimize this risk (121).

The ZoPra combination has been found to improve the speed of ATM nucleo-shuttling by blocking nucleus membrane farnesylation. This results in faster and better DSB signaling, which improves the cell's ability to repair DNA. However, investigations on ZoPra's modulating effect on cancers have shown mixed results; some have showed that ZoPra can be radioprotective on tumors, while others have shown that it can also be radiosensitizing (122).

K. Aim 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 radio protecting effect of Zoledronic acid and Pravastatin, alone and in combination, with a 2 Gy irradiation, on the radio-response of normal human prostate epithelial cell line RWPE-1 in vitro.

CHAPTER II

MATERIALS AND METHODS

A. Cell culture

1. RWPE1: human prostate epithelial cell line.

Human immortalized normal prostate epithelial cell line, RWPE-1 was used and purchased from the American Tissue Culture (ATCC, USA).

B. Cell growth

RWPE-1 cells were cultured and maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 5% heat-inactivated fetal bovine serum (FBSSigma-Aldrich), 1% Penicillin- Streptomycin (Sigma-Aldrich), 0.2% PlasmocinProphylactic (InvivoGen), 1% non-essential amino acids (Sigma, USA), and 1% sodium pyruvate (Sigma, USA). Cells were incubated at 37°C, 5% CO2 humidified incubator.

Typically, the media over the cells was replenished every 4-6 days and cells were splitted after reaching 70% to 80% confluency.

C. 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 all experiments, cells were incubated for 12 hours at 37°C with ZO, and 24 hours with PRA, and followed or not by a 2 Gy irradiation. Later, cells were washed with PBS and culture medium was renewed before irradiation.

D. Irradiation

Cells were irradiated with a 225 kV Precision X-Ray (PXi) irradiator model No X-RAD 225. Irradiation was performed at 2 Gy.min-1 and a 1.5 mm Aluminum filter was used. All cells were irradiated at a dose of 2Gy equivalent to the dose given to patients per irradiation session in clinics

E. Cell Growth Assay/ MTT

The effect of Zo and Pra, alone and in combination, with and without a 2 Gy irradiation, on the viability of RWPE-1, was investigated using the MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay. Cells were plated in triplicates in 100 µl complete media in 96-well culture plates, at a density of 3x103 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 34 was used for the baseline zero. The data are derived from the mean of triplicate wells of three independent experiments.

F. Clonogenic Cell Survival Assay

RWPE-1 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 80% 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. RWPE-1 cells were seeded at a density of 50000 cells per well. After incubation for 14-16 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

G. 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 RWPE-1 cells. Cells were seeded on 12 mm coverslips at the bottom of 24 well plates. When reaching 80% 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 hour 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 hour at 37°C in an incubator, then washed twice with PBS.

Next, cells were incubated with the secondary antibody Alexa Fluor 568 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 laser scanning confocal microscope Zeiss LSM 710 (Zeiss, Germany), and were processed using Zen 2012 image analysis software (blue edition).

H. Micronuclei Assay

The DAPI counterstaining performed during immunofluorescence experiments permitted us to quantify the micronuclei caused by unrepaired chromosomal breaks (123). For each condition, the percentage of cells with micronuclei was counted. Experiments we performed three times.

I. 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 RWPE-1 cell line using MTT assay

The MTT assay was carried out to determine the optimal dose of Zo and Pra, on RWPE-1 cells. 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 RWPE-1 cells on doses 1,3 and 9 μM however Zo exerted significant toxicity on 18 μM. (Figure 4).

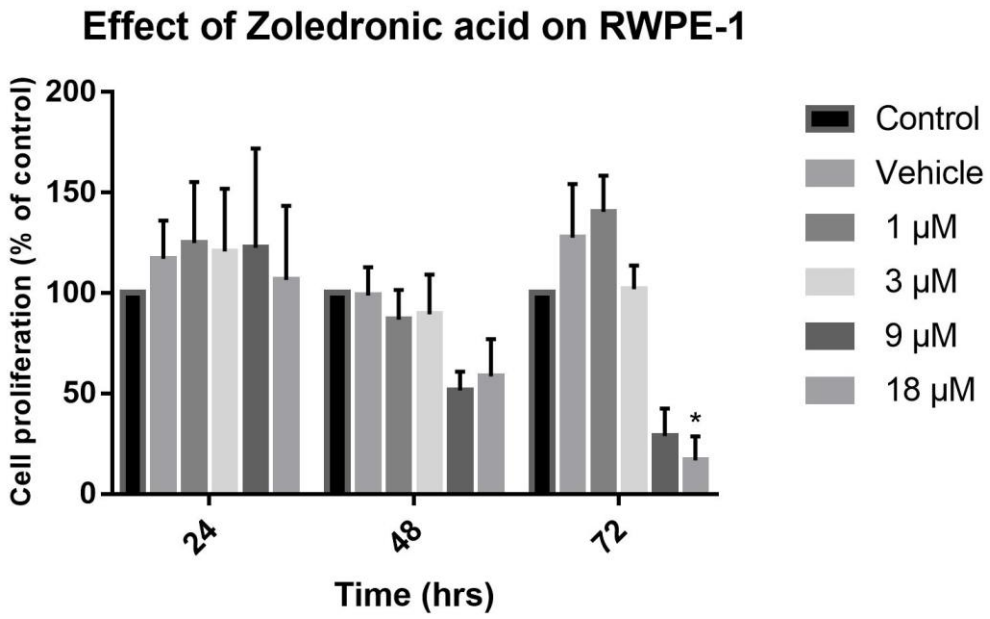


Figure 3: Effect of varying concentrations of Zoledronic acid, on RWPE-1 cell proliferation. After incubation of RWPE-1 cells for 24, 48, and 72 h with or without treatment (Zo), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± SEM. (*) $P < 0.05$.

Increasing doses of Pra on the proliferation of RWPE-1 cells had no significant impact. (Figure 5). Hence, the minimal effective dose of 1 μM Zo and 1 μM Pra was used to carry out further optimization experiments.

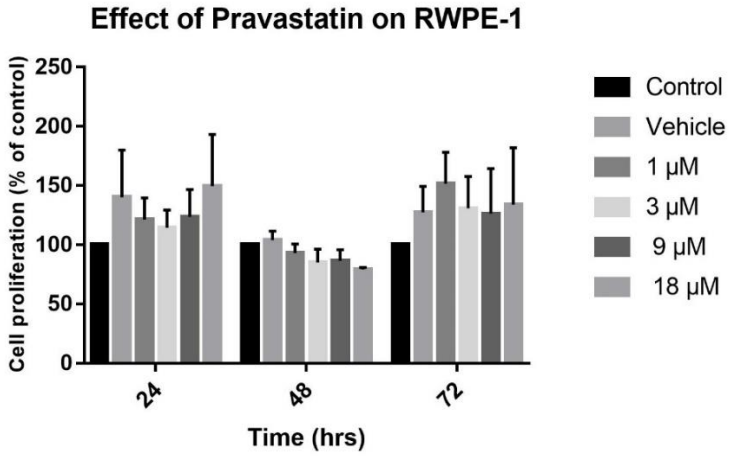


Figure 4: Effect of varying concentrations of Zoledronic acid, on RWPE-1 cell proliferation. After incubation of RWPE-1 cells for 24, 48, and 72 h with or without treatment (Pra), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± SEM.

The cytotoxicity of the combination of 1 µM Zo and 1 µM Pra (1 µM ZoPra) was then assessed. The results show no significance on the cell proliferation of RWPE-1 cells $P > 0.05$ (Figure 6).

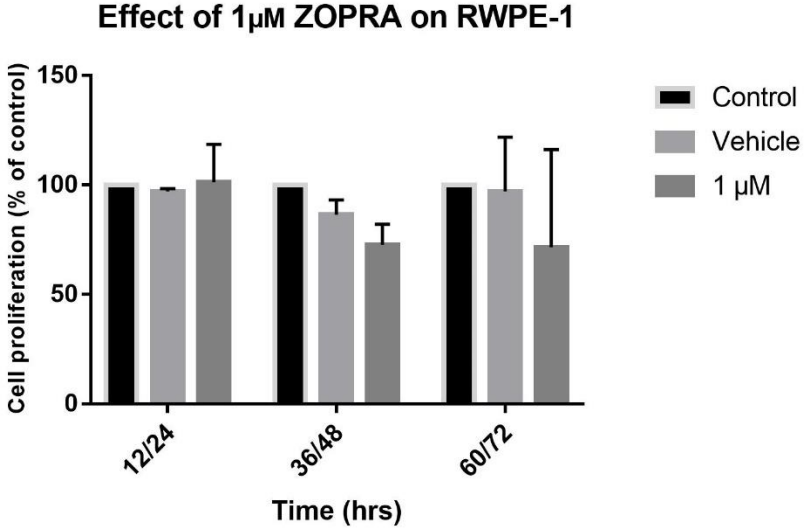


Figure 5: Effect of 1 µM Zo and 1 µM Pra on RWPE-1 cell proliferation. After incubation of RWPE-1 cells for 24, 48, and 72 h with or without treatment (ZoPRA), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± 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 RWPE-1 cells. RWPE-1 cell proliferation was not significantly affected at any time point (Figure 7).

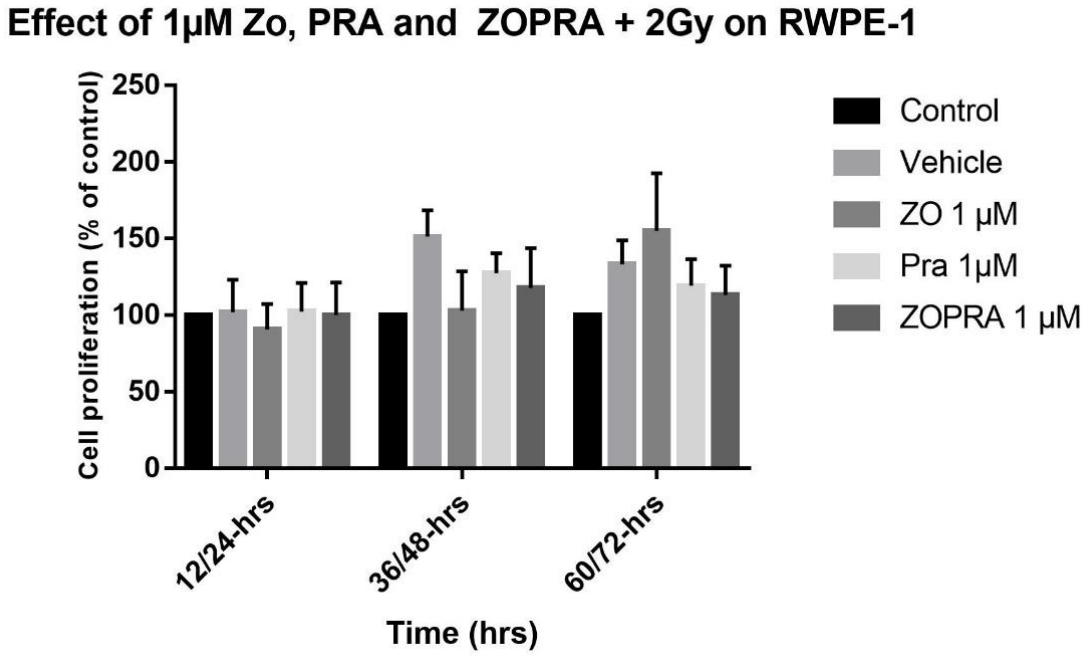


Figure 6: Effect of 1 μM Zo, 1 μM Pra, and 1 μM ZoPra followed by a 2 Gy irradiation on RWPE-1 cell proliferation. After incubation of RWPE-1 cells for 24, 48, and 72 h with or without treatment followed by a 2 Gy irradiation, cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Each plot represents the mean of three independent experiments ± SEM.

B. The effect of Zoledronic acid and Pravastatin, alone and in combination, with 2 Gy irradiation, on the clonogenic survival of RWPE-1 cell line

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. When comparing experimental groups (1 μM Zo, Pra, and ZoPra) to 2 Gy alone, RWPE-1 cell reproductive survival was not significantly affected.

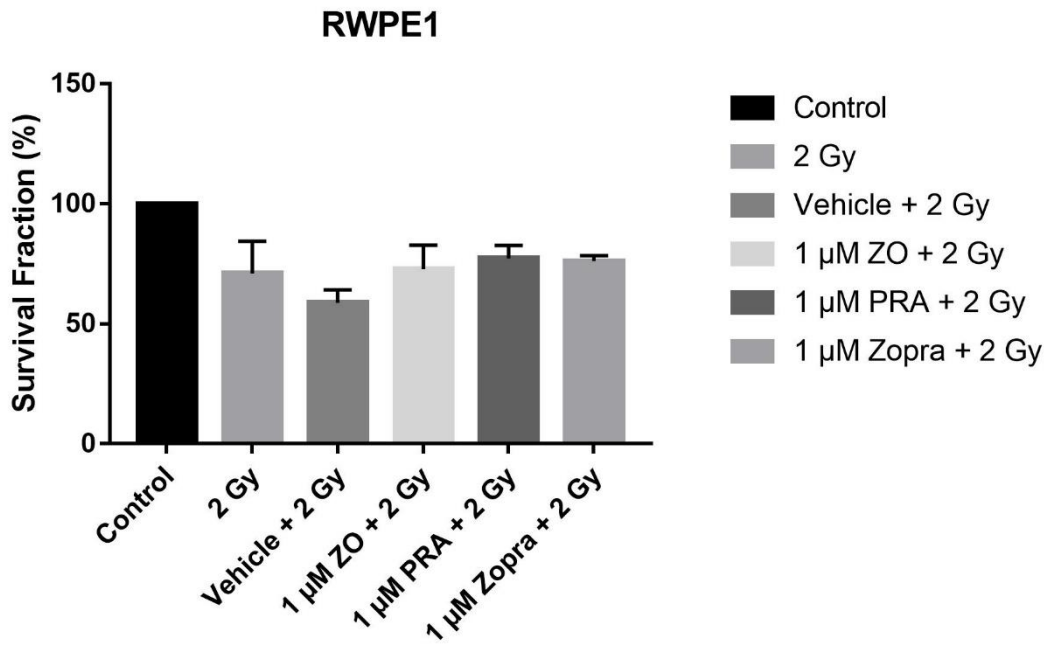


Figure 7: 1 µM ZoPra increase the survival fraction of RWPE-1 cells. Cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 µM Zo, Pra, and ZoPra. 24 hours post-IR, cells were seeded with the predetermined density derived from the plating efficiency experiment and incubated for 14-16 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 ± SEM.

C. Effect of 1 µM ZoPra on residual pATM foci in RWPE-1 cell line.

To assess the effect of ZoPra on the pATM kinetics in RWPE-1 cells, immunofluorescence using anti-pATM was employed. A low number of spontaneous foci(<4) in both untreated and treated groups with 1 µM ZoPra, peak at 60 minutes (1 hour) with 30 ± 5 and 37.5 ± 2.5 foci, resolving to 4.5 ± 0.5 and 2.67 ± 0.33 foci 24 hrs post-IR in both untreated and treated groups with 1 µM ZoPra respectively.

There was no significant effect in pATM dynamics 10 mins post-IR in groups pre-treated with 1 µM ZoPra compared to IR alone. (figure 9)

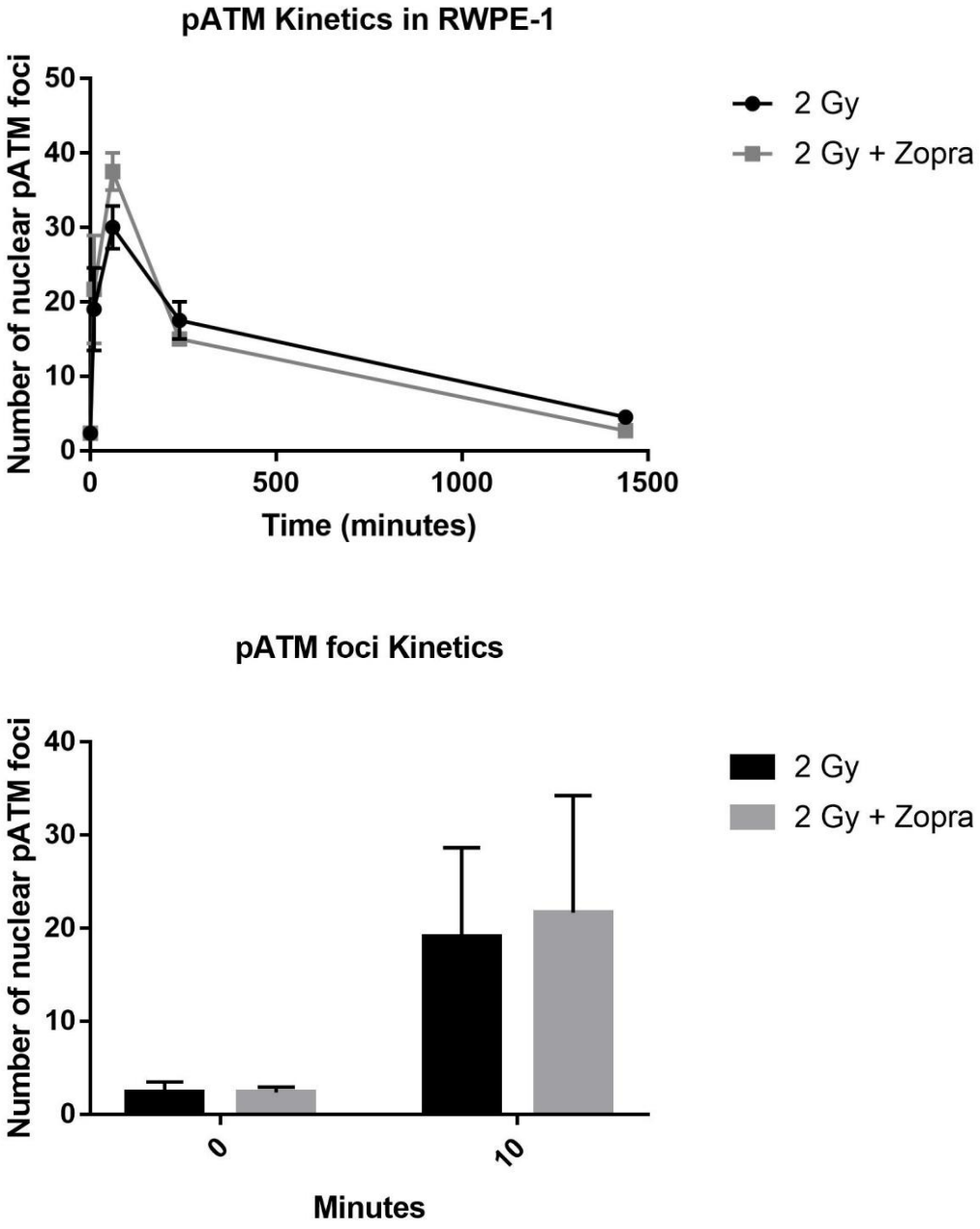


Figure 8: Effect of IR and 1 μM ZoPra on pATM kinetics in RWPE-1 cell line. RWPE-1 cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 μM ZoPra. Cells were fixed 0, 10-, 60-, 240- and 1440-minutes post-IR and stained with anti- pATM to visualize protein kinetics. 30 nuclei were analyzed and pATM foci were counted and plotted. Each plot represents the mean of three independent experiments ± SEM.

D. Effect of 1 μM ZoPra on residual γH2AX foci in RWPE-1 cell line.

To assess the effect of ZoPra on the γH2AX kinetics in RWPE-1 cells, antiγ-H2AX immunofluorescence was employed. RWPE-1 γH2AX kinetics show a low number of spontaneous foci 1.45 ± 0.4 and 1.51 ± 0.6 without IR, which peaks 10 mins post radiation with 65 ± 5 and 71.67 ± 2.33 and declines to 4.62 ± 1.1 and 2.64 ± 0.7 foci 24 hours (1440 mins) post-IR in both untreated and treated groups with 1 μM ZoPra respectively. RWPE-1 cells pre-treated with 1 μM ZoPra show a significant decrease in residual γH2AX foci 24 hrs (1440 mins) post-IR when compared with those only treated with IR ($p<0.0001$) (figure 10).

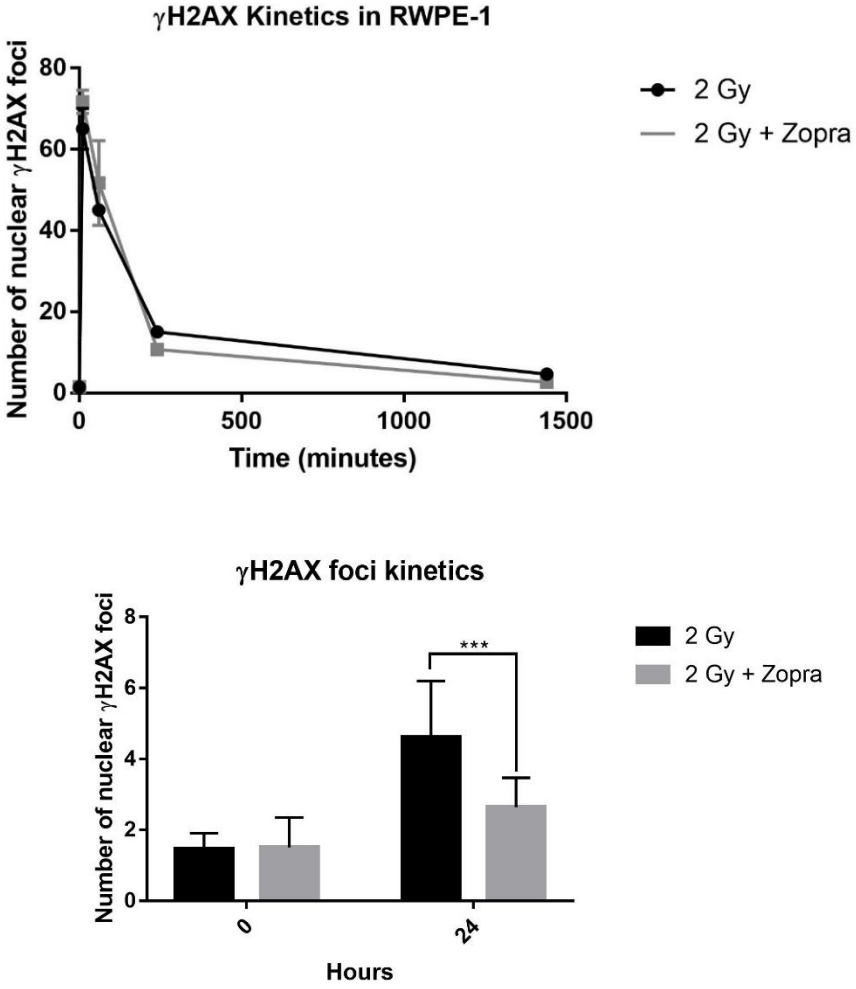


Figure 9: Effect of IR and 1 μM ZoPra on γH2AX kinetics in RWPE-1 cell line. RWPE-1 cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 μM ZoPra. Cells were fixed 0, 10-, 60-, 240- and 1440-minutes post-IR and stained with anti- γH2AX to visualize protein kinetics. 30 nuclei were analyzed and γH2AX foci were counted and

plotted. Each plot represents the mean of three independent experiments ± SEM. (***)P < 0.001 .

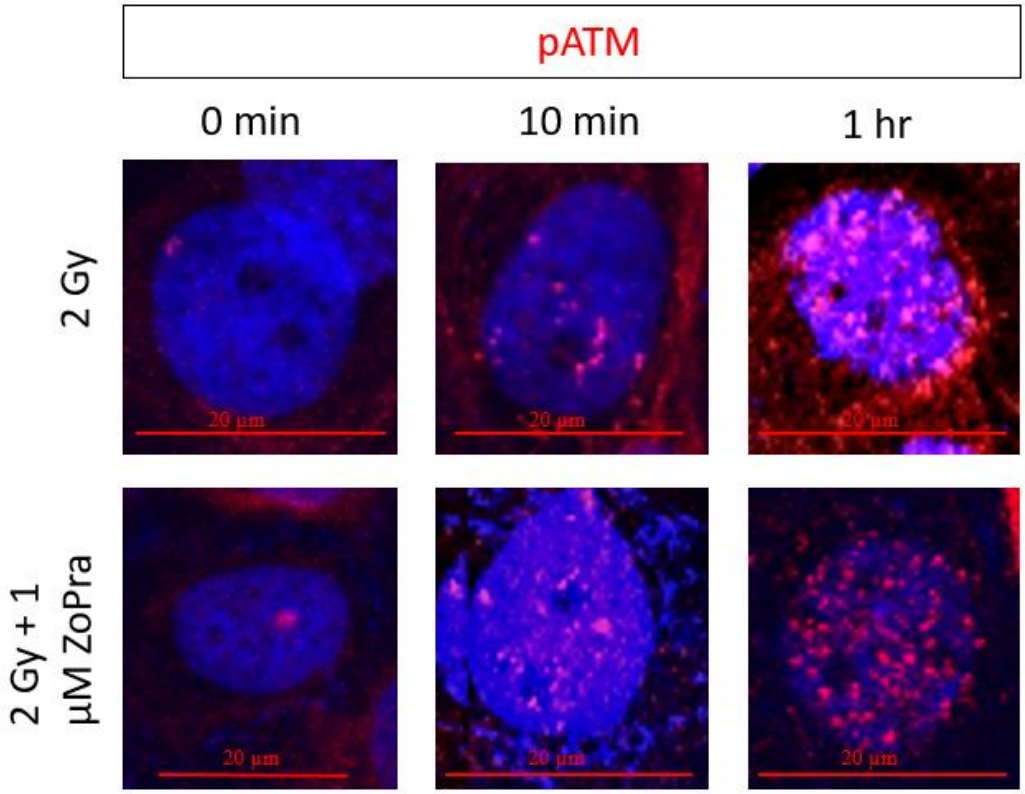


Figure 10: IF images of pATM foci in RWPE-1. Cells were either treated with a 2 Gy IR alone or pretreated with 1 µM ZoPra. They were then fixed on 12mm coverslips 0-, 10- or 60-minutes post-IR. Cells were then stained with anti-pATM monoclonal anti-mouse antibody followed by secondary antibody Alexa Fluor 568 goat anti-mouse IgG. pATM foci in red and nucleus was counterstained using DAPI in blue.

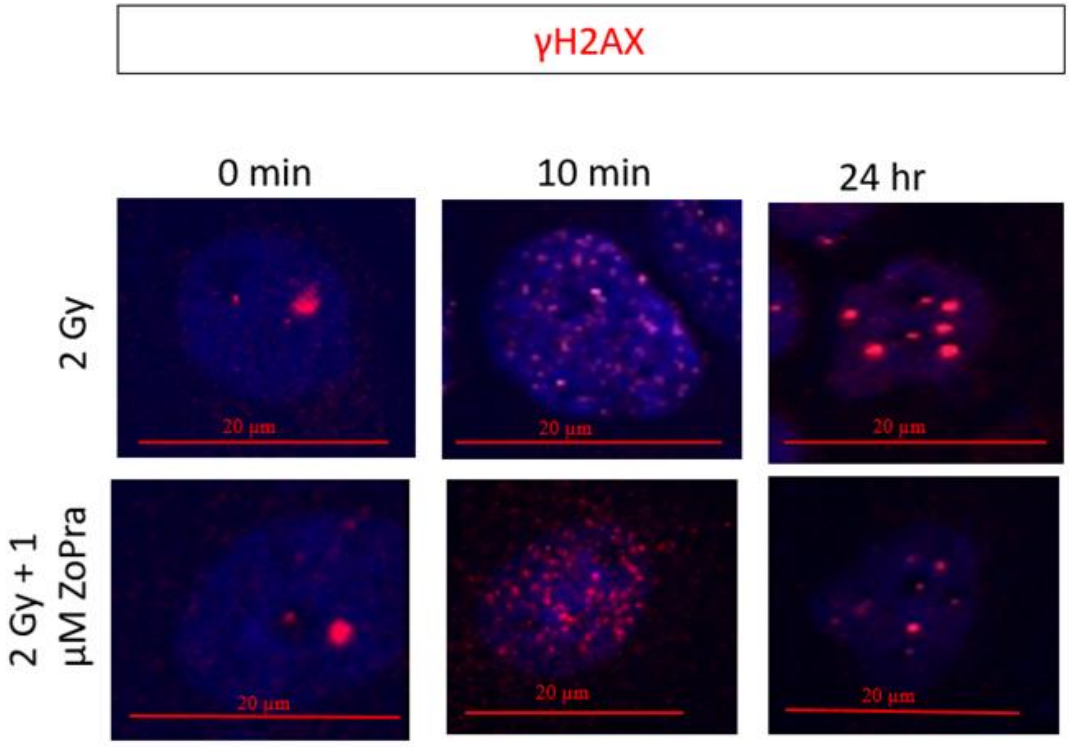


Figure 11: IF images of γH2AX foci in RWPE-1 cells. Cells were either treated with a 2 Gy IR alone or pretreated with 1 μM ZoPra. They were then fixed on 12mm coverslips 0-, 10- or 1440-minutes post-IR. Cells were then stained with antiγH2AX monoclonal anti-mouse antibody followed by secondary antibody Alexa Fluor 568 goat anti-mouse IgG. γH2AX foci in red and nucleus was counterstained using DAPI in blue.

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

The MN was performed 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 RWPE-1 cells, ZoPra did not induce any significant changes in MN% when comparing those pre-treated with ZoPra and those that were not (Figure 11).

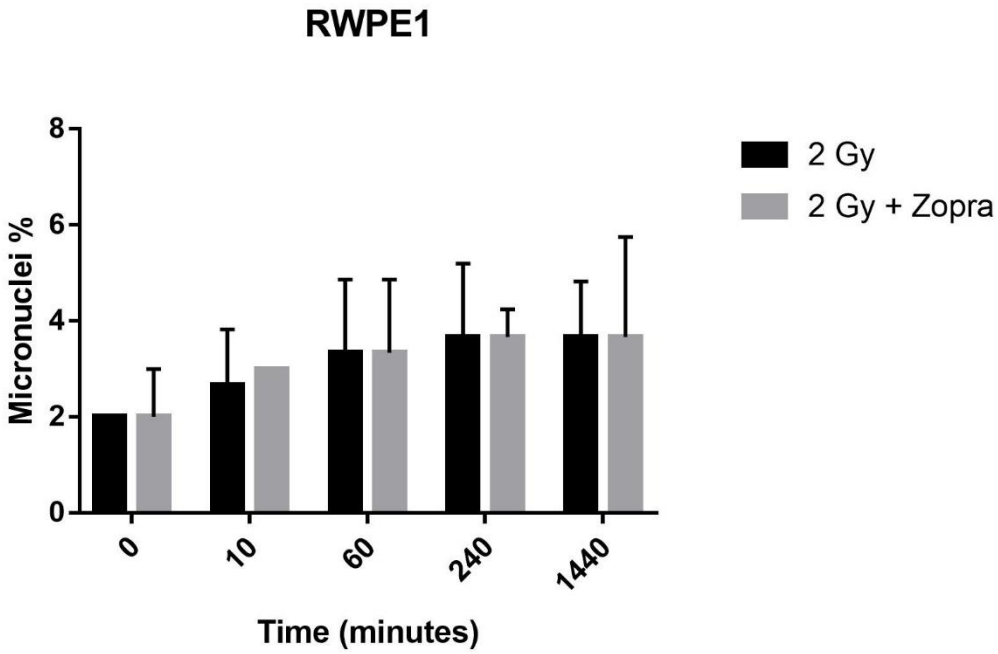


Figure 12: Effect of IR and 1 µM ZoPra on percentage of IR-induced micronuclei in RWPE-1 cell line. RWPE-1 cells were treated with a 2 Gy irradiation with and without pre-treatment with 1 µM ZoPra. DAPI counterstaining performed during immunofluorescence permitted the quantification of micronuclei. Percentage of cells with micronuclei was quantified for each condition. Each plot represents the mean of three independent experiments ± SEM.

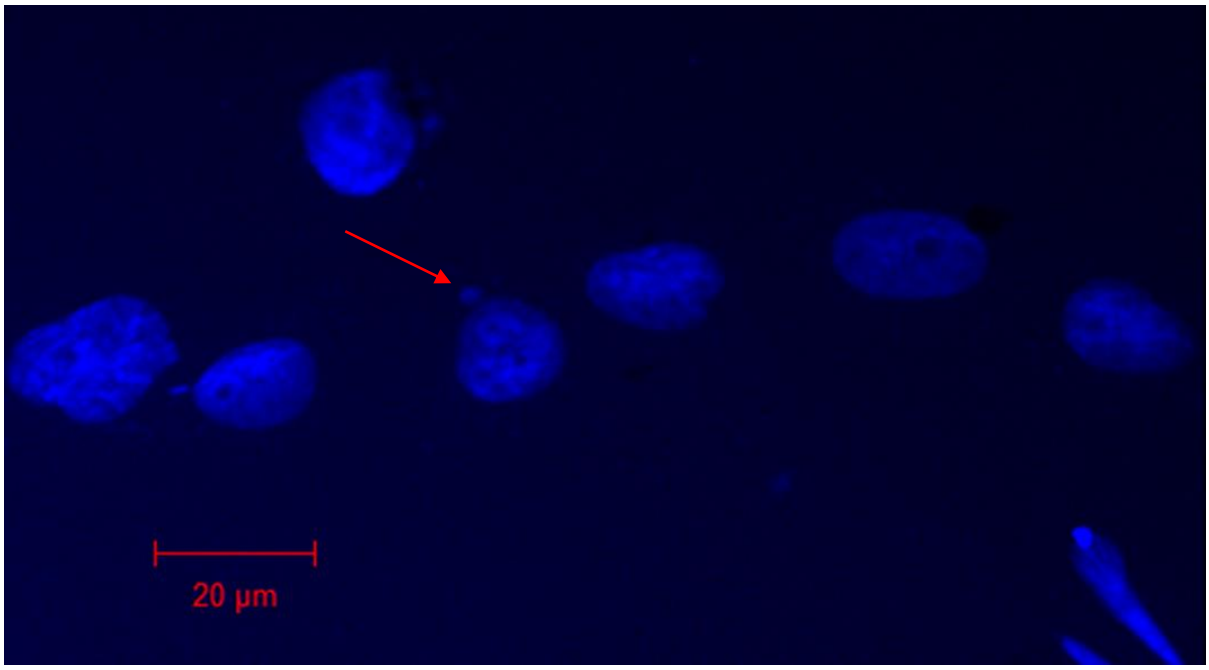


Figure 13: RWPE-1 cells with MN. DAPI counterstaining performed during immunofluorescence experiments to quantify the micronuclei caused by unrepaired chromosomal breaks.

CHAPTER IV

DUSCUSSION

A cells' radioresponse can be described at the cellular and molecular level by cell survival, cell proliferation, and the kinetics of DDR proteins (such pATM and H2AX). The radioprotecting effect of 1 μM Zo, Pra, and ZoPra was tested on the human prostate epithelial cell line RWPE-1. The optimal dose used in all experimental procedures was 1 μM since it was found that its not toxic in RWPE-1 cells. Previous results from our lab and literature review support this claim, in addition to 1 μM being a biological and clinical relevant dose.

Using the clonogenic assay, we examined the impact of 1 M Zo, Pra, and ZoPra on the cell survival of each cell line. In 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 prior to plating. This illustrates the delayed plating method, which is well-known for being employed in radiobiological research since it evaluates a cell's ability to repair IR-induced DNA damage (124). Cells are seeded at a relatively low density predetermined using the plating efficiency technique and allowed to grow for 14-16 days. Cells that retain the capacity to reproduce and form colonies of >50 cells, would have effectively repaired the DNA damage induced by IR. Cells that do not, will lose their ability to reproduce and hence IR would have resulted in their clonogenic death. The target of RT is clonogenic death because when a tumor cell loses its ability to proliferate, it is no longer clonogenically viable (125). Hence the clonogenic assay's outcome reveals RWPE-1 cell line as a radioresistant cell line.

The NHEJ pathway is the primary DDR mechanism that is active in G0/G1 cells after IR.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 demonstrates that the detection of H2AX foci using immunofluorescence is a sensitive way of assessing DNA

repair kinetics and DSB formation (126). It has been demonstrated that γH2AX and pATM foci formation and disappearance kinetics can predict cellular and clinical radiosensitivity (93). Therefore, to further investigate the radio-response of RWPE-1 cell line, 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.

In order to determine the impact of ZoPra on the pATM signaling in DDR, an IF experiment measuring pATM was performed. A reduction in the maximum number of pATM foci was shown to be a reliable indicator of a cell's radiosensitivity (127). 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 RWPE-1 cell line.

The kinetics of H2AX in RWPE-1 cells subjected to a 2 Gy irradiation with and without pre-treatment with 1 μM ZoPra were evaluated using an IF assay. Each unrepaired DSB is represented by a single focus, and the focus was the fraction of residual γH2AX foci 24 hours after IR, which serves as a measure of radiosensitivity. The incubation of RWPE-1 cells with 1 μM ZoPra alone did not affect the number of spontaneous residual foci, hence ZoPra don’t induce DNA damage alone and don’t enhance the signaling pathway. The results showed a lower number of unrepaired DSB (foci) in RWPE-1 cells suggesting that they have been radioprotected when treated with ZoPra. Therefore we can deduce that Zopra enhance DSB recognition and repair of this cell line, with a potential radioprotecting effect on a molecular level. However, this is not yet proven on the cellular scale (clonogenic assay). This might be caused by the fact that the effect, observed on the molecular scale, is not yet enough to be observed on the cellular scale. However, it is noteworthy mentioning that the 2Gy treatment is equivalent to only 1 session of radiotherapy treatment. For prostate cancer, 25-35 sessions are usually required for a full complete treatment. Knowing that the radiation effect

is additive, any difference at the molecular scale for 1 radiotherapy session, will probably have a significant clinical impact after the full treatment.

Further experiments of higher doses or repeated dosages are required to visualize the effect of ZoPra on a cellular level.

Finally, using DAPI counterstaining from the IF experiment, the effect of the ZoPra combination on the percentage formation of irreversibly damaged chromosomal fragments discharged from the nucleus was evaluated. The results of this experiment demonstrates that ZoPra did not have any effect on the percentage of micronuclei in either cell line. The proposed explanation might be that RWPE-1 cells are thought to be radioresistant.

The need of successful therapy is highlighted by the enigmatic etiology of prostate cancer and the inability to manage the majority of its risk factors. More than 50% of PCa patients choose radiotherapy as a standard form of treatment. There are various researches evaluating the impact of statins and bisphosphonates on PCa cells' radioresponse, but no studies have combined the two medications. Given Zoledronic acid is provided to 90% of PCa patients with metastatic disease, evaluating the effects of commercially available, FDA-approved medications, such as Pravastatin and Zoledronic acid, in the hopes of repurposing them was extremely convenient (128, 129).

Our lab team evaluated the impact of a mixture of pravastatin and zoledronic acid on breast cancer cells in vitro in a manuscript that was submitted. By reducing DDR's ability to absorb IR, ZoPra made breast cancer cell lines more radiosensitive and showed that ZoPra could have a radioprotective effect on normal tissues. This led us to target the proteins H2AX and pATM, which are essential for activating NHEJ, the primary form of DDR in G0/G1 cells (130).

The radioprotecting impact of Prostate epithelial cells when treated with ZoPra appeared to be promising in the experiments. Clonogenic and IF tests need to be repeated, on RWPE-1 cells and other cell lines with a different radiosensitive backround, in order to better confirm, support the findings and broaden the spectrum of radiosensitivity. To evaluate the impact of ZoPra on cell viability and DDR protein quantification, other techniques, such as Trypan blue and western blotting, could be employed. In the future, the impact of ZoPra might be evaluated using 3D culture models and human-derived PCa organoids, which more accurately replicate the physiological environment.

In conclusion, Prostate cancer (PCa) is one of the most common types of cancer among men. Radiotherapy is one of the treatments regimen for PCa. However, many patients suffer from unpleasant side effect. Therefore, fine-tuning the effects of radiotherapy on normal surrounding cells is essential. Recently, a combination of bi-phosphonates (zoledronic acid) and statins (pravastatin), ZoPra, was shown to radio-protect normal tissues by enhancing DNA double-strand breaks (DSB) repair mechanism.

The fact that ZoPra was previously shown to have a radio-protective role in the tissues surrounding the tumor, makes it a good candidate to become a therapeutic window-widening drug. Hence, the ZoPra combination has the potential to be a cost-effective treatment in prostate cancer patients undergoing radiotherapy, in addition to the clinical safety of both drugs, widening the therapeutic ratio between local control and normal tissue complications

For future perspective, we expect ZoPra to have a radio-protecting effect on normal prostate and fibroblast cells.

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